The occurrence of microbial hazards and succession of Enterobacteriaceae on fresh-cut fruit during minimal processing and storage

By

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CHAPTER 1: PROBLEM STATEMENT

Today's society is becoming increasingly health conscious and is characterized by a growing interest in the function of food for maintaining consumer health and human well-being (Zink, 2009). Fresh-cut fruits are convenient foods that suit the needs of a healthy lifestyle, combining technical content with an innovative food concept (Food and Agricultural Organization, 2011a). They are an important part of a healthy diet and can decrease the risk of cardiovascular disease and cancer (Allende, Thomas-barberan, Gill, 2006). Fresh-cut tropical fruit on the market today include mangoes and sweet melons (FAO, 2011a).

The market for chilled, fresh-cut fruit has witnessed a global growth in recent years, including developing countries such as South Africa (FAO, 2011a). Growth in the market opportunities will only continue if consumers believe that it is safe with an acceptable shelf life. In order for fresh-cut fruit to be sold locally or exported, there has to be compliance with regulatory requirements including food safety standards and audit standards, compliance with international regulations such as the Codex Alimentarius of the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO).

Although consumption of fresh-cut fruit is beneficial for good health, these foods could be associated with foodborne illness (Alegre, Vinas, Usal, Anguera, Figge, Abdias, 2012). Outbreaks of foodborne illness related to the consumption of fresh-cut fruit, mainly due to *Salmonella* spp and *E. coli* O157:H7, have increased drastically since the 1970's (Food and Drug Administration, 2013). Contamination of fresh fruit and subsequently fresh-cut fruit with human pathogens can occur at several points in the production chain including growth, harvest and minimal processing (Harris, Farber, Beuchat, Parish, Suslow, Garret, Busta, 2003). Although the pH of fresh-cut fruit is thought to be a limiting factor, the growth of *Salmonella enterica* (*S. enterica*) has been reported on fresh-cut mangoes and sweet melon (Freudlund, Back, Sjoberg, Tornquiste, 1987; Leverenzt, Conway, Alavidze, Janisiewicz, Fuchs, Camp, Chighladze, Sulakvelidze, 2001; Bordini, Ristori, Jakabi, Gelli, 2007; Castillo, Martinez-Tellez, Roderiguez-Garcia, 2009). Fresh-cut sweet melon is considered to be a high risk food (Codex Alimentatius Commission, 2011).

In a study that was done to identify disease outbreaks linked to sweet melons occurring between 1950 and May 2011, 85% of outbreaks were identified of which the majority of outbreaks were linked to fresh-cut sweet melon (CAC, 2011). Other than *Salmonella* spp and *E. coli* O157:H7, some members of the Enterobacteriaceae family that is not associated with foodborne diseae such as *Citrobacter* spp, *Klebsiella* spp, *Enterobacter* spp and *Pantoea* spp form a large part of the native microbial community that is naturally present on fresh-cut fruit (Soranio, Rico, Molto, Manes, 2000; Johannessen, Loncarevic, Kruse, 2002; Abdias, Canamas, Asensio, Anguiera, Vinas, 2006). The native microbial community is thought to be of importance in maintaining the health-supporting status of fresh-cut produce (Nguyen & Carlyn, 1994) by outcompeting pathogens for nutrients and physical space and/or by producing antagonistic compounds that reduce the viability of pathogens (Liao & Fett, 2001).

This study aimed to determine the microbiological quality of fresh-cut sweet melons and mangoes during the various stages of minimal processing and storage including whole fruit, cut fruit, packaged fruit and packaged fruit at the end of shelf-life. In addition, the study investigated the change in population dynamics of the Enterobacteriacaea over the various stages of minimal processing and storage of sweet melons. This was done to determine at which stages foodborne pathogenic members of the Enterobactericeae dominate and to study the interaction between different species of the Enterobacteriaceae.

CHAPTER 3: RESEARCH

This study was divided into 2 phases. The first phase aimed at assessing the microbial quality and safety of fresh-cut mangoes and melons from a fresh-cut fruit processing plant during minimal processing including whole, cut and packaged fruit (day 0) and packaged fruit at the end of shelf-life (Figure 8). The second phase aimed to study the succession of Enterobacteriaceae on sweet melons during minimal processing and subsequent storage. Phase 1 involved examining the mango and melon samples for total plate count, Enterobacteriaceae, yeast and mould as well as bacterial pathogens including *S. aureus*, *Salmonella* spp, *L. monocytogenes* and *E. coli* O157:H7. Phase 2 involved identification of the dominant Enterobacteriaceae species on sweet melons during minimal processing (Figure 9).

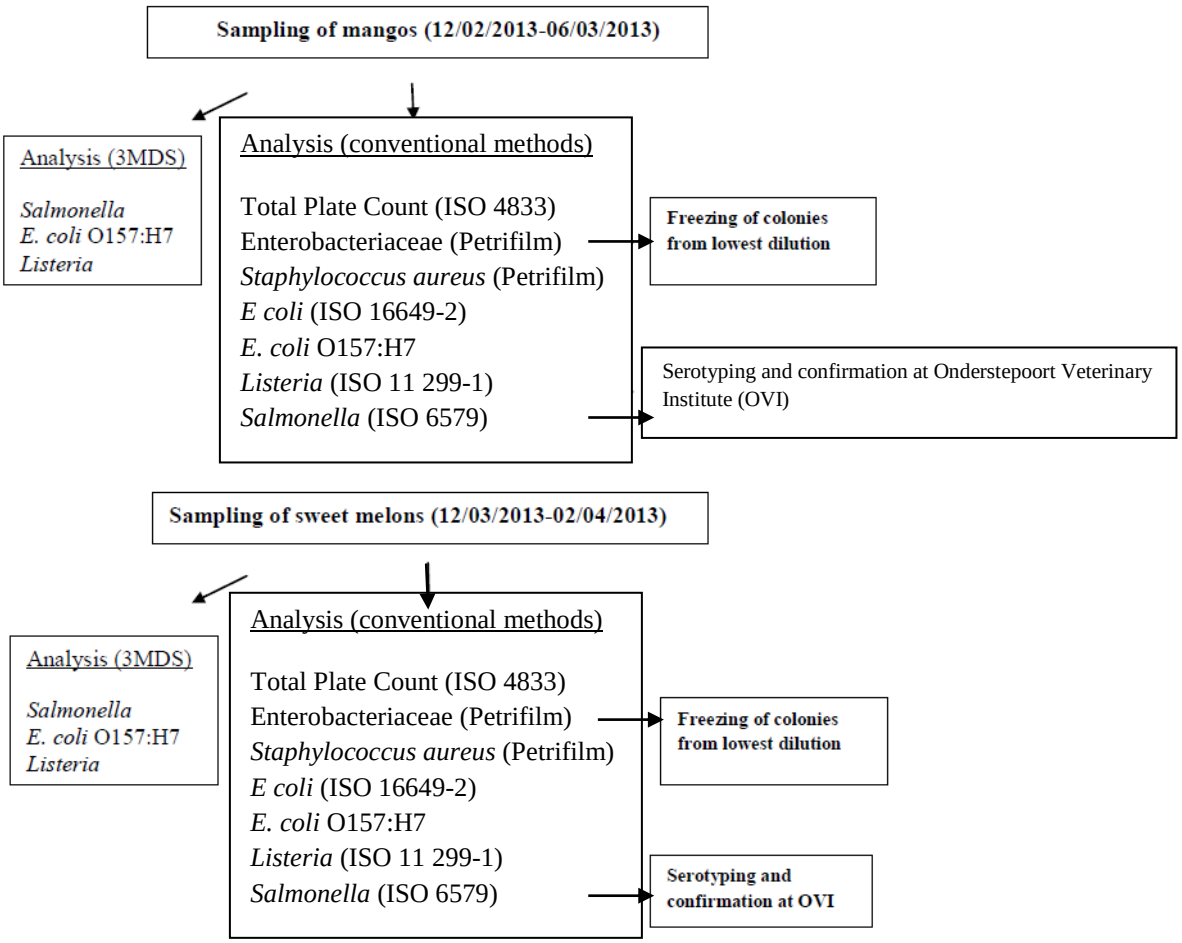


Figure 8: Phase 1 of experimental design showing bacterial analyses of fresh-cut mango and melon samples during minimal processing and storage

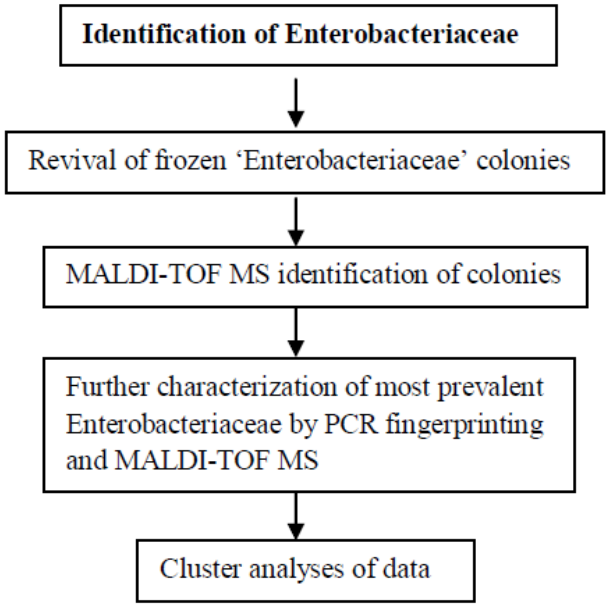


Figure 9: Phase 2 of experimental design showing identification and characterization of dominant Enterobacteriaceae on melon samples during minimal processing and storage

3. THE OCCURRENCE OF MICROBIAL HAZARDS AND SUCCESSION OF ENTEROBACTERIACEAE ON FRESH-CUT FRUIT DURING MINIMAL PROCESSING AND STORAGE AT REFRIGERATED TEMPERATURE

ABSTRACT

In this study, the microbial quality and safety of fresh-cut mangoes and sweet melons were investigated during minimal processing and storage. In addition, the succession of Enterobacteriaceae on sweet melons during minimal processing was studied. For either of mango and sweet melon, whole fruit (n=6), cut fruit (n=6) as well as packaged fruit at the beginning (n=6) and end of shelf-life (n=6) from a fresh fruit processing plant were sampled and analyzed. The samples were analyzed for total plate count (ISO 4833), Enterobacteriaceae (3M Petrifilm), yeast and moulds (3M Petrifilm), *E. coli* (ISO 16649-2), *E. coli* O157:H7 (ISO 16654), *Salmonella* spp (ISO 6579), *L. monocytogenes* (ISO 11290-1) and *S. aureus* (3M Petrifilm). The Enterobacteriaceae species were identified by MALDI-TOF MS and similar species were further characterized by PCR fingerprinting using the $(GTG)_5$ microsatelite and MALDI-TOF MS. The total average plate counts for the fresh-cut mangoes and fresh-cut melons increased significantly ($p<0.05$) during minimal processing and were $<10^6$ $\log_{10}$cfu/g and $<10^7$ $\log_{10}$cfu/g respectively. The average total plate count levels at the end of shelf-life of both fresh-cut fruit were higher than the levels specified in international guidelines. With regards to the pathogens, *S. aureus* was present on the packaged mango and packaged melon samples at the beginning and end of shelf-life. *E. coli* was identified as a dominant Enterobacteriaceae species on the packaged melons at the end of shelf-life, but was not detected during any other preceding processing stage. No *L. monocytogenes* or *Salmonella* spp isolates were detected. The most predominant species identified during minimal processing and storage was found to be *E. cloacae,* which comprised 40% of all identified Enterobacteriaceae isolates (n=90). Cluster analysis of a PCR fingerprint and MALDI-TOF MS profiles of several strains of this species obtained during various stages of processing showed that there is intragenetic variation amongst strains coming from different processing stages. This may suggest that there may be selection or adaption of some strains of *E. cloacae* with certain genotypic or phenotypic characteristics due to the changing conditions at the various minimal processing stages.

The higher levels of spoilage indicators and occurrence of more foodborne pathogens on fresh-cut melon demonstrates that it is more susceptible to microbial spoilage and foodborne pathogens than fresh-cut mango. In addition, it shows that the changing intrinsic (pH, water activity, nutrient content) and extrinsic (gaseous atmosphere composition, temperature, relative humidity) conditions that persist during minimal processing cause a change in the species composition of the Enterobacteriaceae.

3.2.1 INTRODUCTION

Consumers eat more fruit and vegetables as they are increasingly becoming aware of the health benefits associated with it (Abdias *et al.*, 2008). Consumers demand fresh-cut fruit products that are free from chemical presevatives (Zink, 2009). The market for chilled, fresh-cut fruit has shown an increase in recent years. Accordingly, the food industry has responded to this demand with new production practices, creative product development, innovative technology use and skillfull marketing initiatives (FAO, 2011a). Fresh-cut tropical fruit on the market today include melons, mangoes, cantaloupe, watermelon, mangoesteen, rambutan, jackfruit, pummelo, papaya, durian, grapefruit, pineapples and fruit mixes (FAO, 2011a). The increase of production has however been linked to an increase in foodborne diseases associated with the consumption of fresh-cut fruit and some very recent outbreaks involving fresh-cut melon have been reported (CAC, 2011).

Fresh-cut melon is seen as a high risk food as it can support growth of pathogens due to its low acidity (pH 5.2-6.7) and high water activity (0.97-0.99) (Adams & Moss, 2008). Recent outbreaks associated with the consumption of sweet melon have been reported (CAC, 2011). The pathogens associated with these outbreaks were *S.* Typhimurium, Norovirus, *E. coli* O157:H7, *C. jejuni*, *S. sonnei*, *L. monocytogenes*, *Cyclospora* spp and *S. aureus* (CAC, 2011). In 2012, a multistate outbreak of *S.* Braenderup linked to consumption of whole mangoes occurred (CDC, 2012a). Fresh-cut mango has not been associated with disease outbreaks, but a study that was recently conducted found fresh-cut mango bought from a retail store in South Africa to be contaminated with *Salmonella* spp (Harris, 2013). In addition, other studies have found that this pathogen can readily survive and grow within mango pulp (Bordini *et al.*, 2007; Soto *et al.*, 2007).

Exportation of fresh and fresh-cut fruit and vegetables play a crucial role in the South African economy. Outbreaks or even mere detection of foodborne pathogens such as *E. coli*, *Salmonella* spp and *L. monocytogenes* in fresh-cut fruit can cause massive recalls, causing local retailers to suffer great economic losses. In addition, exportation could potentially be affected if the exported produce was found to be of poor microbial quality (Tempelhoff, 2010). Regular microbiological examination and understanding of the risks involved with the production of fresh-cut mango and melon is important to avoid disease outbreaks as well as economic losses.

The Enterobacteriacae family mainly consists of species that are colonizers of the lower gasrointestinal tract such as *E. coli* and *E. faecalis* (Ryan & Drew, 2010). There are species such as *E. asburiae* and *Enterobacter amnigenus* (*E. amnigenus*) that are free-living in the environment and are associated with soil, plants, fruit and vegetables (Hart, 2006; Farmer, Boatwright, Janda, 2007). With regards to humans, all species are opportunisic pathogens and cause disease in immunocompromised individuals such as babies, the elderly or HIV positive individuals (Ryan & Drew, 2010). Two species, *Salmonella* spp and *E. coli* O157:H7, are considered to be significant foodborne pathogens and have been associated with disease outbreaks linked to fresh-cut melon and whole mangoes (CDC, 2012a; CAC, 2011). These two enteric pathogens are of particular concern due to their potential for growth on fresh-cut fruit prior to consumption as well as their low infectious dose (Beuchat, 2002; FDA, 2013).

This study aimed to determine the microbiological quality of fresh-cut sweet melons and mangoes during minimal processing including whole fruit, cut fruit, packaged fruit and packaged fruit at the end of shelf-life. In additon, the study investigated the change in population dynamics of the Enterobacteriaceae over the various stages of minimal processing. This was done to determine at which stages foodborne pathogenic members of the Enterobactericeae dominate and to study the interaction between different species of the Enterobacteriaceae. Fresh-cut sweet melons were chosen for this purpose as there is a higher risk for foodborne diseases to occur with fresh-cut melon than with fresh-cut mango consumption (CAC, 2011; CDC, 2011; CDC, 2012b).

3.2. MATERIALS AND METHODS

3.2.1 SAMPLING

Sampling at the processing plant

Sampling of mangoes ('Kent' cultivar) and melons ('Muskmelon' cultivar) was done three weeks for each fruit on a weekly basis at a minimally processing plant in South Africa. During each sampling session, samples were taken in duplicate at certain stages of the minimal processing procedure, including the unprocessed fruit, the cut fruit and the packaged fruit (n=8) (Fig 9). The unprocessed, cut and packaged fruit were transported under refrigerated conditions to the Department of Food Science at the University of Pretoria and analyzed on arrival. Two packaged fresh-cut products were also analyzed at the end of shelf-life. These were stored at 0°C for 5 days in the Department of Food Science before analysis. Figure 10 shows where samples were collected during minimal processing.

Figure 10: Blue arrows indicate the sampling stages during minimal processing and storage

Sampling at the retail store

In addition to the mango and melon samples that were collected and analyzed from the minimal processing plant, six samples of packaged fresh-cut mango (day 5) and fresh-cut melon (day 5) were purchased at a local store supplied by the same processing plant. All fresh-cut products had reached the end of shelf-life. After purchase, these samples were immediately transported to the Department of Food Science and analyzed on arrival.

SAMPLE PREPARATION FOR ANALYSES

Whole fruit

A whole mango was placed in a stomacher bag containing 225 ml of Buffered Peptone Water (3M). After shaking the bag, the mango was removed and the BPW (3M) was analyzed as described in section 3.2.2.3.

The melons were analyzed by swabbing the entire surface with a sterile swab. This swab was placed in BPW (3M) and analyzed as described in section 3.2.2.3.

Cut fruit

Analyses of the cut fruit were done by placing 25 g of the cut fruit in 225 ml of BPW (3M). The bag was then stomached and the filtrate was used for further analyses as described in section 3.2.2.3.

SWAB COLLECTION FROM MINIMAL PROCESSING OPERATING PERSONNEL

Swabs were taken from the hands of fresh fruit cutting personnel during each sampling session. During each sampling session, 100 cm² of the surface of the hands of three workers was swabbed using ReadiSwab^{TM} in 10 ml of letheen broth (International BioProducts, Bothell, WA). The swab tubes were immediately placed on ice, transported to the Department of Food Science and analyzed on arrival.

3.2.2 DETERMINATION OF PHYSIOCHEMICAL PARAMETERS

Both the temperature and the pH of the samples were measured at the time of analyses. The temperature of the samples was measured with Checktemp 1 (Hanna Instruments, Woonsocket, R1 USA).

The pH of the samples was measured using the 211 Microprocessor pH meter (Hanna Instruments).

3.2.3 BACTERIOLOGICAL DETERMINATION

Total plate count

Method ISO 4833 (International Organization for Standarization, 1991) was used for the enumneration of the aerobic colonies on plate count agar plates (Biolab, Wadewille, South Africa) which were incubated at 37°C for 48 h.

Enterobacteriaceae

Enterobacteriaceae were enumerated on 3M *Enterobacteriaceae* Petrifilm (3M, Maplewood, Minnesota, USA).

Yeast and moulds

Yeast and moulds were enumerated on 3M *Yeast and moulds* Petrifilm (3M)

Escherichia coli

E. coli was enumerated according to the method ISO 16649-2 (ISO, 2001) on TBX Agar plates (Oxoid) that were incubated at 44°C for 18-24 h.

Escherichia coli O157:H7

E. coli O157:H7 was isolated according to the method ISO 16654 (ISO, 2001) but without immunomagnetic separation because reagents were not available. Enrichment was done in Tryptone Soya broth (Oxoid) supplemented with Novobiocin and incubated at 41.5°C for 6 h. *E. coli* O157:H7 were then streaked onto CT-SMAC (Sorbitol Macconkey Agar supplemented with Cefixime and Tellurite) (Oxoid) and incubated at 37°C for 18-24 h. Presumptive *E. coli* O157:H7 colonies were transferred to Nutrient agar (Oxoid) and incubated at 37°C for 24 h. Confirmation of the presumptive *E. coli* O157:H7 isolates was done by MALDI-TOF MS analysis. In addition, these isolates were confirmed with serum agglutination tests. *E. coli* trivalent antiserum 1 (0111 + 055 + 026), 11 (086 + 0119 + 0127), 111(0125 + 0126 + 0128) and IV (0114 + 0124 + 0142) (Biorad) were used.

Salmonella spp

Salmonella spp was isolated according to the method ISO 6579 (ISO, 1993) but without the pre-enrichment step. Enrichment was done in Rappaport-Vassiliaids (RVS) enrichment broth (Oxoid) incubated at 42°C for 24 h. However, MKTT broth as specified by ISO 6579 was not used because reagents were not available. *Salmonella* spp were then isolated on Phenol red/Brilliant Green agar (Oxoid), XLD agar (Oxoid) and Brilliance *Salmonella* agar (Oxoid) and incubated at 35°C for 20-24 h. Presumptive *Salmonella* spp colonies were transferred to Nutrient agar (Oxoid) and incubated for 24 h at 37°C. Confirmation of the presumptive *Salmonella* spp was done at OVI, Pretoria, South Africa.

Listeria monocytogenes

L. monocytogenes was isolated according to the method ISO 11290-1 (ISO, 1998). Primary enricment was done in Half Frazer broth (Oxoid) followed by incubation at 30°C for 24 h.

Secondary enrichment was done in Full Frazer (Oxoid) at 35°C for 48 h. After incubation in the enrichment broth, loopfuls from both the Half Frazer and Full Frazer broth (Oxoid) were plated onto Oxford *Listeria* Selective agar and Palcam *Listeria* agar (Oxoid), respectively, and incubated anaerobically for 48 h.

Presumptive *L. monocytogenes* colonies were transferred to Nutrient agar (Oxoid) and incubated for 24 h at 37°C. Confirmation of the presumptive *L. monocytogenes* isolates was done by MALDI-TOF MS analysis.

Staphylococcus aureus

S. aureus was isolated on 3M *S. aureus* Petrifilm (3M) and confirmed with 3M Petrifilm Staph Express Discs 6493 (3M).

3.2.4 SAMPLING AND STORAGE OF ISOLATED ENTEROBACTERIACEAE

The Enterobacteriacae were obtained from melons during the various stages of minimal processing (see section 3.2.2.1). The dominant species during each processing step were obtained by selecting five colonies from the highest dilution. The colonies were stored in eppendorf tubes supplemented with 10% glycerol to preserve the bacterial cells.

3.2.5 MOLECULAR TYPING OF BACTERIAL PATHOGENS

3.2.5.1 MALDI-TOF IDENTIFICATION OF ENTEROBACTERIACEAE

Enterobacteriaceae isolates obtained in section 3.2.2.1 were analyzed by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Isolates were sub-cultured twice on plate count agar (Oxoid) for 24-48 hours. Purified colonies were picked with sterile toothpicks and placed on a specified spot on a ground-steel MALDI target plate (Sigma-Aldrich, USA) in duplicates and air-dried at room temperature. Samples were then overlaid with 1µlm of HCCA solution (α-cyano-4-hydroxycinnamic acid, 20 µg μl^{-1}), crystallized by air-drying at room temperature and directly screened by MALDI-TOF MS. The MALDI-TOF mass spectra were acquired on an AutoFlex III Smartbeam MALDI-TOF MS instrument (Bruker Daltonics) using the instruments pre-programmed Flex Control 3.0 software (Bruker Daltonics) which stipulated the following parameter settings: linear positive mode, 60Hz laser frequency, 20 kV acceleration voltage, 170 ns extraction delay and a 2000-20 137 m/z range.

For each sample spot (approximately 5 mm in diameter on the ground steel MALDI target plate), an average of 600 shots was delivered at one point, and the final spectrum was an average accumulation of all spectra gathered from at least six different points on a sample spot. The protein molecular detection limit was set in the medium range between 2,000 and 20,000 Da. To validate the accuracy of mass spectral data generated by the MALDI-TOF MS instrument, each batch of samples contains the Bruker bacterial standard (BTS) (Bruker Daltonics).

Identification was performed using the MALDI Biotyper 3.0 software (Bruker Daltonics) which compared the acquired spectra to reference spectra from MALDI Biotyper 2.0 library (Bruker Daltonics). Species identification was considered reliable, when the score calculated by the MALDI biotyper 2.0 exceeded 2, 000.

Creation of a MSP dendrogram to determine the appropriate relatedness of stains of the Enterobacteriaceae species was conducted using the Bruker Daltonics MALDI Biotyper 3.0 software (Bruker Daltonics). Comparative clustering of mass spectra was performed by the ClinProTools 2.1 software (Bruker Daltonics) to statistically evaluate MALDI-TOF mass spectra (Bruker Daltonics). Spectra were normalized and recalibrated, using the respective functionalities of the software.

3.2.5.2 **3M MOLECULAR DETECTION SYSTEM**

In addition to the conventional methods that were used, the 3M Molecular detection system (3M) was used to test all samples for the presence of *Salmonella* spp., *Listeria* spp or *E. coli* O157:H7. For this analyses, 24h 3M enrichment broths where used to determine the presence/absence of *E. coli* and *Salmonella* using the respective 3M MDS kits. The presence of *Listeria* was determined using the *Listeria* specific enrichment broths.

This initial 24 h enrichment step of the stomached fruit samples in 3M buffered peptone water promotes the growth of any *Salmonella* and *E.coli* initially present at low number in the sample and can be directly detected by 3M Molecular Detection System.After the 24 h incubation in the 3M PBW 1µl was transferred to 9µl of 3M *Listeria* enrichment broth and incubated at 37^{o}C for 24 hours, to be used for the *Listeria* detection kit. Twenty micro litres of each of the sample enrichment broths where pipetted into the 3M lysis tubes.

A 3M negative control solution provided in the assay kits was also pipetted into a lysis tube and was treated similar to the samples. The lysis tubes where incubated in a 3M heating block at 105°C for 15 minutes, whereafter the tubes where cooled down rapidly in a 3M prechilled freezer block for 10 minutes. For the amplification step, 20µl of each of the sample lysates where transferred into 3M reagent tubes.

3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 071202, July 2012) and 3M Molecular Detection Assay *E. coli* O157, including H7, (AOAC RI Certificate 071202, July 2012) as well as 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012). Samples were mixed gently by pipetting up and down for five times.

Twenty microlitres of the negative control lysate tubes were transferred to the 3M reagent control and to a reagent tube and were then mixed by pipetting as described previously. The tubes were loaded into the 3M Molecular Detection System and analysed according to the supplier instructions and results were recorded through the computer interphase.

3.2.5.3 PCR FINGERPRINTING OF *E. CLOACAE*

PCR OPTIMIZATION

Since the combination of the $(GTG)_5$ microsatelite primer (Whitesci, Brackenfell, Cape Town, South Africa) and platinum PCR supermix (Invitrogen, Carlsbad, California, USA) for DNA amplification of *E. cloacae* had never been used before, the PCR had to be optimized first. For this purpose, four different concentrations of extracted DNA from *E. cloacae* were amplified with different amounts of primer and DNA. The amount of mastermix and Dimethyl Sulfoxide (DMSO) stayed constant. For amplification, different cycling conditions were used to determine which combination gave the clearest bands during UV visualization of the gels. With regards to the PCR reagents, the mastermix always comprised 90% of the total reaction volume as per company specifications (Invitrogen).

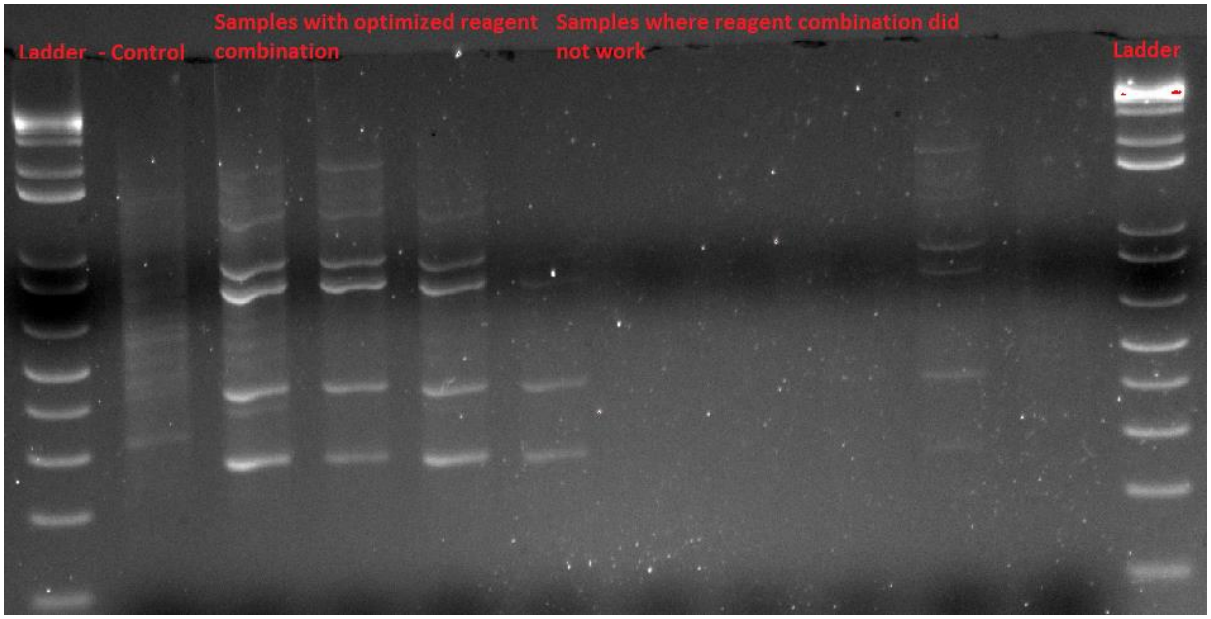


Figure 11: Optimization of PCR for *E. cloacae* (Lane 1, molecular marker; lane 2, negative control; lanes 3-6, optimized concentration of reagents; Lane 12, molecular marker)

DNA EXTRACTION

For DNA extraction, 24 h cultures of *E. cloacae* were used. DNA extraction was performed using a Purelink Genomic DNA Mini Kit (Invitrogen) and a Quick-gDNA MiniPrep kit (Zymo Research, Irvine, CA, USA). The concentration of the extracted DNA samples was measured with a Qubit® 2.0 Fluorometer (Invitrogen).

PCR CONDITIONS

PCR-fingerprinting was performed with $(GTG)_5$ microsatelite primers (WhiteSci) using the modified method from Mohapatra, Broersma, Mazumder (2007). The amplification reactions had lµl DMSO (4%), 1µl DNA, 0.35µl Primer (100µM) and 23 µl of 2X platinum PCR supermix (Invitrogen) to a final reaction volume of 25.35 µl. The PCR program utilized was as follows: 95°C for 5 minutes, followed by 34 cycles of 95°C for 30 s, 40°C for 60 s and 65°C for 3 minutes, with final extension at 65°C for 8 minutes. Finally, the tubes were held at 4°C. The amplification products were standard separated in 2% agarose gels in 1xTBE at 125V for 115 min. The gels were visualized under UV light in a Bio-Rad Gel Doc (Bio-Rad, Hercules, CA, USA).

PCR FINGERPRINT ANALYSIS

The resulting fingerprints were analyzed using the GelCompar II version 5.10 (Applied Maths, Saint-MartensLatem, Belgium) software package. The similarity among digitalized profiles was calculated using the Pearson correlation, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived from the profiles.

3.2.6 STATISTICAL ANALYSES

A single factor Analysis of variance (ANOVA) was conducted to determine if there was a significant increase in the total plate count, Enterobacteriaceae and pH respectively during processing using Statistica Software for Windows Version 7 (Tulsa, Oklohama, USA, 2003). For this analyses, a 95% confidence interval was used. The least significant difference test (LSD test) was used to determine the significant difference between the means of the total plate count, Enterobacteriaceae and pH at the different stages of minimal processing.

The least significant means and standard deviations were also calculated for the total plate count, Enterobacteriaceae and pH during each processing step. The LS means and standard deviations of the total plate count and Enterobacteriaceae was also calculated for the mango and melon samples that were analyzed at the end of shelf-life. Both these tests were performed using Statistica Software for Windows Version 7 (Tulsa, Oklohama, USA, 2003).

A t-test was performed to compare the total plate count, Enterobacteriaceae and pH of packaged mango and melon at the end of shelf-life stored at 5°C to the latter stored at 0°C using Statistica Software for Windows Version 7 (Tulsa, Oklohama, USA, 2003).

A single factor Analysis of varaince (ANOVA) was conducted to determine if there was a significant increase in the levels of *S. aureus* at the end of shelf life in both the mango and melon samples. For this analyses, a 95% confidence interval was used. Analyses were performed using Statistica Software for Windows Version 7 (Tulsa, Oklohama, USA, 2003).

3.3 RESULTS

3.3.1 MICROBIOLOGICAL QUALITY AND pH OF MANGOES AND MELONS OBTAINED FROM A MINIMAL PROCESSING PLANT AND RETAIL STORE

3.3.1.1 Mangoes

Mango samples obtained from a minimal processing plant

The average total plate count of the full, cut, packaged (day 0) and packaged (day 5) mango samples increased significantly ($p<0.05$) during minimal processing and storage and were 3.4 $\log_{10}$cfu/g, 3.4 $\log_{10}$cfu/g, 3.8 $\log_{10}$cfu/g and 4.5 $\log_{10}$cfu/g respectively. The average Enterobacteriaceae counts of the full, cut, packaged (day 0) and packaged (day 5) were 3.3 $\log_{10}$cfu/g, 3.6 $\log_{10}$cfu/g, 3.6 $\log_{10}$cfu/g and 4.3 $\log_{10}$cfu/g respectively. The average pH of the cut, full, packaged (day 0) and packaged (day 5) mango samples were 3.1, 3.4 and 4.3 respectively. Figure 12 shows the change in total plate count, Enterobacteriacae and pH over the various stages of minimal processing of mangoes.

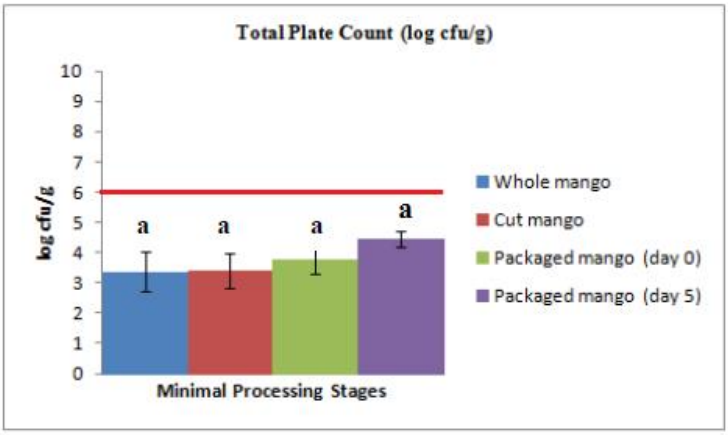
Mango samples obtained from a retail store

The average total plate count and Enterobacteriaceae count of the 6 packaged mango samples analyzed at the end of shelf-life were 5.3 $\log_{10}$cfu/g and 4.7 $\log_{10}$cfu/g respectively. The average pH for these samples was 4.0.

Swabs taken from minimal processing personnel

The average total plate count obtained from the hands of nine fresh-fruit minimal processing personnel was 102 cfu/100cm^2.

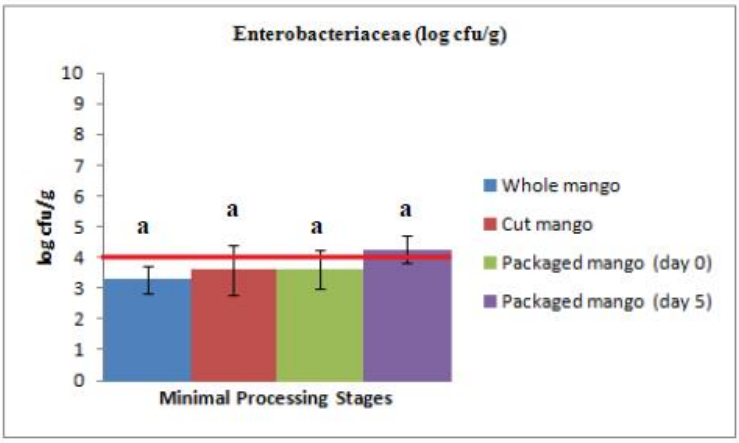
A)



ab ($p \leq 0.05$)

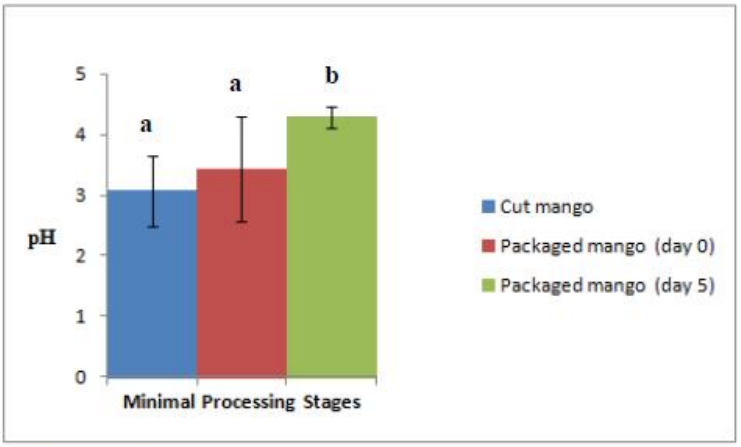
Total plate count should be below this level (Martin-Belloso *et al.*, 2006)

B)



Total plate count should be below this level (HPA, 2009)

C)



ab ($p \leq 0.001$)

Error bars on columns represent standard deviations
Average values with different subscripts differ significantly

Whole mangoes were stored at 26°C; Cut mangoes were stored at <12°C; Packaged mangoes (day 0) were stored at <12°C; Packaged mangoes (day 5) were stored at 0°C

Figure 12: Change in A) total plate count, B) Enterobacteriaceae, C) pH of whole (n=9), cut (n=9), packaged (day 0) (n=9) and packaged (day 5) (n=9) mangoes obtained from minimal processing plant and subsequent storage

3.3.1.2 Melons

Melon samples obtained from a minimal processing plant

The average total plate count of the full, cut, packaged (day 0) and packaged (day 5) melon samples increased significantly ($p<0.05$) and were 3.9 log_{10}cfu/g, 4.7 log_{10}cfu/g, 5.7 log_{10}cfu/g and 6.6$_{10}$log cfu/g respectively. The average Enterobacteriaceae count of the full, cut, packaged (day 0) and packaged (day 5) melon were 3.6 log_{10}cfu/g, 4.1 log_{10}cfu/g, 4.3 log_{10}cfu/g and 5.5 log_{10}cfu/g respectively. The average pH of the cut, packaged (day 0) and packaged (day 5) melon were 5.9, 5.9 and 6.5. Figure 13 shows the change in total plate count, Enterobacteriacae and pH over the various stages of minimal processing of melons.

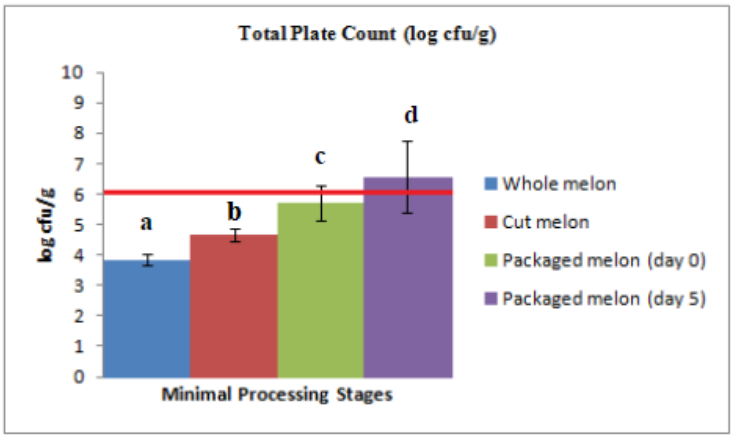
Melon samples obtained from a retail store

The average total plate count and Enterobacteriaceae count of the 6 packaged melon samples analyzed at the end of shelf-life were 5.5 log_{10}cfu/g and 5.1 log_{10}cfu/g respectively. The average pH for these samples was 6.4.

Swabs taken from minimal processing personnel

The average total plate count obtained from the hands of nine fresh-fruit minimal processing personnel was 184 cfu/100cm^2.

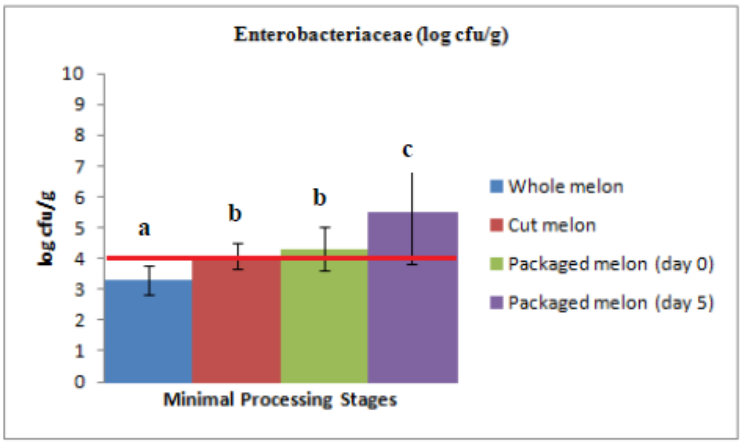
A)



(p≤0.001)

— Total plate count should be below this level (Martin-Belloso *et al.*, 2006)

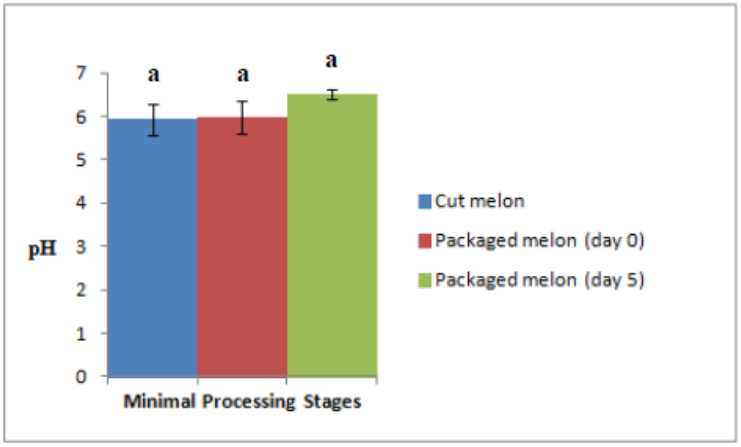
B)



(p≤0.001)

— Total plate count should be below this level (HPA, 2009)

C)



Error bars on columns represent standard deviations
Average values with different subscripts differ significantly

Whole melons were stored at 26°C; Cut melons were stored at <12°C; Packaged melons (day 0) were stored at <12°C; Packaged melons (day 5) were stored at 0°C

Figure 13: Change in A) total plate count, B) Enterobacteriaceae, C) pH of whole (n=9), cut (n=9), packaged (day 0) (n=9) and packaged (day 5) (n=9) melons obtained from a minimal processing plant and subsequent storage

3.3.2 OCCURRENCE OF MICROBIAL HAZARDS ON FRESH-CUT MANGOES AND MELONS OBTAINED FROM A PROCESSING PLANT AND RETAIL STORE

With regards to pathogens screened for in the mango and melon samples obtained from the minimal processing plant, no *E. coli*, *L. monocytogenes* or *Salmonella* spp were detected in any of the mango samples. Fifty percent of the mango samples tested positive for *S. aureus* with all positive samples coming from the packaged fresh-cut product. No *L. monocytogenes* or *Salmonella* spp were identified. In the melon samples, *E. coli* was detected in one melon sample at the end of shelf-life. Fifty-five percent of these samples tested positive for *S. aureus* with all samples coming from the packaged fresh-cut product. Table 8 shows the effect of refrigerated storage at 0°C for 5 days on the average level of *S. aureus* on fresh-cut mangoes and fresh-cut melons obtained from a processing plant.

With regards to pathogens screened for in the mango and melon samples at the end of shelf-life that were obtained from the retail store, no *E. coli*, *L. monocytogenes* or *Salmonella* spp were detected. *S. aureus* was detected in two of the melon samples with the average levels being 1, 60 log cfu/g. Table 9 shows the prevalence and levels of microbial hazards on fresh-cut mango and melon samples obtained from a processing plant and retail store.

Table 8: Effect of refrigerated storage at 0°C for 5 days on the average level of *S. aureus* on fresh-cut mangos (n=6) and melons (n=6) obtained from a processing plant

Samples	Average level of *S. aureus* (log cfu/g)	
Packaged mango	(day 0)	1.2±0.6 (0.5-1.2)[a]
	(day 5)	1.6±0.1 (1.4-1.7)[a]
Packaged mango	(day 0)	1.7±1.5 (1.5-1.8)[a]
	(day 5)	1.9±0.3 (1.5-2.1)[a]

Mean values with different superscripts in cells differ significantly ab ($p \leq 0.05$)

Table 9: Occurrence and levels of microbial hazards on fresh-cut mango (n=6) and melon samples obtained from a processing plant and retail store

	S. aureus		*E. coli*	
	Count (log cfu/g)	Occurrence (%)	Count (log cfu/g)	Occurrence (%)
Cut mango	-	-	-	-
Packaged mango (day 0)	1.2(0.7-1.2)±0.6	33	-	-
Packaged mango (day 5)°	1.6(1.4-1.7)±0.1	66	33	17
Packaged mango (day 5)*	-			
Cut melon	-	-	-	-
Packaged melon (day 0)	1.7(1.5-1.8)±1.5	66	-	-
Packaged melon (day 5)°	1.9(1.5-2.1)±0.3	66	-	-
Packaged melon (day 5)*	1.6(1.4-1.7)±0.1	33	-	-

°Samples obtained at the end of shelf-life from processing plant
***Samples obtained at the end of shelf-life from retail store**
-None detected
No *Salmonella* spp. was detected
No *Listeria* spp. was detected

3.2.3.3 CHANGE IN BACTERIAL COMPOSITION OF SWEET MELONS DURING VARIOUS STAGES OF MINIMAL PROCESSING AND STORAGE

A total of 9 genera and 15 species of the Enterobacteriaceae were identified over all the stages of minimal processing. The composition and diversity of species changed with each processing stage. Table 10 and Figure 14 shows the occurrence and change in composition and diversity of Enterobacteriaceae species during the course of minimal processing. Only two species including *E. cloacae* and *P. agglomerans* were identified from the whole melon. There was an increase in the diversity of species obtained from the whole to the cut melon. Five additional species including *Enterobacter aerogenes* (*E. aerogenes*), *E. asburiae*, *Enterobacter gergoviae* (*E. gergoviae*), *K. oxytoca* and *Leclercia adecarboxylata* (*L. adecarboxylata*) were identified.

The diversity also increased from the cut to the packaged (day 0) product. The highest diversity of Enterobacteriaceae species was found on the packaged (day 0) product. Six additional species including *Citrobacter braakii* (*C. braakii*), *C. freundii* (*C. freundii*), *E. amingenus*, *Raoutella ornithinolytica* (*R. ornithinolytica*) and *S. mascerens* were identified. There was a decrease in number of species obtained from the packaged (day 0) to the packaged (day 5) product. Two species, *E. coli* and *Pantoea anantis* (*P. anantis*), appeared only at the end of shelf-life and no other preceding stage of minimal processing. The most prevalent genera amongst all processing stages was *Enterobacter* spp. Amongst the *Enterobacter* species, *E. cloacae* was the most frequently obtained isolate, accounting for 78% of all *Enterobacter* species and 40% of all the identified Enterobacteriaceae species.

Table 10: Prevalence (%) of dominant Enterobacteriaceae isolates (n=90) isolated from whole, cut, packaged (day 0) and packaged (day 5) melons collected from a fresh fruit processing plant over a period of three weeks

Week	Week 1				Week 2				Week 3				
	WM	CM	PM	PM*	WM	CM	PM	PM*	WM	CM	PM	PM*	Total number of isloates
Sample percentage isolates													
Citrobacter braaki	-	-	10	-	-	-	-	-	-	-	-	-	1
Citrobacter freundii	-	-	80	100	-	-	-	-	-	-	-	-	12
Enterobacter aerogenes	-	33	-	-	-	23	-	-	-	-	-	-	6
Enterobacter amnigenus	-	-	-	-	-	-	10	-	-	-	-	-	1
Enterobacter asburiae	45	22	-	-	33	-	10	12	-	-	50	-	1
Enterobacter cloacae	**60**	**33**	**36**	-	**100**	**46**	**27**	-	**100**	**75**	**100**	**100**	**36**
Enterobacter gergoviae	-	22	-	-	-	-	10	-	-	-	50	-	4
Enterococcus faecium	-	-	-	-	-	-	10	-	-	-	-	-	1
Escherichia coli	-	-	-	-	-	-	-	29	-	-	-	-	4
Klebsiella oxytoca	-	-	-	-	-	-	-	-	-	25	-	-	1
Leclercia adecarboxylata	-	-	-	-	-	31	27	-	-	-	-	-	7
Raotella ornithinolytica	-	-	-	-	-	-	10	-	-	-	-	-	1
Serratia marcescens	-	-	10	-	-	-	-	-	-	-	-	-	1
Panotea agglomerans	20	-	-	-	33	-	-	29	-	-	-	-	6
Panotea anatis	20	-	-	-	-	-	-	40	-	-	-	-	8
Total number of isolates	10	9	10	4	4	13	11	14	4	4	3	4	90
ACC (log_{10} cfu/g)	3.56	4.83	5.2	7.16	3.72	4.42	5.05	7.14	3.97	5	6.04	7.5	

ACC-Aerobic colony count; WM-Whole melon; CM-Cut melon; PM-Packaged melon; PM*-Packaged melon (day 5); - None detected

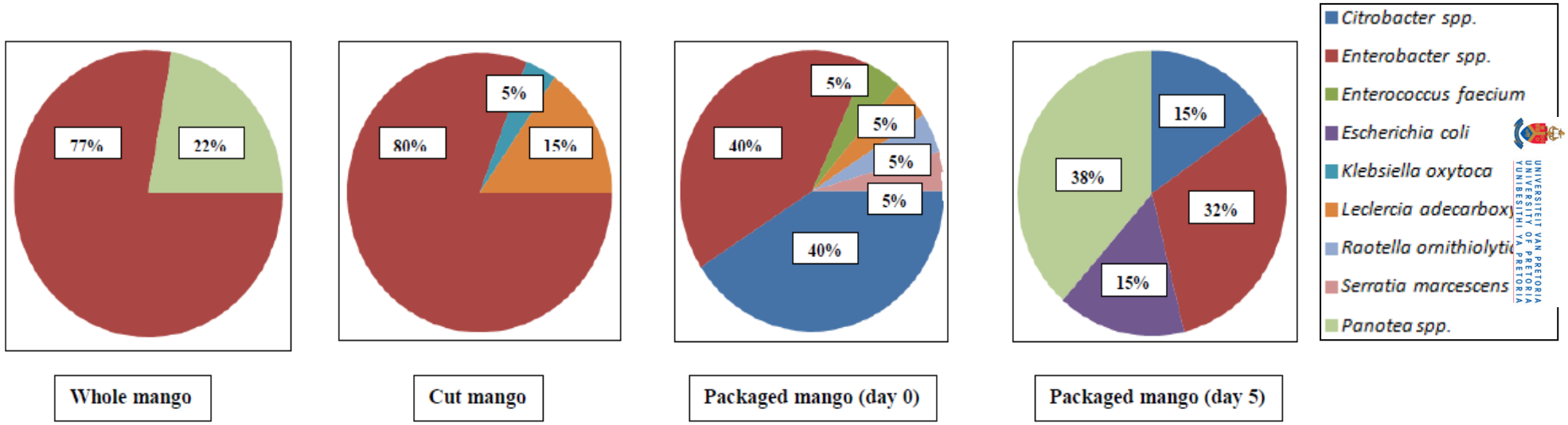


Figure 14: Prevalence (%) of dominant Enterobacteriaceae isolates (n=90) isolated from whole, cut, packaged (day 0) and packaged (day 5) melons collected from a fresh fruit processing plant over a period of three weeks

3.3.4 PCR FINGERPRINT AND MALDI-TOF DENDROGRAM OF *E. CLOACAE* STRAINS TO SHOW VARIABILITY OVER VARIOUS STAGES OF MINIMAL PROCESSING OF MELONS

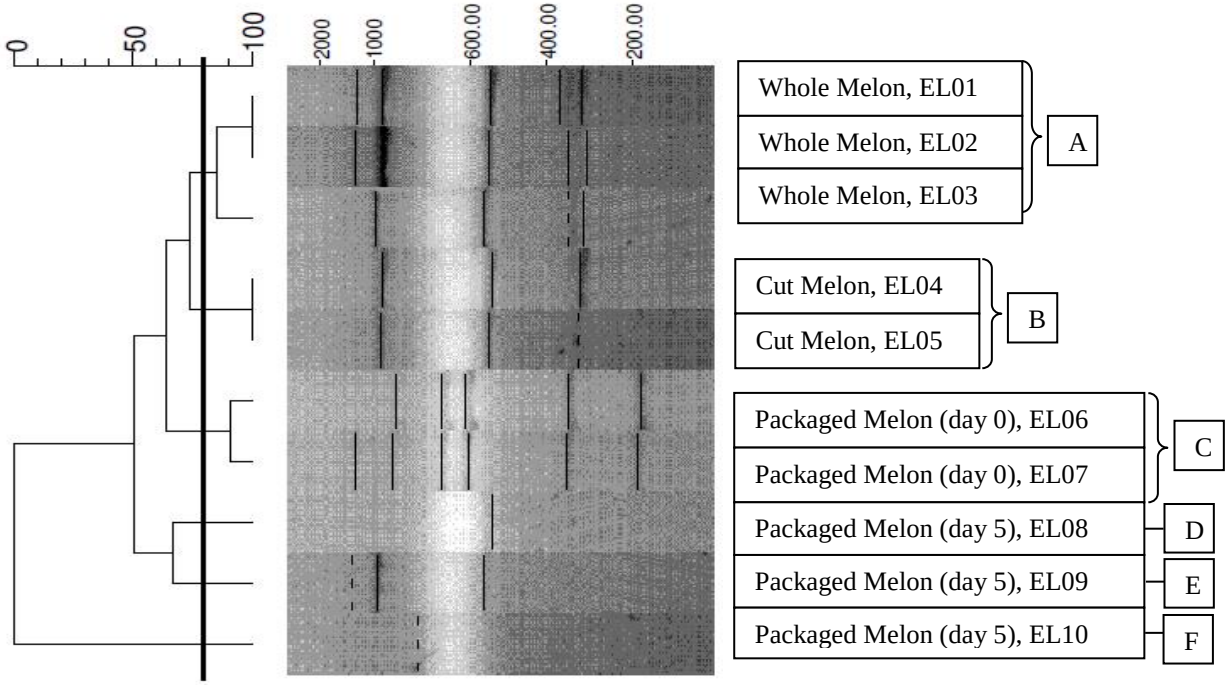


Figure 15: Dendrogram based on cluster analysis of the $(GTG)_5$-PCR fingerprints of *E. cloacae* isolates obtained from various stages of minimal processing

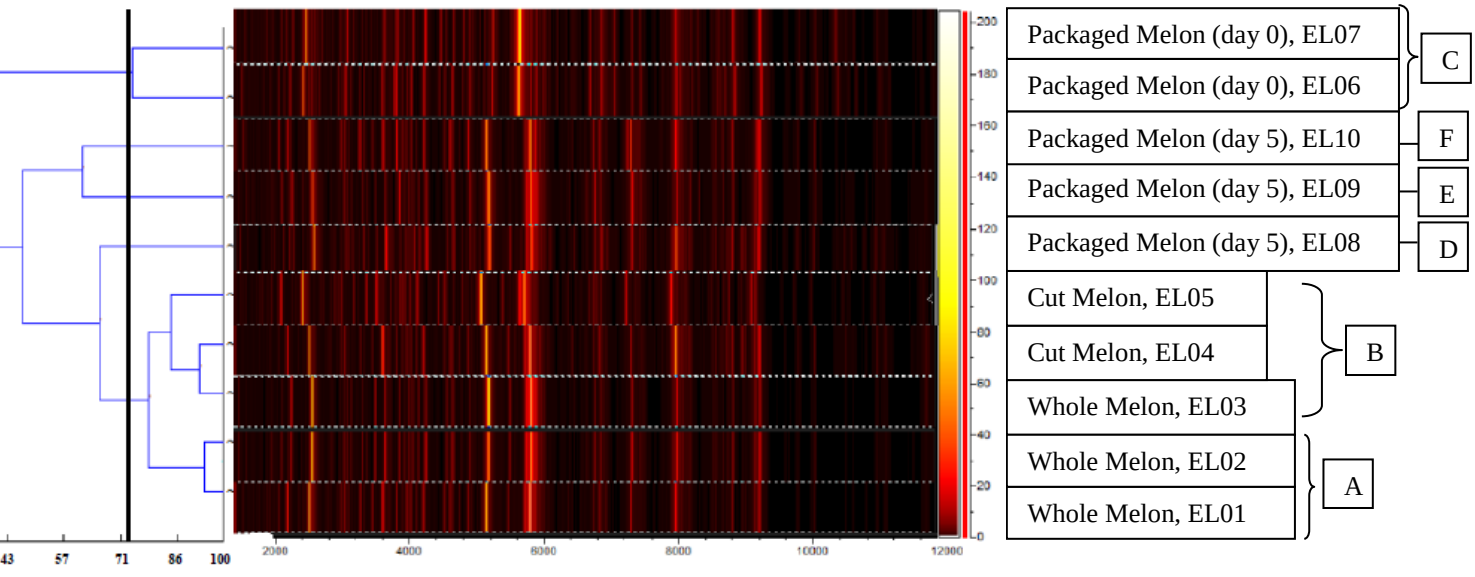


Figure 16: Two-dimensional Unsupervised hierarchical cluster analysis of 12 *E. cloacae* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

Cluster analyses were performed on the $(GTG)_5$ patterns followed by the formation of a dendrogram (Figure 14). Two distinct clusters, cluster 1 and cluster 2, could be observed. Within cluster A, six different clusters could be observed. Isolates with > 80% similarity were regarded as similar strains. This yielded 6 groups, A-F. Group A consisted of 3 strains that were closely similar, with all isolates coming from the whole melon. Group B consisted of two strains that were identical, with both isolates coming from the cut melon. Group C consisted of two strains which were closely similar (85%) with both strains coming from the packaged melon. Group D, E and F consisted of a single strain that was isolated from the packaged melon at the end of shelf-life.

The molecular profile acted as a benchmanrk for determination of similarity between *E. cloacae* MALDI-TOF MS profiles. When this was considered, isolates greater than 72% were regarded as similar groups. Compared to the dendrogram obtained from the PCR fingerprint, similar groupings of *E. cloacae* were observed in the dendrogram generated from the MALDI-TOF MS profiles. The dendrogram of the MALDI-TOF MS profiles yielded six groups, A-F. Group A consisted of 2 strains that were closely similar, with both isolated from the whole melon. Group B consisted of 3 strains with one strain originating from the whole melon and two strains from the cut melon. Group C consisted of 2 strains both coming from the packaged (day 0) melon. Group D, E and F consisted of a single strain that was isolated from the packaged melon at the end of shelf-life. Figure 15 shows the generated dendrogram and associated mass spectrometry profiles of the different *E. cloacae* isolates.

3.3.5 MALDI-TOF DENDROGRAMS OF ENTEROBACTERIACEAE STRAINS TO SHOW VARIABILITY OVER VARIOUS STAGES OF MINIMAL PROCESSING OF MELONS

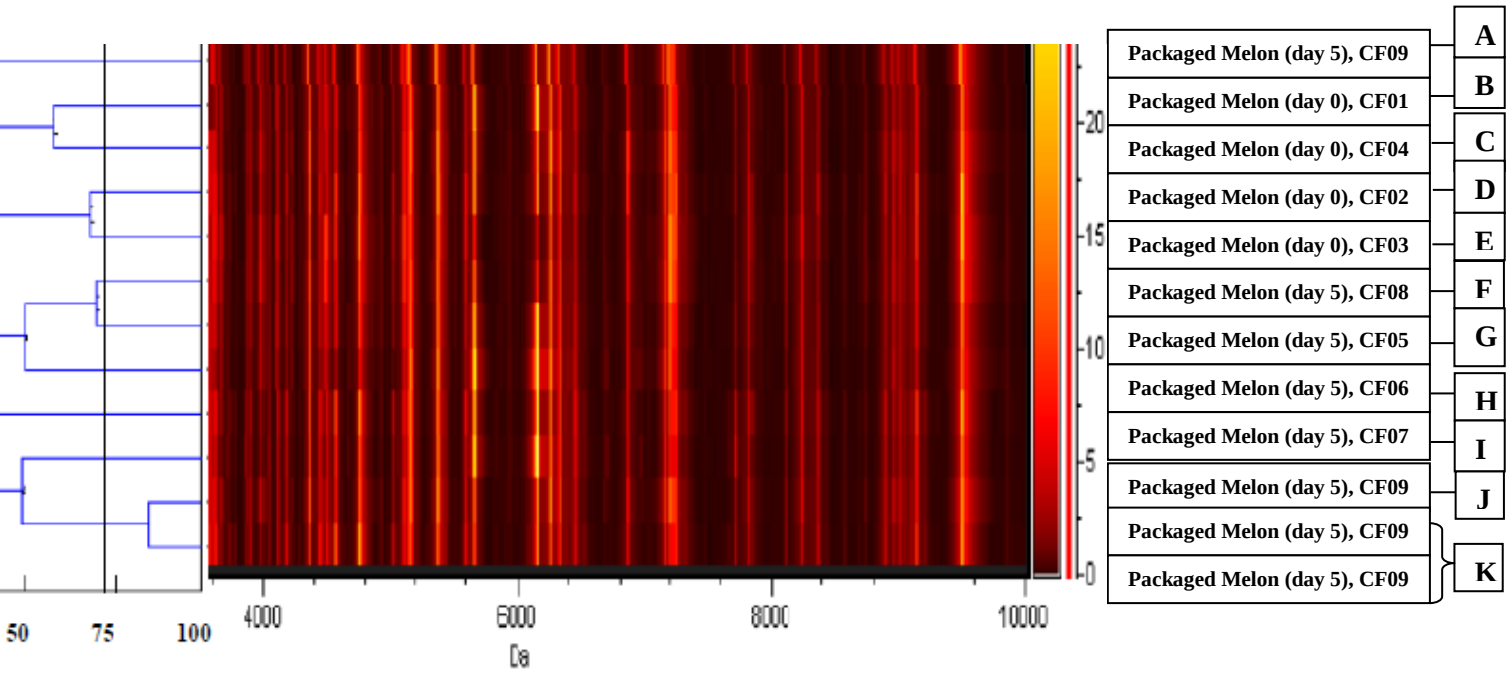


Figure 17: Two-dimensional Unsupervised hierarchical cluster analysis of 9 *C. freundii* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

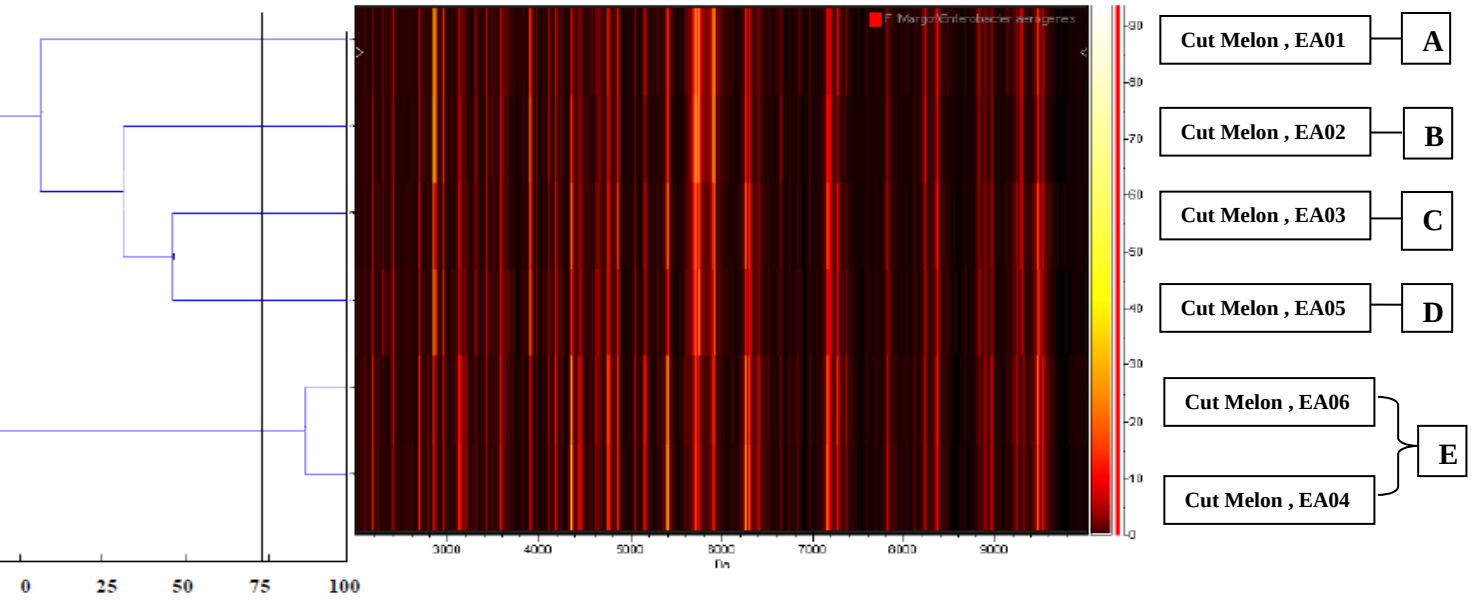


Figure 18: Two-dimensional Unsupervised hierarchical cluster analysis of 6 *E. aerogenes* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

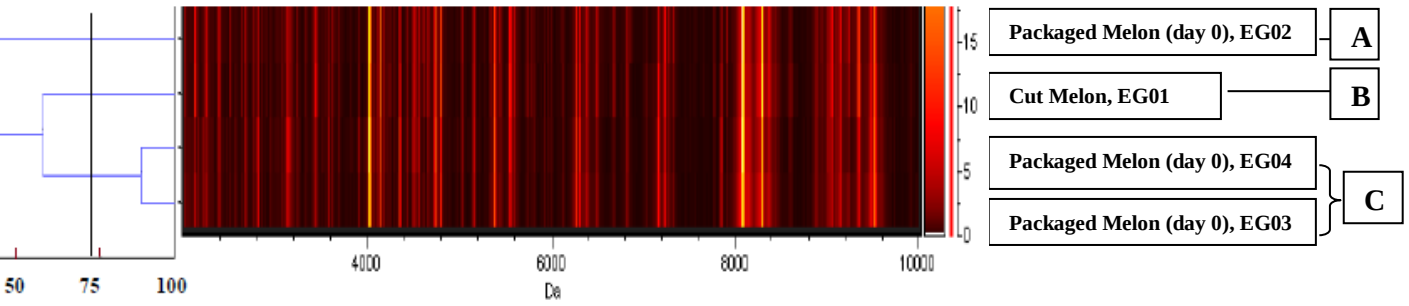


Figure 19: Two-dimensional Unsupervised hierarchical cluster analysis of 4 *E. gergoviae* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

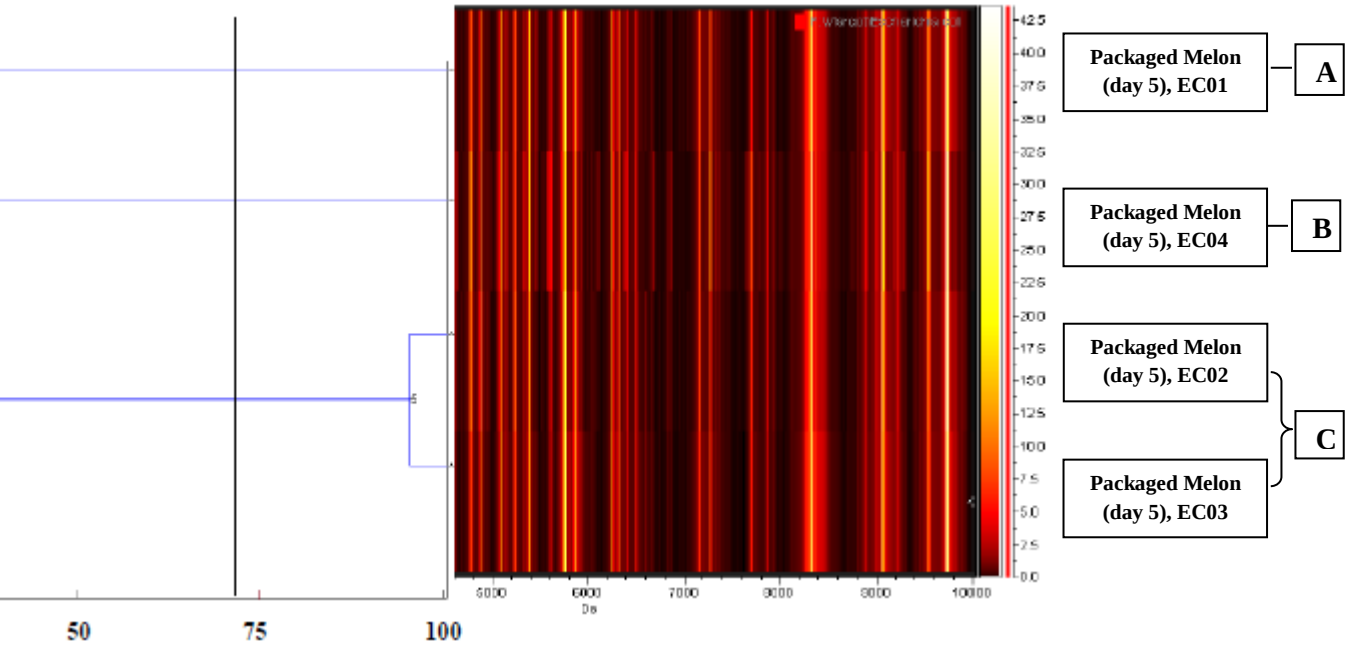


Figure 20: Two-dimensional Unsupervised hierarchical cluster analysis of 4 *E. coli* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

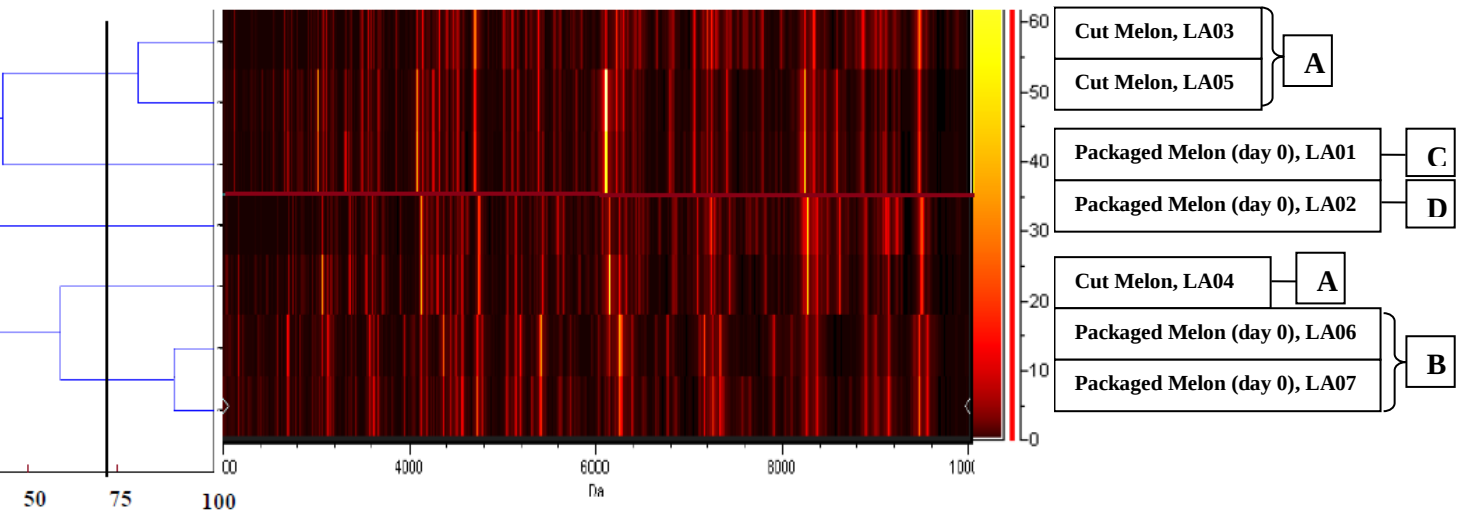


Figure 21: Two-dimensional Unsupervised hierarchical cluster analysis of 7 *L. adecarboxylata* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

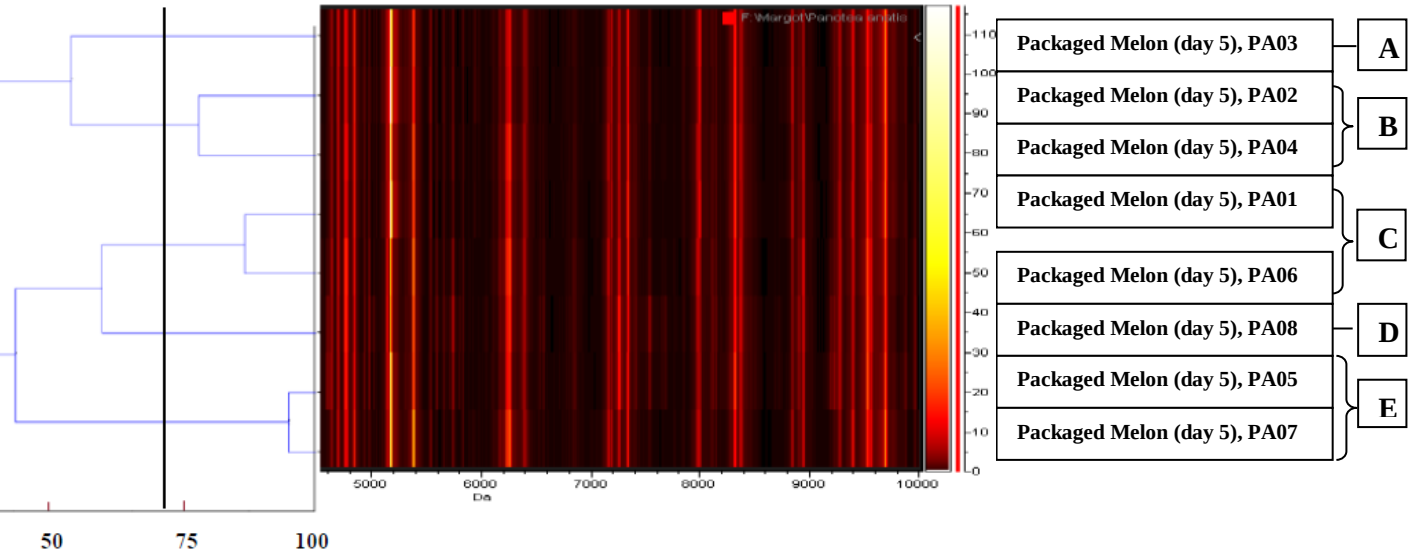


Figure 22: Two-dimensional Unsupervised hierarchical cluster analysis of 8 *P. ananatis* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles

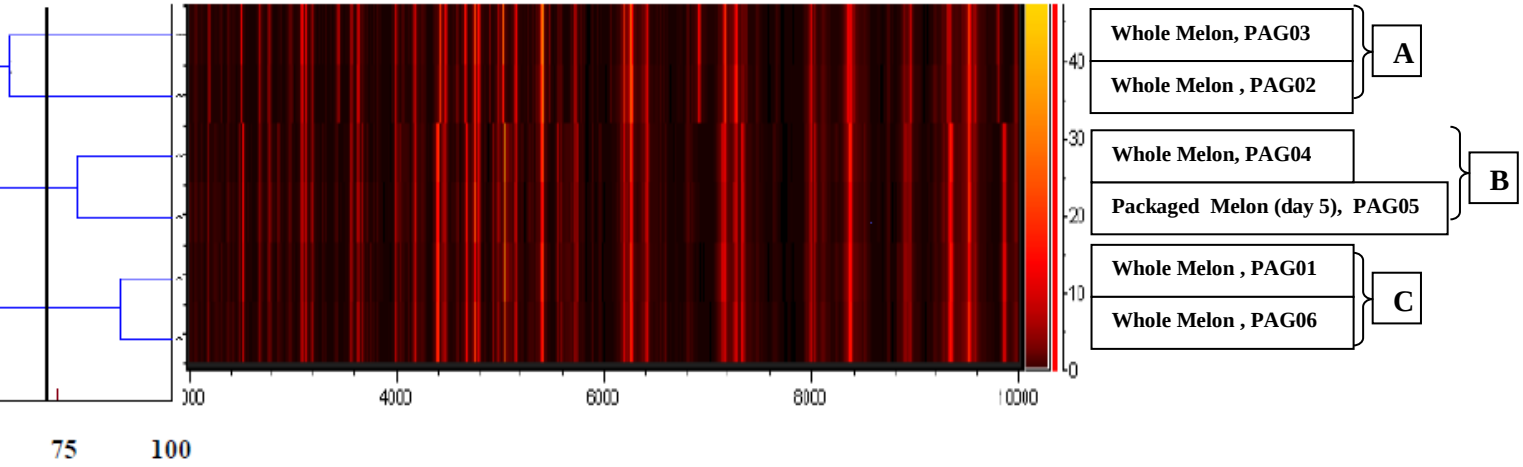


Figure 23: Two-dimensional Unsupervised hierarchical cluster analysis of 6 *P. agglomerans* isolates (506 spectra) versus 100 m/z values (rows) dendrogram and associated chromatic gel view of the mass spectra profiles.

Citrobacter freundii

For the cluster analysis of *C. freundii* strains, two distinct clusters, cluster 1 and cluster 2, could be observed. Within cluster 1, nine different clusters could be observed. Within cluster 2, five different clusters could be observed. Isolates with >72% similarity were regarded as similar strains. This yielded 11 groups, A-K. Group A-H consisted of a single strain with all isolates coming from the packaged melon (day 0). Group I and J consisted of a single strain with both isolates originating from the packaged melon at the end of shelf-life. Group K consisted of two strains that were closely similar (80%) with both isolates coming from the packaged melon at the end of shelf-life (Figure 17).

Enterobacter aerogenes

For the cluster analysis of *E. aerogenes* strains, two distinct clusters, cluster 1 and cluster 2 could be observed. Within cluster 1, three different clusters could be observed. Isolates with >72% similarity were regarded as similar strains. This yielded 5 groups, A-E with all groups originating from the cut melon. Group A, B, C and D consisted of a single strain. Group D consisted of two strains which were closely similar (80%) (Figure 18).

Enterobacter gergoviae

For the cluster analysis of *E. gergoviae* strains, two distinct clusters, cluster 1 and cluster 2 could be observed. Within cluster 2, two clusters could be observed. Isolates with >72% similarity were regarded as similar strains. This yielded 3 groups A-C. Group A consisted of a single strain that was obtained from the packaged melon (day 0).

Group B consisted of a single strain that was obtained from the cut melon. Group C consisted of two strains that were closely similar (80%) with both strains originating from the packaged melon (Figure 19).

E. coli

For the cluster analysis of *E. coli* strains, two distinct clusters, cluster 1 and cluster 2 could be observed. Within cluster 2, two clusters could be observed. Isolates with >72% similarity were regarded as similar strains. This yielded 3 groups A-C. Group A and B consisted of a single strain both of which were obtained from the packaged melon at the end of shelf-life. Group C consisted of two strains that were closely similar (80%) with both strains originating from the packaged melon at the end of shelf-life (Figure 20).

Leclercia adecarboxylata

For the cluster analysis of *L. adecarboxylata* strains, two distinct clusters, cluster 1 and cluster 2 could be observed. Within cluster 1, three clusters could be observed. Within cluster 2, four clusters could be observed. Isolates with >72% similarity were regarded as similar strains. This yielded 5 groups, A-E. Group A consisted of two strains that were closely similar (80%) with both strains coming from the cut melon. Groups B and C consisted of a single strain with both isolates coming from the packaged melon (day 0). Group D consisted of a single strain obtained from the cut melon. Group E consisted of two strains that were closely similar (80%) with both strains originating from the packaged melon (Figure 21).

Pantoea ananatis

For the cluster analysis of *P. ananatis* strains, of two distinct clusters, cluster 1 and cluster 2 could be observed. Within cluster 1, three clusters could be observed. Within cluster 2, four clusters could be observed. Isolates with >72% similarity were regarded as similar strains.

This yielded 5 groups, A-E with all groups originating from the packaged melon at the end of shelf-life. Group A consisted of a single strain. Group B consisted of two strains that were closely similar (78%). Group C consisted of two strains that were closely similar (85%). Group D consisted of a single strain. Group E consisted of two strains that were closely similar (95%) (Figure 22).

Pantoea agglomerans

For the cluster analysis of *P. agglomerans* strains, two distinct clusters, cluster 1 and cluster 2 could be observed. Within cluster 2, three clusters could be observed. Isolates with >72% similarity were regarded as similar strains. This yielded 4 groups, A-D. Group A and B consisted of a single strain both of which were obtained from the whole melon. Group C consisted of two strains that were closely similar (78%) with one strain originating from the whole melon and the other from the packaged melon. Group D consisted of two strains that were closely similar (90%), with both originating from the whole melon (Figure 23).

3.4 DISCUSSION

The current study set out to determine the microbiological quality and safety of fresh-cut mangoes and melons collected from a fresh fruit processing plant during all stages of minimal processing and during subsequent storage at refrigerated conditions. Similar levels of total plate count and significant increase thereof during storage of fresh-cut packaged mango and melon over a period of 5 days at <8°C has been demonstrated by other studies (Chien *et al.*, 2007; Munira, Rosnah, Zaulia, Russly, 2013; Silveira, Aguayo, Artes, 2013). The mean total plate count of the packaged mango at the end of shelf-life is acceptable for consumption but unacceptable for the packaged melon because the microbial guidelines for food safety state that the aerobic colony count for ready-to eat-food should be $<10^6$ cfu/g (Martin-Belloso, Soliva-Fortuny, Oms-Olimu, 2006).

The level of Enterobacteriaceae that was found in all fruit samples analyzed corresponds to previous studies in which the level of Enterobacteriaceae on fresh-cut mango and sweet melon was analyzed (Abdias *et al.*, 2008; Silveira *et al.*, 2013). The mean Enterobacteriaceae count of the packaged fresh-cut mango and melon samples at the end of shelf-life is unacceptable for consumption because the microbial guidelines for food safety state that the Enterobacteriaceae count for ready-to eat-food should be $<10^4$ cfu/g (New Zealand Food Safety Authority, 2005; Health Protection Agency, 2009). The high total plate count above guidelines coupled with the high varibility in Enterobacteriaceae count even beyond guidelines indicates inconsistent process controls in the food safety management system in both the production of fresh-cut mangoes and melons.

The significant difference in total plate count and Enterobacteriaceae count between the mangoes and the melons during all stages of minimal processing was probably due to the difference in pH noted for both fruit. In contrast to sweet melon which has amongst the most alkaline pH values amongst fruit (Adams & Moss, 2008), mango is considered an acidic fruit ($pH \leq 4.4$) for which microbial growth is known to be slow (Panagiotis & Nychas, 2011). In the mango samples, the pH ranged between 3.08 and 4.30 which is characteristic for the 'Kent' cultivar (Dea, Brecht, Cecilia, Baldwin, 2010) and increased during processing which corresponds to findings in the literature (Robbani, Sharfuddin, Rabbani, 1996).

In the melon samples, the average pH was one unit greater than that of the mangoes and ranged between 5.94 and 6.50 which is has also been shown for the 'Muskmelon' cultivar (Parveen *et al.*, 2012). It also increased during processing which is similar to the literature (Munira *et al.*, 2013). The increase in pH of the fresh-cut mango and melon samples during storage may have been due to physiochemical changes. When fresh-cut fruit is stored, the same physiochemical reactions occur as during fruit ripening but only at a faster rate. Organic acids, such as malic and citric acids that are present in the fruit are utilized for respiration leading to an increase in pH (Jha *et al.*, 2010).

In both the mango and melon samples, there was an increase in the mean total plate count between the whole fruit and the cut fruit even though there is a critical control point (CCP) involving washing of the whole fruit with chlorinated water (37-50 ppm) with a pH of 7 and a temperature of 20°C for a contact time less than 5 minutes. Since this is one of only two critical control points with regards to microbial safety in the minimal processing procedure, a reduction in total aerobic colony count was expected. Generally, chlorine washes at permitted concentrations ranging between 50-200 ppm with contact times less than 5 minutes give total plate count reductions between 0-3 log cfu/g (Klaiber, Baur, Wolf, Hammes, 2005; Lavelli, Pagliarini, Ambrosoli, Minati, Zanoni, 2005; Nou & Lou, 2010) but are incapable of completely removing or inactivating microorganisms on fresh produce (Abdias *et al.*, 2008).

It has been demonstrated that the survival of microbial populations during surface decontamination of mango and melon populations is enhanced due to protection of microbial cells that are buried within the stem end or scar (Beuchat, 1998; Ngarmsak, Delaquis, Tiovonen, Tipvanna, Buncha, 2006). According to literature, there is an inconsistency in the results obtained from testing the efficiency of chlorine for total plate count reduction and pathogen inactivation (Har & Pereira, 2013).

The reason why the chlorine wash had a greater effect on reducing the microbial population on the mangoes than on the sweet melons may be due to the differences in physical and chemical properties of the fruit surfaces. Sweet melons have a rough, netted surface with vein tracts (Gerchikov *et al.*, 2008; Parveen *et al.*, 2012) which encourages film attachment of bacterial cells and shields them from sanitizing agents (Har & Pereira, 2013).

In contrast, mangoes have a waxy, smooth surface, which is less suited for microbial attachment (Fernandes *et al.*, 2014). Therefore, loose bacterial cells are easier removed from mangoes than from melons resulting in higher population reductions (Har & Pereira, 2013).

(Har & Pereira, 2013) demonstrated that a chlorine wash with a concentration of 200 ppm at pH of 7 and a temperature of 20°C for a contact time less than 5 minutes reduces *S. aureus* and *E. coli* populations present on tropical fruits with less than l $\log_{10}$cfu/g and are incapable of completely removing or inactivating these pathogens. Since a concentration of only 37-50 ppm was used in this particular study, these identified pathogens could therefore have been present on the whole unprocessed fruit, survived the chlorine wash and were subsequently transferred to the finished fresh-cut product during the processing operation.

The significant difference in both total plate count and Enterobacteriaceae count between the packaged melon and packaged mango samples stored at 0°C and those stored at 5°C at the end of shelf-life is probably due to the difference in storage temperature. Lower temperatures are known to slow down or completely inhibit growth. The majority of mesophilic microbes are still able to proliferate at 5°C but below this temperature growth is generally slow or completely inhibited (Huang, Hwang, Phillips, 2011).

The levels of *S. aureus* that were detected on all analyzed packaged mango and melon samples at the end of shelf-life are acceptable according to the requirements stated by the guidelines of microbiological analyses for ready-to-eat food, being between 20-<100 cfu/g^{-1} (CFS, 2007; NZFSA, 2005). The presence and survival of this pathogen in these fruit is probably due to its ability to grow over an extensive range of environmental conditions. It can grow at 6°C (Schelin, Wallin-Carlquist, Cohn, Lindqvist, Barker, Radstrom, 2011) which falls within the temperature range at which fresh-cut fruit is stored and a pH range of 4-10 (Schelin *et al.*, 2011) which has been determined to be the range of the analyzed mango and melon samples. In a study assessing the microbiological quality of whole fruit which included melons, *S. aureus* was found to be the most prevalent species (Eni, Oluwawemitan, Solomon, 2010) which indicated that this pathogen can survive and possibly grow on fruit surfaces. In both the mango and melon samples, the levels of *S. aureus* did not increase significantly from the time the fruit was packaged to the end of the shelf-life. This is probably due to the fact that *S. aureus* is considered to be a poor competitor of the native microflora of foods (Panagiotis & Nychas, 2011).

Even though the levels of this pathogen adhere to the microbiological guidelines and it did not increase significantly during storage, the presence of *S. aureus* in these products is a concern due to its ability to grow and produce staphylococcal enterotoxins over an extensive range of environmental conditions, even if these are not favourable for its growth (Panagiotis & Nychas, 2011; Schelin *et al.*, 2011). Only 100 ng of enterotoxin produced by this pathogen is required to cause staphylococcal food poisoning (Asao, Kumeda, Kawai, Shibata, Oda, Haruki, 2003). *S. aureus* associated with fresh-cut sweet melons has been has been reported as being responsible for causing disease outbreaks (CAC, 2011). No reports of disease outbreaks with the consumption of mangoes associated with *S. aureus* could be found.

The identification of *E. coli* at the end of shelf-life is a concern as microbial guidelines specify that it should not be present (HPA, 2009). *E. coli* is considered to be a significant foodborne pathogen being the aetological agent responsible for most disease outbreaks linked to fresh and fresh-cut produce (FDA, 2013). This pathogen is a particular threat in the food industry due to its potential for growth prior to consumption and low infectious dose (Beuchat, 2002; FDA, 2013). *E. coli* associated with fresh-cut sweet melons has been has been reported as being responsible for causing disease outbreaks (CAC, 2011). No disease outbreaks associated with the consumption of mangoes contaminated with *E. coli* has been reported. The presence of *E. coli* indicates that there must have been faecal contamination along the minimal processing line (Martinez, Pachepsky, Shelton, Whelan, Zepp, Molina, Panhorst, 2013). *E. coli* was only detected at the end of shelf-life and not at any other preceding processing stage which implies that it may have been present in the earlier stages of minimal processing, but below detection levels. The detection of *Salmonella* spp at the end of shelf-life and no other preceding processing stage in fresh-cut lettuce has been demonstrated by others (Perry-Hanson & Buys, 2010). In both cases, the pathogens must have been present in preceding processing stages. They were probably not detected then because their levels were too low. The intrinsic (pH, Aw, nutrient availability) and extrinsic (gaseous atmosphere, relative humidity, temperature) conditions during subsequent processing stages became more favourable for their growth and after storage. This led to their proliferation to detectable levels at the end of shelf-life.

All bacteria isolated in this study have previously been isolated from fruits and vegetables in other studies (Dunn, Hall, Altamirano, Dietrich, Robinson-Dunn, Johnston, 1995; Adebolu & Ifesan, 2001; Tambekar & Mundhada, 2006; Olayemi, 2007; Uzeh, Alade, Bankole, 2009). The identification/isolation of non-faecal coliforms such as *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp and *Pantoea* spp from the mango and melon samples, which had formed presumptive colonies on chromogenic agars, is not of public health concern as they exist commonly in water, soil and vegetation and have long been recognized in fresh fruit and vegetables (Soranio *et al.*, 2000; Johannessen *et al.*, 2002).

In a study that was conducted to examine the microbiological quality of apples in the European Union (EU), strains of *Citrobacter* spp, *Enterobacter* spp and *Klebsiella* spp were isolated (Abdias *et al.*, 2006). Similarly, a study which aimed to determine the bacteriological quality of a variety of fruit and vegetables showed that *Citrobacter* spp, *Enterobacter* spp and *Klebsiella* spp were most pre-dominant non-faecal coliforms identified (Johannessen *et al.*, 2002).

Ngarmsak, Delaquis, Tiovonen, Ngarmsak, Ooraikul, Mazza (2006) identified *P. agglomerans* as a dominant bacterial species from fresh-cut mango. O'Connor-Shaw *et al.* (1995) conducted a study to identify the Enterobacteriaceae on processed mango and obtained thirty-four isolates of *K. pneumoniae*, followed by seven isolates of *E. cloacae* and two isolates of *K. oxytoca* and *P. agglomerans.* The high incidence and prevalence of *K. pneumoniae* on fresh fruit and vegetables, is due to its capability to metabolize certain polyalcohols and to fix nitrogen (Temme, Zhao, Voigt, 2011). Rao & Rao (1983) demonstrated that *Klebsiella* spp is not easily removed from the surface of vegetables/fruit/fresh produce by washing with tap water. *Klebsiella* spp is able to grow at a low pH of 3.6, which falls within the pH range determined for the mangoes in this study (O'Connor-Shaw *et al.*, 1995).

Change in population dynamics of Enterobacteriaceae composition on fresh-cut apples and fresh-cut lettuce due to changing environmental factors including storage temperature and atmosphere composition has been demonstrated (Alegre, Abdias, Anguera, Oliveira, Vinas, 2010). To my knowledge, no studies have characterized the succession of Enterobacteriacae during the course of minimal processing of sweet melons.

The reason *E. cloacae* was the most frequently isolated dominant species during all stages of minimal processing may be as a result of selection and or adaption of this specie due to the changing conditions of minimal processing. *E. cloacae* is a well known plant pathogen and is considered to be amongst the most frequently isolated species of Enterobacteriaceae from fruit and vegetables (Farmer *et al.*, 2007). Adaptation or selection of a species facilitates its colonization and survival (Leverenz *et al.*, 2006). A PCR fingerprint with the $(GTG)_5$ microsatellite showed that there is genetic variability between *E. cloacae* isolates that were obtained from the various minimal processing steps. This succession may be due to adaptation of this species which gives it a competitive interspecies advantage and could explain why it was the most frequently identified isolate. Adaptation of strains to changing conditions during industrial production with PCR fingerprinting has also been demonstrated by others (Capece, Salzano, Romano, 2003; da Silva-Fiho, dos Santos, do Monte Resende, de Morais, Simones, 2005).

Other than *E. cloacae*, some of the identified species including *C. freundii, E. aerogenes, E. gergoviae, E. coli, L. adecrboxylata, P. ananatis* and *P. agglomerans* were present only at one or at the most two stages during the minimal processing procedure. For most species, MALDI-TOF MS analyses revealed a high degree of variability amongst strains of the same specie over changing conditions of minimal processing. Like *E. cloacae, L. adecarboxylata* and *P. agglomerans* however also showed a high degree of similarity of strains obtained from the same minimal processing stages. This suggests that for these species, there may also be adaptation and or selection of certain strains to the changing conditions of minimal processing.

The fact that similar trends of grouping with PCR fingerprinting using the $(GTG)_5$ microsatellite and MALDI-TOF MS of *E. cloacae* indicates that MALDI-TOF MS can be regarded as a useful tool in both identification and resolution of strain similarities over different minimal processing stages of fruit.

3.5 CONCLUSION

With regards to the microbial spoilage indicators, both the average total plate count and Enterobacteriaceae levels were significantly higher in the melon samples than in the mango samples. The level of both these indicators was above the microbial guidelines for the fresh-cut melon samples at the end of shelf-life. With the mango samples, only the Enterobacteriaceae count was above the microbial guidelines.

S. aureus had a higher prevalence (66%) in the melon samples than in the mango samples (50%). *E. coli* was detected in the fresh-cut melon samples at the end of shelf-life, but not in any of the mango samples. The higher levels of spoilage indicators and higher prevalence of more foodborne pathogens on fresh-cut melon than fresh-cut mango suggest that fresh-cut melons may be more susceptible to microbial spoilage and foodborne pathogens.

The composition of the Enterobacteriaceae changed over the various stages of minimal processing and storage of sweet melons. *E. coli*, a significant foodborne pathogen, predominated at the end of shelf-life and was not detected in any preceding processing stage. This shows that the current food safety management system is therefore not validated for *E. coli*. In addition, it also demonstrates the inefficiency of end-point testing in ensuring food safety. The pre-dominance and possible adaption of *E. cloacae* over the various stages of minimal processing shows that there may be potential to use this species as a biocontrol agent on fresh-cut melons.

CHAPTER 2: LITERATURE REVIEW

2.1 FRESH-CUT FRUIT INDUSTRY

The market for refrigerated fresh-cut fruits has shown a significant growth in recent years and this was stimulated by the demand of consumers for convenient, healthy and ready-to-eat foods (Zink, 2009). In response to this, the food industry has responded with new production practices, creative product development and the innovative use of technology (FAO, 2011a). The growing interest amongst consumers for new and exotic tastes has resulted in an increase in international trade and several tropical countries have responded by producing fresh-cut products for export (FAO, 2011a). These products are fruit or vegetables that have been fresh-cut into a completely usable, packaged product that offers consumers high nutrition, flavour and convenience while maintaining freshness (International Fresh-cut Produce Association, 2000).

Fresh-cut tropical fruit that can be bought include melons, mangoes, papaya, rambutan, mangoesteen, pineapples and mixed combinations (FAO, 2011a). These products especially attract consumers as they are nutritious, fresh, ready-to-eat and are usually more affordable. Therefore a wide variety of fresh-cut fruits has been developed to provide consumers with ‘quick’ and convenient healthy foods (Ahvenainen, 1996). The production of fresh-cut fruits needs very little product transformation but there must be investment in management systems, technology, equipment and stringent control in food safety practices to make sure that the products have a high quality (FAO, 2011a).

Developing countries like South Africa have undergone many demographic changes in the past two decades. With population growth, urban centres are expanding on all continents. Parallel to this, traditional food supply chains and food habits have been changing to keep up with all these changes (FAO, 2011b). Growth in the market opportunities will continue if consumers believe that fresh-cut fruit is safe with an acceptable shelf-life.

Tropical fresh-cut fruit face significant challenges in entering export markets including failure to meet permitted pesticide residue levels, allowance levels of microbial contamination, food safety standards, food laws of the importing country, quality standards of the buyer as well as compliance with international regulations such as the Codex Alimentarus of the World Health Organization (WHO), and the Food and Agriculture Organization of the United Nations (FAO). Large exportation deals from South Africa to other countries could be affected if foodborne pathogens are detected on fresh-cut fruit. This is a big concern for major retailers in the country (Tempelhoff, 2010). Therefore, contamination with foodborne pathogens should be avoided as far as possible.

Although there have been many previous studies done on whole fruit, vegetables and fresh-cut vegetables, not many studies have been conducted on the microbial quality of fresh-cut fruit (Olaimat & Holley, 2012). There have been studies in Singapore, Switzerland, Brazil, USA, Spain, Australia, etc. but little research has been conducted on fresh-cut mangoes and melons from retail supermarkets in South Africa for *Salmonella* Typhimurium (*S.* Typhimurium), *L. monocytogenes* and *E. coli* (Abdias, Usal, Anguera, Solsona, Vinas, 2008; Althaus, Hofer, Corti, Julmi, Stephan, 2012; Olaimat & Holley, 2012; Seow, Agoston, Phula, Yuk, 2012).

2.2 MINIMAL PROCESSING

Firstly, the unprocessed fruit undergoes two washing stages: the first entails washing the whole fruit with water to remove pre-harvest contaminants such as soil and pesticide residues and a second wash of the peeled fruit to remove tissue fluids as well as to reduce microbial contamination. Figure 1 shows the various stages of minimal processing. The procedure of minimal processing can vary between different processing plants (Corbo, Speranza, Campaniello, D'Amato, Sinigaglia, 2010). The sensory and microbial quality of the water used for the washing must be good and its temperature low (below 5°C). Washing can prolong the shelf-life of fresh-cut fruit for a few days (Allende *et al.*, 2006). After washing, the unprocessed fruit are subjected to a single or several unit operations including peeling, cutting, slicing and shredding. Some fruit like mango and melon require peeling before they are cut. There are various ways in which this can be performed including mechanical, chemical and high pressure stem peeling but the preferred method is hand peeling with a sharp knife (Corbo *et al.*, 2010).

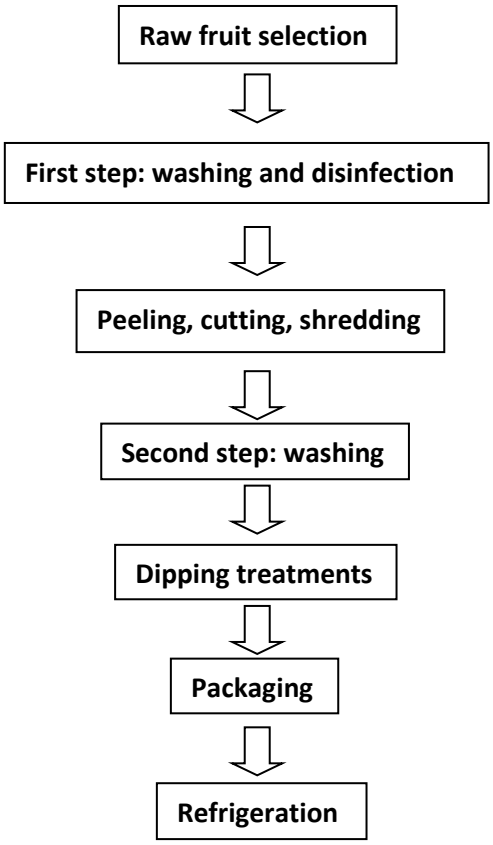


Figure 1: Flow diagram of fresh-cut fruits (Corbo *et al.*, 2010)

These operations cause the formation of lesions in the tissue, enzymatic browning, texture decay, an increase in respiration rate, an increase in ethylene production, weight losses, undesirable volatile production, support rapid microbial growth and therefore significantly reduce the shelf-life (Chien, Sheu, Yang, 2007).

After the mechanical operations have been performed, the fresh-cut fruit is packaged. There are various ways in which this can be done including modified atmosphere packaging (MAP), moderate vacuum packaging (MVP), equilibrium modified atmospheric packaging (EMAP) and active or smart packaging (Siddiqui, Chakrabotry, Ayala-Zavala, Dhua, 2011).

2.3 TROPICAL FRUIT: MANGOES AND MELONS

2.3.1 MANGO

Mango (*Mangifera indica* L.) is a tropical export crop and is produced in 90 countries around the world. In 2008, mango comprised about 40% of global tropical fruit production and this is higher than any other tropical fruit (FAO, 2011a). Asia is responsible for 77% of global mango production and America and Africa account for about 13% and 9% respectively (FAO, 2011a). Mango is of significant economical importance for South Africa and is produced in high numbers. During the 2010/2011 marketing season, the total gross value of mangoes was R0.18 billion which represents 7.7% of the gross value of South African tropical fruits (Department of Agriculture, 2012). Figure 2 illustrates the total gross value of production (GVP) for mangoes over the last 10 years.

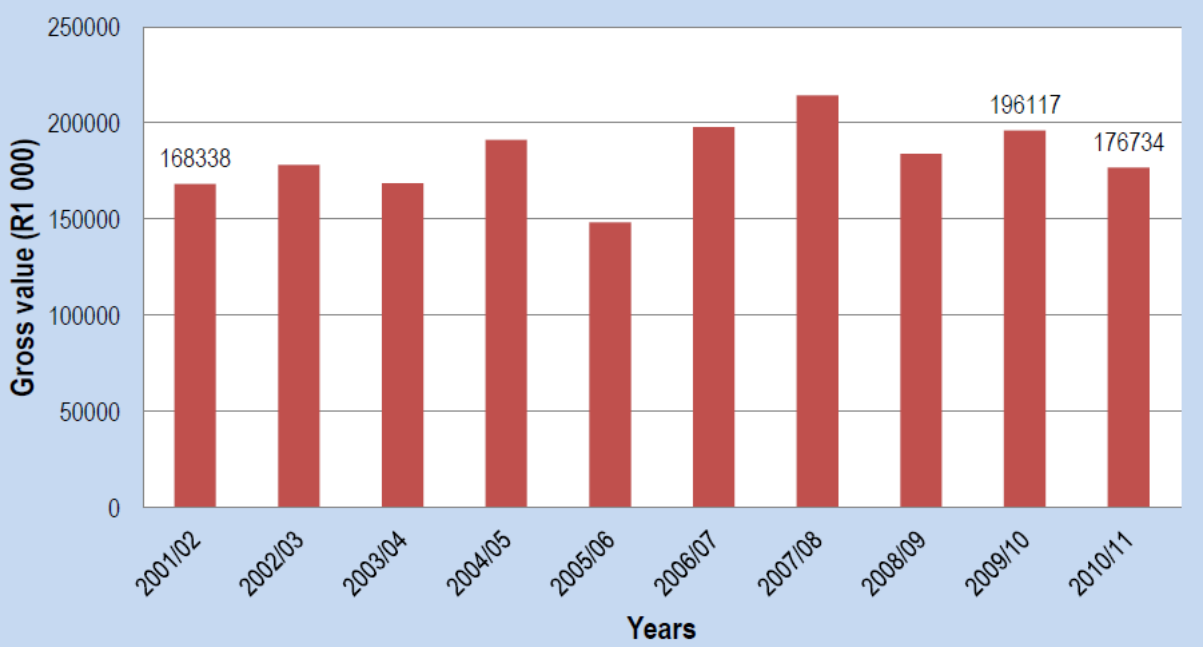


Figure 2: Gross value of production for mangoes in South Africa during 2001/02-2010/11 (DOA, 2012)

The majority of South Africa's annual mango crop is processed and sold through national fresh produce markets and only a small majority of the produced mangoes are exported. A total of 1449 tons of mango was exported from South Africa in 2011. The mango industry in South Africa is important for providing employment in mango production and processing (DOA, 2012).

It provides direct employment for several support industries where the mangoes are grown. Direct employment in the industry in 2011 was estimated to be at 2 900 with about 17400 dependents (DOA, 2012).

The popularity and worldwide production increase of mangoes can be linked to the health benefits associated with mangoes which in turn are linked to its nutritional content and physiological composition. Mango fruit is rich in pre-biotic dietary fibre, vitamins A, C and E, minerals and polic acid (United States Department of Agriculture, 2014a), antioxidants (Carlsen, Halvorsen, Holte, Bohn, Dragland, Sampson, Willey, Senoo, Umezono, Sanda, Barikmo, Behre, Willett, Phillips, Jacobs, 2010) and polyphenols (Gorrinstein, Zemser, Haruenkit, Chuthakorn, Grauer, Martin-Belloso, Trakhtenberg, 1999). Studies have shown that a vitamin A deficiency in developing countries can be prevented by the consumption of mango (Drammeh, Marquis, Funkhouser, Bates, Eto, Stephensen, 2002). It has been shown that mango juice and polyphenolic extracts from mangoes show anticarcinogenic effects by the induction of apoptosis of certain cancer cells (Noratto, Bertoldi, Krenek, Talcott, Stingheta, Mertens-Talscott, 2010).

The physiochemical characteristics within a mango cultivar changes during the ripening process of the fruit because organic acids, such as malic and citric acid that are present in the fruit are utilized as substrates for respiration during the course of ripening (Jha, Narsaiah, Sharma, Singh, Bansal, Kumar, 2010). During ripening, the total soluble solids content and pH of the fruit pulp increase while the titratable acidity in the fruit decreases. The acidity of the pulp of ripe mangoes ranges from 0.12-0.38% titratable acidity (Jha *et al.*, 2010).

Mango is a versatile fruit when it comes to processing. Mango flesh can be processed by canning, juicing, drying, freezing or cut and there is evidence that the processed mango fruit market is increasing (Sauco, 2004). More than 1000 different cultivars of mango are available worldwide (Schamili, Fatahi, Hormaza, 2012). The different cultivars differ from each other with regards to certain characteristics including morphology, flesh colour and titratable acidity (Padda, Amarante, Garcia, Slaughter, Mitcham, 2011). With regards to export, the most popular mango cultivars are Kent, Keitt, Tommy Atkins, and Haden. These cultivars have fruit that have a red blush, are less fibrous, firmer and are more suited for long-distance transportation than other types of cultivars (Sauco, 2004).

2.3.2 MELONS

Melon (*Cucumis melo* L.) belongs to the family Curcubitaceae and is originally from the Middle East (Maynard, Dunlap, Sidori, 2001). There are two genera of melons that are commonly consumed: *Citrulius* in which *C. lanatus* includes the well known watermelons and *Cucumis* in which *C. melo* includes many melon varieties, some commonly known as muskmelon or by other varietal names (CAC, 2011).

Amongst the *C. melo* varieties there are some significant differences in the melon rind including the smooth skinned honeydew, crenshaw and casaba melons, the netted skinned cultivars like the cantaloupe (also commonly known as sweet melon), Santa Claus (or Christmas melon) as well as some partly netted varieties like the Persian melon, Chinese Hami melons and Charentals (CAC, 2011).

There has been cross breading amongst various melon cultivars to improve the suitability for commercial production as well as consumer appeal (CAC, 2011). The global melon production worldwide increased from 2008 to 2009. In 2009, 2,336,782 more tonnes were produced than the previous year (FAO, 2011a). Since 2001, the overall production has increased by 42%. China is the leading country in melon production (51%) followed by Turkey (6%), United States and Spain. Similar to the global increase, South Africa's production of sweet melons has also shown an increase from 4371 tonnes produced in 2000/1 to 7588 tonnes in 2010/11. The average price of the fruit also showed a parallel increase from 2182 R/t in 2000/1 to 5264 R/t in 2010/11 (DOA, 2013).

Sweet melons are a very important export product of sub-Saharan Africa and over the past few years, there has been an increase in the export trend of horticultural exports. Countries that export melons in sub-Saharan Africa include Ghana and South Africa (FAO, 2005). For both these countries, the boost of horticultural earnings is very important to their economy. Table 1 shows the world production of watermelons and other melon types in 2008 and 2009.

Table 1: World production of melons (tonnes) in 2008 and 2009 (CAC, 2011)

Year	other melons	watermelons	Total
2008	27,637,248	98,439,589	126,076,837
2009	27,726,563	100,687,056	128,413,619

(CAC, 2011)

The flesh of melons can be processed into a variety of products. It can be processed to fresh-cut fruits, desserts and to juice (Saftner & Lester, 2009). The fruit pulp is refreshing, has a sweet pleasant aroma, bright colour, firm flesh texture and a sugar content above 10% (de Melo, Narain, Bora, 2000; Seko, 2004**;** Gusimi & Webner, 2005).

2.4 FOODBORNE DISEASES ASSOCIATED WITH WHOLE AND FRESH-CUT MANGOES AND MELONS

2.4.1 FOODBORNE DISEASES LINKED TO FRESH-CUT FRUIT

With the increased global production of fresh-cut fruit in recent years, there has also been an increase in food-borne illness associated with the consumption thereof. Recent disease outbreaks linked to the consumption of whole and fresh-cut melon and mango has been reported (Sivapalasingam, Friedman, Cohen, Tauxe, 2004; Centre for Disease Control, 2008; CAC, 2011; CDC, 2011; CDC, 2012a; CDC, 2012b).

There is a higher risk involved for disease incidence to occur with fresh-cut fruit than unprocessed fruit. Fresh-cut fruit have more risk factors that are of importance during their production: in addition to the factors/conditions that concern the contamination of fresh produce with indigenous pathogens during cultivation or at harvest, the cutting or slicing operation in the minimal processing plant also has an influence (FAO, 2011a). The processing operations can result in the transfer of pathogenic bacteria that are present on and within the unprocessed fruit surface. Fresh-cut fruit is generally eaten raw and therefore any pathogen contamination would signify a hazard for foodborne illness (Danyluk & Schaffner, 2011).

Foodborne illness that occurs as a result of consumption of fresh-cut fruit depends on various factors. The product must be contaminated with a pathogen and must survive at sufficient levels to cause disease. The infective dose differs between different pathogens. At one end of the scale, *Gardia lamblia* (*G. lamblia*) and *Shigella* spp require a minimum of 10 cells to start an infection. Less than 1000 organisms of *L. monocytogenes* can cause disease (Danyluk & Schaffner, 2011; Olaimat & Holley, 2012). On the other hand, *S. aureus* and *Vibrio cholerae* (*V. cholerae*) require $10^8/10^9$ cells for an infection to develop (Schmid-Hempel & Frank, 2007). Pathogens which have a very low infective dose don't have to multiply but simply have to survive in sufficient numbers to cause illness. Some pathogens have to multiply in order to achieve certain numbers sufficient to cause illness (Leggett, Cornwallis, West, 2012). When fresh produce has high numbers of microbial pathogens, it is often consumed because it is not perceived as being spoiled. Therefore, specifications that require very low microbial counts may (in some instances) compromise produce safety.

A wide diversity of pathogens and vehicles for dissemination has been associated and documented in produce-related outbreaks including fresh-cut fruit. A review of outbreaks in the USA from 1973-1997 demonstrated that parasites such as *Cryptosporidium* spp were responsible for 16% of the outbreaks, viruses such as hepatitis A for 20% and bacterial pathogens such as *Salmonella* spp and *E. coli* O157:H7 for 60% of the outbreaks (Sivapalasingam, Friedman, Cohen, 2004). Amongst the bacterial pathogens, *Salmonella* spp was the most prevalent pathogen, accounting for half of the bacterial related outbreaks (Sivapalasingam *et al.*, 2004). In addition to bacterial pathogens, pathogenic protozoa such as *Cryptosporidium* spp and *Giardia* spp have also been isolated from surface, treated effluent and drinking water (Duhain, 2011). Although the majority of outbreaks caused by *Cryptosporidium* spp and *Giardia* spp have been associated with drinking water, *Cryptosporidium* spp oocysts have also been identified on the surface of raw fruit and vegetables in both developing and developed countries (Robertson, Johanessen, Gjerde, Lnocarevic, 2003). The encysted form of the pathogen can survive a long time in the environment due to the nature of its outer wall. In addition, *Cryptosporidium* spp oocysts and *Giardia* spp oocysts have been reported to be resistant against chlorine at the concentration and times which is usually used for the disinfection of vegetables.

Therefore, vegetables which are considered to be safe after chlorine treatment are not, could potentially still carry *Cryptosporidium* spp (Chaidez, Soto, Gortares, Menka, 2005; Duhain, 2011) and could therefore cause foodborne infections.

2.4.2 FOODBORNE DISEASES LINKED TO WHOLE AND FRESH-CUT MELON

Melon consumption has become a significant health concern in some countries with significant negative consequences for trade in this commodity (CAC, 2011). Between 1950 and May 2011, 85 outbreaks associated with melon were identified and they occurred mainly in North America. The most common disease causing agent was *S. enterica* (47.1%) followed by Norovirus (22.4%), *E. coli* 0157:H7 (5.9%), *Campylobacter jejuni* (*C. jejuni*) (3.5%), *Shigella sonnei* (*S. sonnei*) (2.41%), *L. monocytogenes*, *Cyclospora* spp as well as a combination of *S. aureus* and *Bacillus cereus* (*B. cereus*). The number of cases reported varied between 2 to 600, the actual case numbers being >100 higher. Three deaths were reported in 3 salmonellosis outbreaks and a listeriosis outbreak (CAC, 2011). Table 2 shows the food vehicles and table 3 the contributing factors of the 85 foodborne illness outbreaks linked to melon occurring between 1950-May 2011 in North America.

Table 2: Food vehicles associated with 85 foodborne illness outbreaks occurring between 1950-May 2011, where melons were implicated (CAC, 2011)

Food vehicles including melons	Number of outbreaks (%)
cantaloupe	21(24.7)
honeydew	2(2.4)
watermelon	14(16.5)
melons (not specified)	5(5.9)
meal/dish including cantaloupe	25(29.4)
meal/dish including honeydew	16(18.8)
meal/dish including watermelon	14(16.5)
meal/dish including melons (not specified)	9(10.5)

Table 3: Contributing factors, where identified, among 85 outbreaks of foodborne illness associated with melons between 1950-May 2011 (CAC, 2011)

Food vehicles including melons	Number of outbreaks (%)
Pre-harvest transportation contination	3(3.5)
Unwashed melons	4(4.7)
Pre-cut and/mixed dish	12(14.1)
Infected food handler	10(11.8)
Poor hygiene, bare hands	8(9.4)
Cross-contamination	2(2.4)
Poor temperature control	13(15.3)

The data pertaining to disease outbreaks linked to melons collected during 1950-May 2011 strongly suggests that sweet melon is a high risk food and that pre-cut/mixed dish has the greatest potential for transferring disease. In 2008, a multistate outbreak of *Salmonella* Lichfield (*S.* Litchfield) was linked to the consumption of whole sweet melons which affected 51 people across 16 states (CDC, 2008). In 2011, a multistate outbreak of *L. monocytogenes* was linked to the consumption of whole sweet melons from Jensens farms, Colorado (CDC, 2011). In 2012, a multistate outbreak of *S.* Typhimurium, *Salmonella* Newport (*S.* Newport) and listeriosis was linked to the consumptions of whole sweet melons. The *S.* Typhimurium and *S.* Newport outbreak sickened 261 persons over 24 states and three deaths were reported (CDC, 2012b).

Infections caused by *Salmonella* spp are considered to be amongst the most prevalent recognised communicable diseases caused by bacteria worldwide (Pui, Wong, Chai, Tunung, Jeyaletchuni, Noor Hidaya, Ubong, Farinazleen, Chea, 2011a). The various *Salmonella* cause different clinical syndromes including gastroenteritis. Gastrointestinal infections are linked to the *Salmonella* serotypes that occur commonly in animals and humans. The severity of the symptoms can range from asymtopmatic carriage to severe diarrhoea. Other more common serotypes include *Salmonella* Virchow (*S.* Virchow), *Salmonella* Infantis (*S.* Infantis) and *S.* Newport (Adams & Moss, 2008). Systemic disease is caused by host-adapted serotypes that are more invasive leading to systemic disease in their host. In humans, this is applicable to typhoid and paratyphoid bacilli, (*Salmonella* Typhi) *S.* Typhi, and *Salmonella* Paratyphi (*S.* Paratyphi), which cause enteric fever and septicaemic diseases (Pui, Wong, Chai, Nillian, Ghazali, Cheah, Nakaguchi, Nishibuchi, Radu, 2011b).

Together with *Salmonella* spp, *E. coli* O157:H7 poses an equally great concern amongst health and food safety professionals due to its ability to cause foodborne illness (Olaimat & Holley, 2009). *E. coli* O157:H7 is regarded as being responsible for the most produce-related outbreaks in recent decades (Berger, Sodha, Shaw, Griffin, Pink, Hand, Frankel, 2010). *E. coli* O157:H7 has the ability to produce toxins termed vero- or shiga toxins which ultimately result in haemolytic-uremic syndrome (HUS) (Newell, Koopmans, Verhoef, Duizer, Aidara-Kane, Sprong, Opsteegh, Hangelaar, Threefal, Scheutz, Van der Giessen, Kruse, 2010). These organisms are also often referred to as Shiga toxin producing organisms (STEC). The symptoms from infection by this particular group can range from non-bloody (mild) to bloody stools which contain no leukocytes (Benenson, 1995).

The pathogenicity of EHEC has been linked to certain virulence factors: shiga toxins, adherence factors, locus of enterocyte effacement (LEE) and intimin (Welch, 2006). There are two known shiga toxins including shiga toxin 1 (*Stx 1*) and shiga toxin 2 (*Stx 2*). Adherence factors help with attachment to sites within hosts such as the small intestine. The toxins and adherence factors are coded by pathogenicity islands on the genome such as LEE (Welch, 2006). Pathogenicity in humans occurs through attachment of adherence factors to the gut and formation of lesions, local arrangement of the host cell cytoskeleton and secretion of liquid and electrolyte into the gut (Weiss, Schmidt, Stober, 2011). *E. coli* 0157:H7 is seen as a significant pathogen causing disease in developing countries, in particular the southern hemisphere which includes South Africa (Beutin, 2006).

The main virulence factors of *L. monocytogenes* are a pore-forming cytotoxin, listerolysin O and invasion-associated surface proteins termed listerolysin O (Ryan & Drew, 2010). *L. monocytogenes* can cause a variety of diseases which can range from a mild chill to premature birth of children or miscarriage, as well as meningitis in newborn children. Septicaemia and meningitis occur in adults that have an impaired immune system like those that suffer from leukaemia or cancer. Infection occurs in healthy adults and children, but this is very rare (Dietrich, Karst, Fischer, Wehland, Janseh, Leger, 2006).

2.4.3 FOODBORNE DISEASES LINKED TO WHOLE AND FRESH-CUT MANGO

The first documented outbreak of foodborne illness linked to whole mango contaminated with *Salmonella* spp occurred in 1999 which sickened 78 people and caused the death of two (Sivapalasingam *et al.*, 2004). In 2012, a multistate outbreak of *Salmonella* Braenderup (*S.* Braenderup) linked to consumption of mangoes occurred. This caused a total of 127 persons to become ill across 15 states of America, but no deaths were reported. The mangoes that caused this outbreak were sourced from Agricola Daniella in Mexico (CDC, 2012a). According to the FDA, this outbreak caused Fresh Del Monte Produce, a US-based manufacturer of fresh-cut fruit and vegetables, to recall 1600 bowls of fresh-cut mangoes due to possible *Salmonella* spp contamination (FDA, 2012). Up to date, there has been no disease outbreaks associated with fresh-cut mango. A recently conducted study however found fresh-cut mango from an outlet store to be contaminated with *Salmonella* spp (Harris, 2013). In addition, the survival and proliferation of this pathogen within mango pulp has been demonstrated (Bordini, Ristori, Jakabi, Gelli, 2007).

Fresh-cut mango could therefore be a health risk to consumers. Table 4 shows the outbreaks related to whole mango and whole melon from 2002-2012 in Canada and USA.

Table 4: Outbreaks related to whole mango and sweet melon from 2002-2012 in Canada and USA

Period	Pathogen	Vehicle	Source
2008	*S.* Lichfield	Sweet melon	CDC, 2008
2011	*L. monocytogenes*	Sweet melon	CDC, 2011
2012	*S.* Typhimurium	Sweet melon	CDC, 2012b
	S. Newport	Sweet melon	
	L. monocytogenes	Sweet melon	
2012	*S.* Braenderup	Mango	CDC, 2012a

2.5 ASSOCIATION OF WHOLE AND FRESH-CUT MANGOES AND MELONS WITH FOODBORNE PATHOGENS

Fresh-cut melons and mangoes can become contaminated with foodborne pathogens at any point along the food production chain in a similar way as other fresh-cut fruit (CAC, 2011). In addition, there are specific extrinsic and intrinsic characteristics of melons and mangoes that influence the risk of contamination and the potential for growth and survival of pathogens that are important considerations in assessing and managing food safety risks (CAC, 2011). The extrinsic factors include suitability of the fruit surface for bacterial attachment and internalization of pathogens through the fruit surface. The intrinsic factors that affect bacterial growth include pH, water activity, and nutrient content.

2.5.1 INTRINSIC FACTORS AFFECTING PATHOGEN GROWTH

The peeling, cutting or shredding operations that are performed during minimal processing injures the fruit which exposes a favourable environment for the pathogens (Harris, Farber, Beuchat, Parish, Suslow, Garret, Busta, 2003) and therefore microbial growth is greater on fresh-cut products than on unprocessed fruit (Ayala-Zavala, Rosas-Dominguez, Vega-Vega, Gonzales-Aguilar, 2010a; Ayala-Zavala & Gonzales-Aguilar, 2010b).

The increase in microbial growth on fresh-cut products is associated with damaged tissues and broken cells. Cellular disruption leads to release and intermixing of enzymes and nutrients that can be used by microorganisms to grow (Siddiqui, Chakrabotry, Ayala-Zavala, Dhua, 2011). The intrinsic factors of fruit including water activity, pH and nutrients affect the stability of microorganisms (Khetarpaul, 2006).

Water activity

The water activity (Aw) of food refers to the amount of moisture that is available to micro-organisms for growth. Bacteria proliferate in food with a high water activity, a water activity being around 0.99. Many will not grow below 0.95 and some bacteria can survive below 0.75 (Adams & Moss, 2008). Yeasts and moulds can tolerate low levels of water activity, some as low as 0.62 (Khetarpaul, 2006).Therefore, the spoilage of fruit and vegetables is more often as a result of yeasts and mould than bacteria. Both mango and melon have a high Aw of about 1 and this therefore makes them suited to support bacterial growth (Adams & Moss, 2008).

pH

Micro-organisms can grow and proliferate only within a certain pH range. The majority of bacteria prefer to grow in food that has a pH close to neutrality. Low pH generally inhibits microbial growth. Yeast and moulds prefer acidic food (pH 4 to 4.5) (Khetarpaul, 2006). The pH of sweet melons is between 6.2-6.7 and is therefore less acidic in comparison to other fruits (CAC, 2011). The pH of mangoes is between 3.98 and 5.08 depending on cultivar (Harris *et al.*, 2003). This fruit is considered to be an acidic fruit (Adams & Moss, 2008). Despite its low pH, it has been demonstrated that some bacterial pathogens can survive and grow on mango pulp (Bordini, Ristori, Jakabi, Gelli, 2007). Bordini *et al.* (2007) demonstrated the presence of *Salmonella* spp in mangoes (var. Tommy Atkins) produced in Brazil and also showed that when mango pulp was inoculated with *Salmonella* spp, the inoculums increased. The inoculated mango was kept at 22°C and 8°C respectively. In both cases, the inoculums increased but a higher increase was observed at 22°C than at 8°C.

The low acidity of sweet melon pulp readily supports the growth of foodborne pathogens. If contaminated sweet melon pulp is stored under elevated temperatures, *Salmonella* spp, *E. coli* O157:H7 and *L. monocytogenes* can reach high concentrations without overt visual signs of spoilage within 24 h (Golden, Rhodehamel, Kautter, 1993; Del Rosario & Beuchat, 1995; Li, Friederich, Danyluk, Harris, Schaffner, 2013; Danyluk, Friederich, Schaffner, 2014).

It has been demonstrated that *S.* Sonnei is able to grow rapidly when it is injected into the pulp of watermelon and can reach 8-9 log cfu/g in three days at 20 or 30°C (Castillo, Martinez-Tellez, Roderiguez-Garcia, 2009). The majority of inoculation studies have been based on *S. enterica* serovars inoculated onto sweet melon, honeydew as well as watermelon pulp. Generally the inocula survived at 4 or 5°C, the growth was retarded at 10°C but from 20°C the inocula reached hazardous levels in 4-6 h up to seven days depending on the initial contamination level (Escartin, Ayala, Lozano, 1989; Golden, Rhodehamel, Kautter, 1993; Ukuku & Sapers, 2001). *S. enterica* can survive on sweet melon pulp stored at 5°C and increase up to 2 log units when stored at 10°C (Leverenz, Conway, Alavidze, Janisiewicz, Fuchs, Camp, Chighladze, Sulakvelidze, 2001). Inoculation studies have shown that sweet melon pulp readily supports the growth of *E. coli* O157:H7. Some studies have been carried out to determine the survival and growth of *E. coli* O157:H7 on sweet melon pulp. It was found that the inocula survived at 4°C, growth was retarded at 10°C but from 20°C the inocula reached hazardous levels in 4-6 hours up to seven days depending on the initial contamination level (Escartin, Ayala, Lozano, 1989; Golden *et al.*, 1993; Ukuku & Sapers, 2001). Danyluk *et al.* (2014) found that *L. monocytogenes* inocula increased by 4 log units following storage for fifteen days at 5°C. In addition, it was found that the growth rate of this pathogen on sweet melon pulp increases with temperature.

2.5.1 EXTRINSIC FACTORS AFFECTING PATHOGEN GROWTH

Pathogen survival on fruit surfaces

Different fruit types and cultivars can have a different abundance of bacterial groups on their surface (Critzer & Doyle, 2010) and farming and storage conditions can influence the composition of the bacterial groups (Ponce, Aguero, Roura, Del Valle, Moreira, 2008; Granado, Thurig, Kieffer, Peterini, Fliessbach, 2008; Lopez-Velasco, Welbaum, Boyer, Mane, 2011).

There is a range of environmental factors that can influence microbial composition such as pH which can vary across produce types (Nguyen & Carlin, 2000; Gram, Ravn, Rasch, Bruhn, Christensen, 2002; Kroupitski, Goldenberg, Belausov, Pinto, Swartsberg, Ganot, 2011). Generally, pathogens that are present on the fruit surface will survive but not grow (Harris *et al.*, 2003) and due to the naturally hostile environment pathogen levels on the fruit surface tend to decline due to its low nutritional content and water activity (Harris *et al.*, 2003). A number of studies have however indicated that *Salmonella* spp and *E. coli*, once attached to the fruit surface, are capable of replicating to relatively high levels on or within the fruit (Warriar, Spaniolas, Dickinoson, Wright, Waltes, 2003; Jablasone, Warriner, Griffiths, 2005).

Plant microbiota interactions are important in colonization or inhibition of enteric pathogens on fruit surfaces. Two epiphytes, *Wausteria paucula* (*W. paucula*) and *Enterobacter asburiae* (*E. asburiae*), were found to interact differently with *E. coli* O157:H7 on fresh-cut produce. *E. asburiae* decreased *E. coli* O157:H7 by 20-30 fold whereas *W. paucula* enhanced survival by sixfold (Cooley, Chao, Mandrel, 2006).

Pathogen attachment to fruit surfaces

The attachment of bacteria has been a problem in the food industry and has become a challenge for fresh produce processing industries. The mechanisms by which pathogenic bacteria attach to fruit surfaces are quite complex (Ells & Hansen, 2006). Three mechanisms have been put forward namely biofilm formation, the use of flagella and fimbriae as well as interaction with other bacteria (Critzer & Doyle, 2010). Figure 3 shows some of the different mechanisms through which enteric foodborne pathogens may attach to fruit. Biofilm formation on fruit surfaces and equipment in fruit processing facility is a major concern as cross-contamination of bacterial pathogens to the fresh-cut product has been demonstrated (Tang, Pui, Wong, Noorlis, 2012). A biofilm is formed when bacterial cells attach to one another and/or adhere to a living inert contact surface. The attached bacterial cells are enclosed in a self-produced polymeric matrix. Biofilms are regarded as very dangerous biological structures because they can become a persistent source of contamination (Hout & Michels, 2010). The organisms can increase their ability to colonize and survive in harsh conditions if they are able to form this biofilm (Monier & Lindow, 2003).

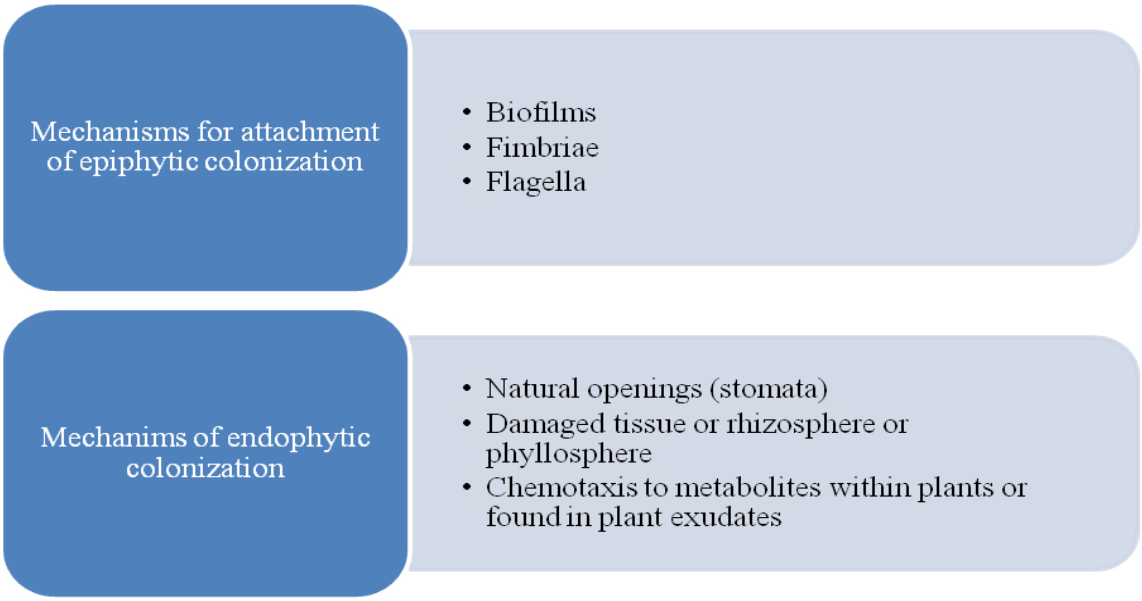


Figure 3: Colonization of fresh fruits by enteric foodborne pathogens (Critzer & Doyle, 2010)

Some fruits possess characteristic surfaces that allow for a stronger attachment and colonization by bacteria (Bastos, Soares, Andrande, Arruda, 2005). The physical and chemical properties of food contact surfaces contributing to microbial adhesion include hydrophobicity, and topography (Araujo, Bernardes, Andrande, Fernandes, Sa, 2009).

Sweet melons have a hydrophilic, veined, rough and 'netted' surface (Gerchikov, Keren-Keiserman, Perl-Treves, Ginzberg, 2008; Parveen, Muhammad, Asghar, Khan, Salam, 2012). This allows the attachment of bacterial cells to inaccessible sites within the sweet melon netting and/or the formation of bacterial biofilms that contain a protective matrix of exopolysaccharide (Annous, Burke, Sites, 2004; Annous, Solomon, Cooke, Burke, 2005). Biofilm formation of *Salmonella* Poona (*S.* Poona) on sweet melon surface has been demonstrated (Annous, Solomon, Cooke, Phillips, 2013). Figure 4 shows attachment and biofilm formation by *S.* Poona cells inside the netting on the sweet melon surface.

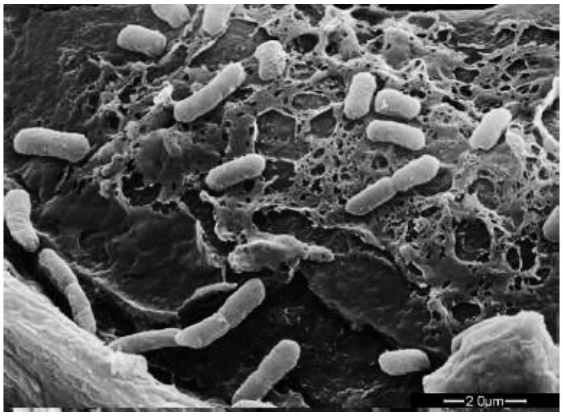


Figure 4: Attachment and biofilm formation by *S.* Poona cells inside the netting on the sweet melon surface (Annous, Solomon, Cooke, Phillips, 2013)

The surface of mangoes is hydrophilic and rough and is suited for bacterial attachment (Fernandes, Sao Jose, Zerdas, Andrade, Fernandes, 2014). Adhesion to the mango surface by *S. enterica* and *E. coli* O157: H7 has been demonstrated (Fernandes *et al.*, 2014). In addition, studies have shown that the mango surface enhances the attachment and resistance of *Salmonella* spp (Ukuku & Fett, 2006).

There are various mechanisms by which *S.* Enterica can attach to fruit including the formation of non-hemagglutinating pili, fimbriae, and flagella (Berger, Sodha, Shaw, Griffin, Pink, Hand, Frankel, 2010; Patel & Sharma, 2010). Biofilm formation of *S.* Typhi on mango surfaces has been demonstrated (Tang *et al.*, 2012). Figure 5 shows the attachment and biofilm formation of *S.* Typhi on a mango surface.

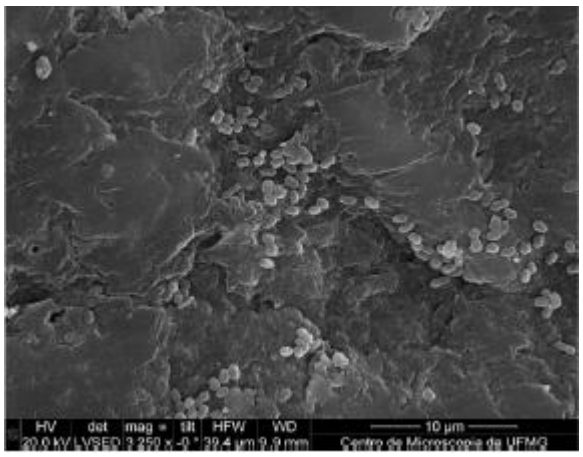


Figure 5: Attachment and biofilm formation by *S.* Typhi cells on the mango surface (Tang *et al.*, 2012).

Internalization of pathogens through the fruit surface

Internalization of pathogenic microorganisms from the external environment to deeper within the fruit flesh has been demonstrated. This can either occur by infiltration into fruit with contaminated wash water or entry of pathogens through openings that are present on the fruit surface (Penteado, Eblen, Millar, 2004; Bordini *et al.*, 2007; Soto, Chavez, Baez, Martinez, Chaidez, 2007; Castillo *et al.*, 2009). During infiltration, bacteria are pulled into the internal fruit tissues along with water (Deering, Mauer, Pruitt, 2012). This process is enhanced as a result of a negative temperature difference between the fruit and the water (FAO, 2011a).

In mangoes, infiltration occurs through the stem scar (Bordini *et al.*, 2007) and in melons it occurs primarily through the ground spot where the netting is underdeveloped and secondarily through the stem scar (Castillo *et al.*, 2009). Internalization of *Salmonella* spp and *E. coli* O157:H7 has also been demonstrated by entry of these pathogens through the natural openings present on the fruit surface (Deering *et al.*, 2012). Several studies suggest that these pathogens are associated with the natural openings in the fruit surface (Dong, Iniguez, Ahmer, Triplett, 2003; Duffy, Ravva, Stanker, 2008; Itoh, Sugita-Konishi, Kasuga, Jwaki, Hara-Kudo, Saito, 2008; Shaw, Berger, Feys, Knutton, Pallen, Frankel, 2008; Gomes, Da Silva, Moreira, Castell-Perez, Ellis, Pendelton, 2009; Kroupski *et al.*, 2009). Figure 6 shows the major routes of entry for bacteria into the fruit interior.

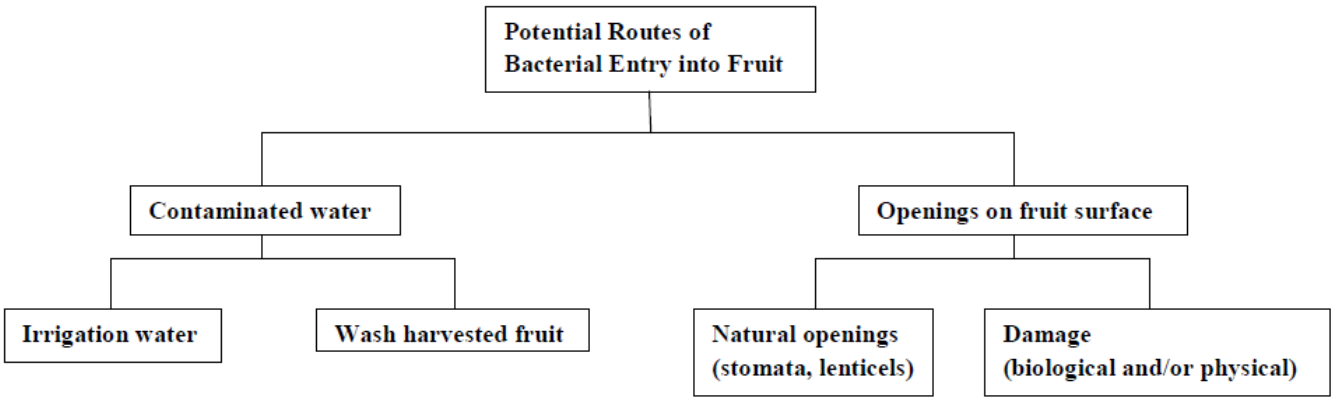


Figure 6: Outline of the major routes of entry for bacteria into the fruit interior (Deering *et al.*, 2012)

2.6 BACTERIAL CONTAMINATION SOURCES OF FRESH-CUT FRUIT

Contamination of fruit and subsequently fresh-cut fruit with pathogens can occur during pre- and post-harvest practices (Gil, Selma, Lopez-Galvez, Allende, 2013). Several authors have reviewed the potential sources of pathogen contamination and preventative approaches to their control pre-harvest for fresh produce in general (Doyle & Erickson, 2008). There are various pre-harvest factors that could potentially lead to bacterial contamination of fresh fruit including cultivar and climatic conditions, soil, irrigation water, animal activity, human activity and wastes, insects, dust, equipment associated with growing and harvesting and reconstituted fungicides and insecticides (Table 5)

Table 5: Sources of pathogenic microorganisms on fresh fruit and vegetables (Beuchat & Ryu, 1997; Steele & Odumeru, 2004; Beuchat, 2006)

Preharvest	Postharvest
Feces	Faeces
Soil	Human handling (workers, consumers)
Irrigation water	Harvesting equipment
Water to apply fungicides, insecticides	Transport to cultivation (fields to packing shed)
Air (dust)	Wild and domestic animals (including fowl and reptiles)
Wild and domestic animals	Insects
Insects	Air (dust)
Human handling	
	Wash and rinse water
	i) Sorting, packing, cutting and further processing equipment
	ii) Ice
	iii) Transportation vehicles
	iv) Improper storage (temperature)
	v) Improper packaging (including new packaging technologies)
	vi) Cross-contamination (other foods in storage, preparation and display areas)
	vii) Improper display temperature
	viii) Improper handling after wholesale or retail purchase

2.6.1 PRE-HARVEST SOURCES OF CONTAMINATION

Soil

Soil is a common environment for many human pathogens including *L. monocytogenes.* Pathogens can enter the soil environment from various sources, but organic fertilizers such as animal manure is the most common source, especially if it is not adequately aged or treated before application (Jacobsen & Bech, 2012).

The survival of *Salmonella* spp in soils is dependent on various factors including temperature, soil type, presence of plants, exposure to sun (UV), protozoan predation and the initial number of organisms present. *S.* Typhimurium can survive in soils for up to 332 days in manure-amended soils (Islam, Morgan, Doyle, Phatak, Millner, Jiang, 2004; Holley, Arrus, Ominski, Tenuta, Blank, 2006; You, Rankin, Aceto, Benson, Toth, Dou, 2006). *S.* Typhimurium has been detected in soil in melon growing fields (Gallegos-Robles, Morales, Alvarez-Oieda, Osuna-Garcia, Martinez, Morales-Ramos, Fratamico, 2009). Since melons grow within soils, there is a greater risk for the transfer of foodborne pathogens as compared to mangoes, which grow on mango trees and are therefore not directly in contact with the soil.

Irrigation water

Irrigation water has been noted as a source of bacterial contamination on fresh fruit (Ijabadeni, Minnaar, Buys, 2011; Gemmel & Schmidt, 2012). *E. coli* and *S.* Typhimurium have been shown to be common human pathogen contaminants of irrigation water and subsequently cause foodborne disease from consumption of this contaminated produce (Levantesi, Bonadonna, Briancesco, Grohmann, Toze, Tandoi, 2012). The transfer of *S.* Typhimurium to sweet melon rind via furrow and drip irrigation systems has been demonstrated (Suslow, Sbodio, Lopez, Wei, Tan, 2010). A number of studies that have been carried out on the bacteriological quality of irrigation water in South Africa have reported on its deteriorating microbiological quality (Obi, Green, Bessong, De Villiers, Hoosen, Igumbor, Potgieter, 2004; Kinge, Ateba, Kawadza, 2010; Ijabadeni *et al.*, 2011; Gemmel & Schimdt, 2012). There are various environmental factors which contribute to the contamination of irrigation water with bacterial pathogens including agriculture, wildlife and human inputs (Pachepsky, Shelton, Mclain, Patel, Mandrel, 2011). Figure 7 shows the mechanisms by which irrigation water can become contaminated.

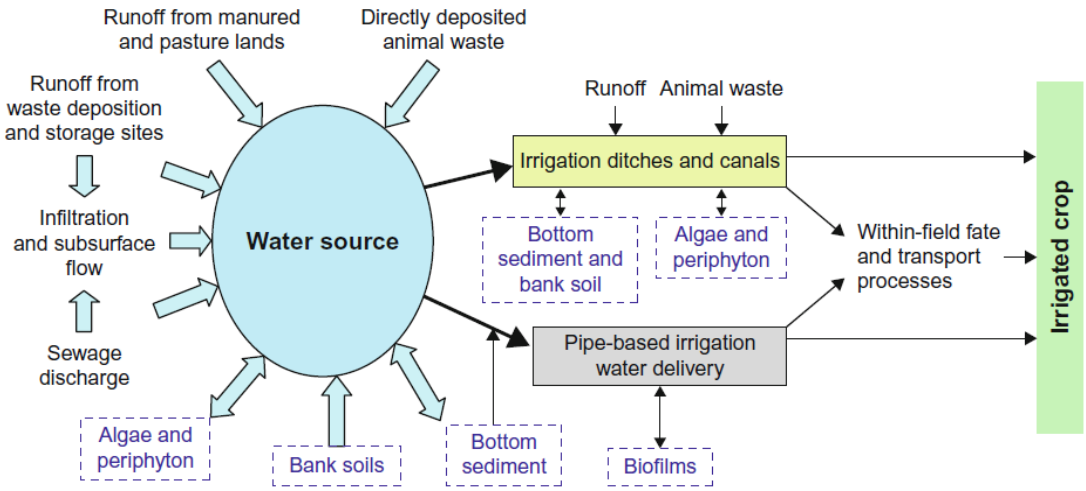


Figure 7: Mechanisms by which irrigation water can become contaminated (Pachepsky *et al.*, 2011).

Human and animal sources

Animals can contaminate irrigation water and under favourable conditions bacterial numbers can increase which place irrigated crops under risk of contamination (Matthews, 2009). Sewage and ruminant manure have been identified as being the main sources of pathogens including *Salmonella* spp and *E. coli* O157:H7 (Olaimat & Holley, 2009). Cattle that are infected with *E. coli* O157:H7 have been reported to shed up to 10^7 cfu/g of faecal material and these cattle are referred to as 'super shedders' (Solomon & Sharma, 2009). Whether irrigation water and soil presents an actual food safety risk depends on the interaction of human pathogens in the farm environment with fruit surfaces, adhere to them, survive environmental stresses in the field and subsequent exposure to the sanitizing processes (Sapers & Doyle, 2009). People working with fruit are known to be a source and direct contact vector of microorganisms that are a significant public health concern. Hygiene practices, from land preparation, planting, weeding, and pruning, to harvest influence whether produce becomes contaminated from direct human transfer (Gil, Selma, Suslow, Jacxens, Uyttendale, Allende, 2013). The hands of field workers working in a melon vine have been shown to be contaminated with *E. coli* (Castillo, Mercado, Lucia, Martinez-Ruiz, Ponce, De Leon, Murano, Acuff, 2004).

From the above it is clear that pathogens can survive in the pre-harvest environment of fruit that will be used for minimal processing. Certain factors allow for easier access for pathogens to fresh fruit including cultivation soil containing pathogens, soils fertilized with manure and the use of contaminated or untreated water for irrigation purposes. These pre-harvest factors are not the only factors that play a role in the fresh-cut fruit product. After harvest, the produce encounters many opportunities to contaminate. Pre-harvest bacterial contamination of fresh fruit that is to be minimally processed should be limited in order to reduce the risk to the final consumer.

2.6.2 POST-HARVEST SOURCES OF CONTAMINATION

Factors that play a role in contamination during post-harvest practices include post-harvest handling, processing, retail food service operation and consumer handling (Gil *et al.*, 2013). Post-harvest handling addresses the activities that occur in the field, namely harvest and field packing.

These operations involve considerable contact between fresh produce and workers (handlers), tools, equipment, surfaces, water or ice, and field environment (soil, dust and insects) (Beuchat, 1996). This poses a risk of contamination of fresh fruit since the post-harvest environment is full of microbes and potential pathogens (Olaimat & Holley, 2012).

Processing facility

Several factors of the processing facility can influence the microbial quality of fresh-cut fruit including adequate management of temperatures, distance from supply facility, sources of insects, rodents or foul odours, presence of airborne dust, collection of dirt, sanitary drains, window ledges, relative humidity, ventilation, processing personnel and equipment hygiene (FAO, 2011a). Microbial communities in fresh-produce processing facilities comprise diverse microbial species. They can include bacteria proficient at forming biofilms, which can potentially protect food spoilage and pathogenic bacteria that allow them to survive in processing plants (Gibson, Taylor, Hall, Holah, 1999; Bridier, Braindet, Thomas, Dubois-Brissonet, 2011). *Salmonella* spp, *L. monocytogenes*, *E. coli* O157:H7 and *S. aureus* have been noted to form biofilms in food processing plants (Gunduz & Tuncel, 2006).

Employee hygiene and particularly good hand hygiene is crucial in food contamination and the subsequent risk of foodborne illness (Rediers, Claes, Kinnerk, Peeters, Willems, 2008). Hand contact surfaces have been identified as high potential exteriors for contamination by pathogens. Examples of these are toilet facilities, handknobs, room door knobs and keyboards (FAO, 2011a; Letho, Kuisma, Maatta, Kymalainen, Maki, 2011). *S.* Typhimurium can contaminate fresh fruit through contact via handling by infected workers (FAO, 2011a).

Post-harvest sanitation mechanisms such as washing with chlorine cannot remove all bacterial pathogens from fresh produce (Johnston, Jaykus, Moll, Anciso, Moora, Moe, 2006; Solomon & Sharma, 2009). Generally, chlorine washes at permitted concentrations ranging between 50-200 ppm with contact times less than 5 minutes give total plate count reductions between 0-3 log cfu/g (Klaiber, Baur, Wolf, Hammes, Carle, 2005; Nou & Lou, 2010). *B. cereus* can survive washing with chlorine and can germinate again once favourable conditions are presented (Elhariry, 2011).

Chlorine washing can reduce *S. aureus* and *E. coli* present on fruit surfaces by about 1 log but does not entirely remove these pathogens (Har & Pereira, 2013). If pathogens are transferred to fruit prior to minimal processing, they will be present in the final product. Quality control of fruit that will be used for minimal processing therefore has to begin at primary production and continue throughout production so that the low level risk of contamination that can be present on farms is reduced or is not increased prior to consumption (CAC, 2011).

From the above, it can be seen that fresh-cut fruit can become contaminated with human pathogens and parasites whilst growing in the field or during harvesting, post-harvest handling, processing and distribution. To minimize the risk associated with microbial hazards of fresh-cut fruit, producers and processors have several detailed schemes or codes of practice and regulations available.

2.7 ENTEROBACTERIACEAE ON FRESH-CUT FRUIT

The Enterobacteriaceae is a large group of gram negative, rod shaped, non-spore forming, and facultative anaerobic bacteria. Currently, the family consists of at least 34 genera, 149 species and 21 subspecies (Ryan & Drew, 2010). Some species including *Serratia marcescens* (*S. marcescens*) (Donnenberg, 2010) are wide-spread in the environment such as soil, water and plants, but are not a common component of the human faecal flora (Donnenberg, 2010). There are some species including *E. cloacae* that occur both in the environment and the lower intestinal tract of humans and animals (Ryan & Drew, 2010).

All species of the Enterobacteriaceae are pathogenic to either humans, plants or both (Table 6). With regards to humans, the majority of species are opportunistic pathogens that are responsible for nosocomial infections in hospitals, with the victims mostly being immunocompromised individuals (Ryan & Drew, 2010).

Table 6: Examples of Enterobacteriaceae species pathogenic to humans and plants

Specie	Host	Disease	Source
E. cloacae	Humans	Neonatal septicemia	Anthony & Prasad, 2011
E. cloacae	Plants (onion)	Bulb decay	Zaid, Bonasera, Beer, 2011
E. cloacae	Plants (mulberry)	Bacterial wilt	Zho, Wang, Xie, Zhou, Zhao, Praphat, 2010
Salmonella	Humans	Enteric fever	Pui *et al.*, 2011b
Shigella	Humans	Shigellosis	Bennish & Albert, 2011
Pantoea	Plants	Bulb rot	Coutinho & Venter, 2009
E. coli	Humans	Diharrea	Ryan & Drew, 2010
Klebsiella	Humans	Enteric fever	Morais, Daporin, Bao, Campello, 2009
Citrobacter	Humans	Nosocomial infection	Ryan & Drew, 2010
Serratia	Humans	Keratitis	Posluszny, Conrad, Halerz, Shankar, Gamelli, 2011

Two species, *Salmonella* spp, and *E. coli* O157:H7, are significant foodborne pathogens that are commonly associated with disease outbreaks linked to microbialy contaminated fresh-cut fruit (Berger *et al.*, 2010). Although more attention is often paid to the pathogenic properties of partiuclar genera of the Enterobacteriaceae, some genera constitute a spoilage group when conditions favour their growth (Ercolini, Russo, Torrieri, Villani, Villani, 2006; Nychas, Paramitholis, Dougeraki, 2011). *Enterobacter* spp, *Pantoea* spp and *Serratia* spp are considered to be important species with regards to spoilage of fresh-cut fruit (Allende *et al.*, 2006). The Enterobacteriaceae family is known to colonize plants, fruit and vegetables (Abdias *et al.*, 2008).

A recent study that was done showed that the majority of species that were isolated from the surface of a variety of fruit and vegetables belong to the Enterobacteriaceae family, with *Pantoea agglomerans* (*P. agglomerans*) being the most frequently isolated species (Left & Frierer, 2013). The study also showed that different fruit and vegetable types harboured different amounts of Enterobacteriaceae (Left & Frierer, 2013). Members of the Enterobacteriaceae that are associated with fruit surfaces share characteristics which enable them to survive the extreme conditions they are exposed to including low nutritional content, low water activity and extreme temperature fluctuations (Harris *et al.*, 2003). They are capable of fruit surface adhesion and colonization, can fix nitrate required for growth and can metabolize carbohydrates provided by their host (Vessey, 2003; Lugtenberg & Kamilova, 2009). Some members including *Enterobacter* spp and *Klebsiella pneumoniae* (*K. pneumoniae*) are plant growth-promoting-(rhizo)bacteria (PGPR) and produce plant beneficial phytohormones which enhances plant growth (Vessey, 2003; Lugtenberg & Kamilova, 2009).

The mechanical operations that take place during minimal processing of fresh fruit such as cutting can transfer Enterobacteriaceae species that are present on the unprocessed whole fruit surface to the cut, final product (FAO, 2011a). This occurs even though unprocessed fruit is washed with sanitizers such as chlorine prior to the minimal processing procedure (FAO, 2011a). Several studies have indicated the presence of Enterobacteriaceae on fresh-cut fruit (Rico, Martin-Diana, Barat, Barry-Rian, 2007; Ergun, Jeong, Huber, Cantliffe, 2007). O'Connor-Shaw *et al.* (1995) isolated *P. agglomerans*, *E. cloacae* and *Klebsiella oxytoca* (*K. oxytoca*) from fresh-cut mangoes. The native microbial community that is present on fresh-cut fruit is thought to play a critical role in keeping the health-supporting status of fresh-cut fruit (Nguyen & Carlin, 1994). They compete with pathogens for physical space and nutrients as well as by producing antagonistic compounds such as lytic agents, antibiotics, bacteriocins, protein exotoxins and other secondary metabolites which reduce the viability of pathogens (Liao & Fett, 2001). These organisms have the advantage of being part of the naturally established microbial community on the target produce. This may facilitate their survival and colonization on the fresh-cut fruit when applied in the appropriate numbers (Leverenz, Conway, Janisiewicz, Abdias, Kurtzman, Camp, 2006).

It has been demonstrated that an Enterobacteriaceae species, CPA-6, isolated from apples controls foodborne pathogens on fresh-cut apples and peaches. In this study, fresh-cut apple and pear slices were inoculated with 10^5 log cfu/g *E. coli* O157:H7 and 10^6 log cfu/g of CPA respectively and incubated at 20°C or 5°C. CPA-6 effectively inhibited the growth of, or reduced, in some cases below the limit of detection, *E. coli* O157:H7 populations on both fruit incubated for two days at 20°C and 5°C respectively (Alegre *et al.*, 2012). In another study, the microflora of fresh-cut lettuce and spinach were evaluated to inhibit *E. coli* O157:H7. The majority of microorganisms isolated that were inhibitory to *E. coli* O157:H7 were genera belonging to the Enterobacteriaceae family including *Pantoea* spp, *Klebsiella* spp and *Enterobacter* spp (Johnston, Harrison, Morrow, 2009).

Additional studies are required to determine the mechanisms by which foodborne pathogens can be controlled by the natural microflora. This could help in developing effective competitive inhibition microflora that inhibits enteric pathogen colonization and survival (Critzer & Doyle, 2010).

2.8 THE ENTEROBACTERIACEAE AS INDICATOR ORGANISMS

Food is often examined microbiologically to determine its shelf-life, safety for human consumption or whether it conforms to established microbiological criteria (Adams & Moss, 2008). Indicator organisms are often used for this purpose as they demonstrate that food has been exposed to conditions that could have introduced hazardous microorganisms (Sengupta & Saha, 2013) and it eliminates the need to test for particular pathogens which is difficult and time consuming since they are often present in low numbers (Scott, Rose, Jenkins, Farrah, Lukasik, 2002). Coliforms are frequently used as indicator organisms. These bacteria are capable of lactose fermentation in the presence of bile at 37°C. They include organisms of faecal origin such as *E. coli* but also organisms that are not of faecal origin such as *Citrobacter* spp and *Enterobacter* spp (Sengupta & Saha, 2013). Faecal coliforms, a subset of the total coliform group, are also commonly used as indicator organisms. They have the same properties as the coliforms except that fermentation can take place at 44.5° -45.5°C (Sengupta & Saha, 2013).

Neither the examination for coliforms nor faecal coliforms can indicate the presence of lactose-negative organisms of which some including *Salmonella* spp, *Shigella* spp and *E. coli* O124 are considered to be significant foodborne pathogens (Berger *et al.*, 2010). Therefore, the test for coliforms and faecal coliforms could give a false reassurance of food safety. The Enterobacteriaceae family has been proposed as an alternative indicator to the coliform group and is increasingly being used. It consist of about 20 genera including *E. coli*, all members of the coliform group and foodborne pathogens such as *Salmonella* spp, *Shigella* spp and *Yersinia* spp (Berger *et al.*, 2010). Testing for the entire Enterobacteriaceae is therefore considered to be more inclusive for pathogenic bacteria than testing for the coliform or faecal coliform group (Sengupta & Saha, 2013). The Enterobacteriaceae counts are considered to be useful indicators of hygiene and post-processing contamination of heat processed foods. They give an indication of the hygienic quality of the food rather than faecal contamination and therefore these counts say more about the general microbiological quality rather than the health risks that are associated with a product (Tortorello, 2003). The test for Enterobacteriaceae is not applicable to fresh, fresh-cut fruit and vegetables as it is expected that these food products have an inherently high Enterobacteriaceae count (Centre for Food Safety, 2007).

2.9 MICROBIAL SUCCESSION ON FRESH-CUT FRUIT

Microbial succession can be defined as the way in which microbial communities change over time following the colonization of a new environment (Frierer, Nemergut, Knight, Craine, 2010). Depending on the environment, there are different categories of microbial succession that can occur including autotrophic, endogenous heterotrophic and exogenous heterotrophic (Frierer *et al.*, 2010). Endogenous heterotrophic succession is likely to be found on food products including fresh-cut fruit (Ercolini, Mauriello, Blaiotta, Moschetti, 2004; Haruta, Ueno, Egawa, Hashiguchi, Fuji, Nagano, 2006). In this type of succession, the initial community development on the fruit surface can be fast (Redford & Frierer, 2009). Succession on the fresh-cut fruit product is fuelled by organic carbon derived from the substrate itself (Ercolini *et al.*, 2004; Haruta *et al.*, 2006). There are some important sources of environmental (non-resource) stress that can shape microbial diversity during succession including water availability, shear stress, UV radiation, antibiotics/antimicrobials, temperature, salinity, redox status (particularly O_2) and pH (Frierer *et al.*, 2010). Certain processes can lead to microbial succession (Table 7).

Table 7: Processes that lead to microbial succession

Process	**Examples where the process may apply to microbial succession**
Dispersal limitation (Foster & Tilman, 2003)	Inoculation of food products, development of communities in water pipes and other semi-isolated habitats
Resource depletion via uptake (competition) (Tilman, 1985)	Microbial uptake of nutrients, substrate catabolism, light interception
Facilitation between community members that reduce environemtal stress (Callaway & Walker, 1997)	Biofilm production in mixed microbial communities, metal detoxification
Facilitation via increasing availability of limiting resources (Chapin, Walker, Fastie, Sharma 1994)	N_2-fixation, phosphatise production, syntrophic
Increasing environmental stress (Westman & Whitaker, 1975)	Acidification by fermenting bacteria, production of antibiotics, generation of heat by microbial activity during composting

Several studies have investigated microbial succession on a wide variety of food products including cheese, alcoholic beverages, cured meats, vinegar, cacao fermentation and fresh-cut produce etc. (Ercolini *et al.*, 2004; Haruta *et al.*, 2006). Babic, Roy, Watanda, Wergin (1996) studied the changes in microbial population on fresh-cut spinach. No previous study has yet investigated the succession of Enterobacteriaceae on fresh-cut melons or fresh-cut mangoes.

2. 10 DIAGNOSTIC TECHNIQUES USED FOR THE ENTEROBACTERIACEAE

The diagnostic techniques used for Enterobacteriaceae identification and characterization include both culture-based and molecular-based methods. The ISO 21528-1 and 3M *Enterobacteriaceae* Petrifilm (3M) are examples of culture based methods that are used frequently (Paulsen, Borgetti, Schopf, Smulders, 2008). Generally, the disadvantage of culture-based diagnostic methods is that they are time consuming and laborious. In addition, many microorganisms do not grow rapidly in standard culture media and hence are not detected (Dowd, Wolcott, Sun, McKeehan, Smith, Rhoads, 2008). On the other hand, molecular methods allow for the rapid identification of bacteria to the strain level (Yildirim[1], Yildirim[2], Kocak, 2011). Molecular diagnostic tools that are often used for Enterobacteriaceae characterization include PCR, DNA sequencing and the development of ribosomal clone libraries (Tuttle, Mostow, Mukherjee, Hu, Melton-Kreft, Ehrlich, Dowd, Ghannoum, 2011).

In addition to the previously mentioned molecular techniques, Matrix associated desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid, accurate and cost effective method of microbial characterization and identification. This technology produces characteristic mass spectral fingerprints, that are unique signatures for each microorganism and are therefore ideal for microbial identification at the genus and species levels and has the potential to be used for strain typing and identification (Croxatto, Prod'hom, Greub, 2012). MALDI-TOF has been used to characterize a wide variety of microorganisms including bacteria, fungi, and viruses (Giebel,Worden, Rust, Kleinheinz, Robbins, 2010). Several studies have shown the potential of this methodology for rapid and accurate characterization and identification of Enterobacteriaceaea (Hrabak, Chudackova, Walkova, 2013; Pavlovic, Konrad, Iwobi, Sing, Busch, Huber, 2012).

2.11 CONCLUSION

There has been a global increase in the production and consumption of fresh-cut fruit such as mango and melon. Consumers are increasingly becoming aware of the health benefits linked to the latter. There has however also been a parallel increase in foodborne disease linked to consumption of fresh-cut melon, which is regarded as a high risk food commodity. No disease outbreaks have been associated with fresh-cut mango but there have been outbreaks linked to whole mango. Inoculation studies have shown that mango and melon pulp supports the growth of foodborne pathogens. Melon pulp is however more susceptible to spoilage and pathogenic microorganisms than mango pulp due to the intrinsic differences of these fruit. This may suggest that fresh-cut melon is more susceptible to spoilage and pathogenic microorganisms than fresh-cut mango. This difference could be an important consideration for the food safety management systems of minimal processing industries in ensuring microbiological safety of these products. Fresh-cut fruit play an important role in the South African economy as it is an important export trade. Large exportation deals could be lost if it is found that the fresh-cut fruit are of poor microbiological quality.

The Enterobacteriaceae is one of the most dominant microbial families associated with fruit and vegetables. These organisms are opportunistic pathogens, particularly to immunocompromised individuals such as the elderly, infants and HIV-infected people. Some bacteria, including *E. coli* O157:H7 and *Salmonella* spp, are significant foodborne pathogens.

To my knowledge, no study has investigated the succession of Enterobacteriaceae over the course of minimal processing and subsequent storage. This could point out at what minimal processing stages foodborne pathogens belonging to the Enterobacteriaceae dominate and demonstrate the interaction between the different species of the Enterobacteriaceae.

2.12. HYPOTHESES AND OBJECTIVES

2.12.1 HYPOTHESIS

1. Fresh-cut melon will be more prone to survival of foodborne pathogenic microorganisms such as *Salmonella* spp, *E. coli* O157:H7, *L. monocytogenes* and spoilage microorganisms than fresh-cut mangoes due to the difference in the intrinsic properties of these fruit. Fresh-cut mango has a lower pH than fresh-cut sweet melon (5.2-6.7) (Harris *et al.*, 2003). There is thus a higher risk for foodborne diseases to occur with fresh-cut melon than with fresh-cut mango consumption (CAC, 2011; CDC, 2011; CDC, 2012b).

2. The changing extrinsic (i.e. temperature) and intrinsic (i.e. pH) factors persisting over the various stages of minimal processing and subsequent storage of sweet melons will lead to a change in the species composition of the Enterobacteriaceae. This is one of the most dominant microbial families associated with fresh fruit and vegetables (Left & Frierer, 2013). It is known that changing environmental conditions facilitate microbial succession (Frierer *et al.*, 2010).

2.12.2 OBJECTIVES

1. To determine the microbiological quality and safety of fresh-cut mango and sweet melon during minimal processing and subsequent refrigerated storage.
2. To determine the effect of minimal processing and subsequent refrigerated storage of fresh-cut melon on the species composition of the Enterobacteriaceae.

CHAPTER 4: GENERAL DISCUSSION

4.1 CRITICAL REVIEW OF METHODOLOGY

This study set out to assess the microbiological safety and quality of fresh-cut melons and mangoes from a minimal processing plant. In addition, it aimed to determine the succession of Enterobacteriaceae on sweet melons during the various stages of minimal processing and storage. Fruit samples were collected in duplicate at various stages of the minimal processing and storage: whole fruit, cut fruit, packaged fruit at the beginning and end of shelf-life. In addition, fresh-cut mango and fresh-cut melon samples purchased at a local retail store were analysed at the end of shelf-life.

For microbiological analyses purposes, both molecular and conventional methods were used. With regards to the conventional methods, culture-based methods and petrifilm was used. The culture-based methods required a lot of laboratory equipment, large amounts of medium, and several days to detect the target pathogens mainly due to multiple enrichment steps (Stewart & Gendel, 1998). This is problematic as rapid and reliable methods are of vital importance in food surveillance studies in order to prevent outbreaks and to detect these organisms in the early warning and notification systems (Yeni, Acar, Polat, Soyer, Alpas, 2014). In addition to the above mentioned problems, the culture-based methods provided difficulties with microbial enumeration and pathogen identification and characterization. Enumeration was difficult due to overlapping colonies and the formation of smears on agar plates. In contrast, clear, individual colonies were observed on petrifilm.

A) **B)**

Figure 24: Total plate count on 3M petrifilm (A) and total plate count agar plate (B) respectively

The use of petrifilm also had drawbacks. Dominant Enterobacteriaceae colonies that were obtained were very small and this made downstream analyses of the isolates difficult. Due to their small size, colonies were individually picked off by toothpicks for freezing purposes and no duplicates could be made. The use of VRBG agar plates to obtain Enterobacteriaceae colonies may have allowed for the development of larger colonies and duplicates could have been made of these obtained isolates.

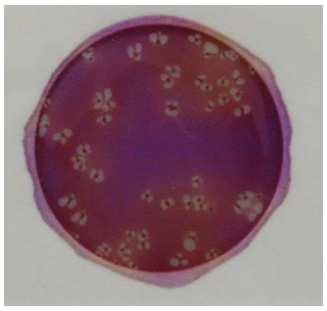


Figure 25: Enterobacteriaceae colonies obtained on 3M petrifilm

The culture-based methods, even though they are internationally validated, provided false positive results during identification and characterization of common bacterial pathogens such as *Salmonella* spp., *E. coli* and *L. monocytogenes.* Therefore, MALDI-TOF MS had to be used to confirm some of the bacterial isolates. Isolates identified as *Salmonella* spp on mango samples on two of the three weeks of sampling were serotyped as: *K. pneumoniae, Pseudomonas aerigunosa* (*P. aerigunosa*), *C. freundii* and *E. cloacae.* Isolates identified as *Salmonella* spp on the melon samples on all three weeks of sampling, were identified as *K. pneumoniae, C. braakii, C. freundii, E. cloacae* and *E. asburiae.* The use of genotypic methods involving PCR may have helped to accuratly screen for bacterial pathogens during the study hence saving time and other resources.

No *E. coli* was detected with the conventional culture-based ISO 16649-2 method which involved plating out on TBX agar plates. *E. coli* was however detected as the dominant species following MALDI-TOF MS identification after isolation from petrifim. This questions the effectivity of ISO 16649-2 to detect *E. coli* in a food sample. The obtained *E. coli* isolates were not characterized further, but this could have been done by serological testing.

The combination of genotypic methods such as molecular based methods involving PCR with the standard, culture-based methods may have been beneficial in this study. Molecular methods allow for sensitive and rapid detection of several pathogens.

The results can be obtained in shorter times as in a day such as in real-time multiplex PCR (de Boer, Ott, Kesztyus, Kooistra-Smid, 2010). In addition, molecular methods give reliable, high throughput, reproducible and specific results (Klein, 2002; Lindstrom, Keto, Markkula, Nevas, Hielm, Korkeala, 2001). With regards to the molecular methods used in this study, the kits that were used for the 3M Molecular Detection System were faulty which resulted in all the positive controls being invalid. Therefore, all results obtained from using this machine were invalid.

Not all of the dominant Enterobacteriaceae isolates could be identified by MALDI Biotyper 3.0 software (Bruker Daltonics). This software compares the acquired mass spectra from isolates to reference spectra from MALDI Biotyper 2.0 library (Bruker Daltonics). For some of the isolates, there were no reference spectra in the library and hence they could not be identified. The PCR for the *E. cloacae* isolates was optimized, but further optimization could result in an even clearer fingerprint. For the optimized PCR, the DNA concentration had to be between 20-80 ng/µl. The DNA extracts of some isolates were lower than this required concentration. In addition, the extracted DNA was continuously frozen and thawed for PCR purposes. Therefore a vague or no fingerprint was observed for come *E. cloacae* strains.

4.2 RESEARCH FINDINGS AND FUTURE WORK

Fresh-cut mangoes and melons collected from a fresh-cut fruit processing plant were contaminated with bacterial pathogens including *S. aureus* and *E. coli*, both known to cause disease in humans. In addition, the total plate count and Enterobacteriaceae levels on these products at the end of shelf-life were higher than levels specified in microbiological guidelines (HPA, 2009; Martin-Belloso, Soliva-Fortuny, Oms-Olimu, 2006).

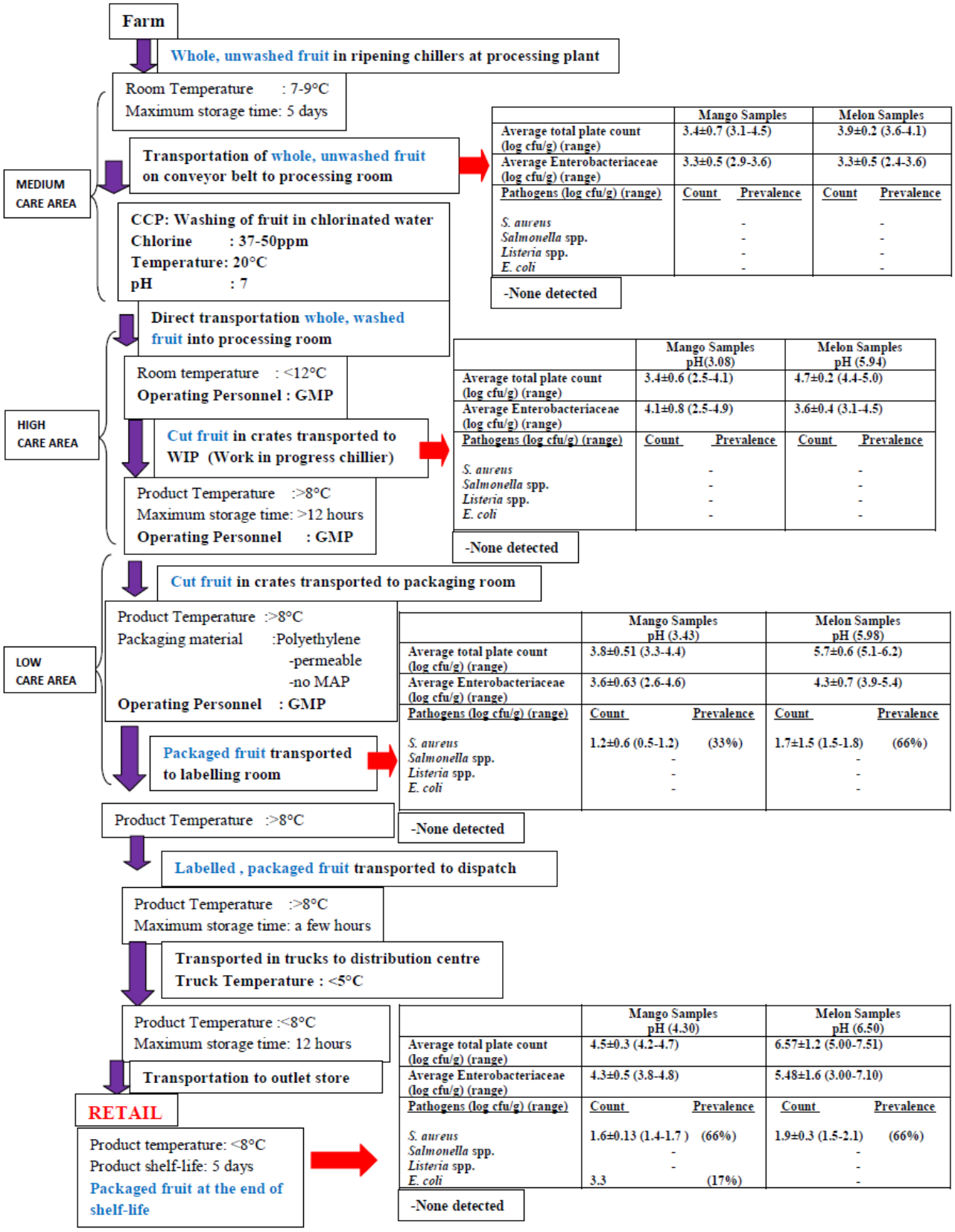
The species composition of the Enterobacteriaceae changed over the course of minimal processing and storage of sweet melons. Figure 24 gives an overview of microbiological data obtained over the various stages of minimal processing and storage of sweet melons. The higher level of spoilage indicators and higher prevalence of more foodborne pathogens on fresh-cut melon shows that it is more susceptible to microbial spoilage and foodborne pathogens than fresh-cut mango. This is probably due to the difference in the intrinsic properties including the pH of the respective fruit. Sweet melons were found to have a significantly higher pH throughout all stages of minimal processing and storage than mangoes.

In both the mango and melon samples, a high degree of variability in the levels of spoilage indicators was noted at certain stages of minimal processing and storage. For example, the average total plate count for the packaged melon (day 0) was 5.72 $\log_{10}$cfu/g. The upper limit of the range (6.20 $\log_{10}$cfu/g) from which this average was obtained is already above the microbiological criteria. In this case the consumer would therefore be exposed to a product that has an unacceptable total plate count before the product had reached the stipulated end of shelf-life.

In this experiment, the packaged fresh-cut fruit samples (day 0) were stored at 0°C for 5 days and not between 5-10°C as would be the case in the real-life retail scenario. Knowing that a temperature increase of 5°C leads to a significant increase in total plate count and Enterobacteriaceae, it is alarming to consider what the indicator counts may have been if their packaged fresh-cut fruit had been stored between 5-10°C. The presence of *E. coli* at the end of shelf-life on the fresh-cut melon indicates that the current implemented food safety management system with regards to this pathogen is not efficient (Sawe, Onyango, Kamau Njage, 2014).

Currently implemented food safety management system

Microbiological analyses results



	Mango Samples		Melon Samples	
Average total plate count (log cfu/g) (range)	3.4±0.7 (3.1-4.5)		3.9±0.2 (3.6-4.1)	
Average Enterobacteriaceae (log cfu/g) (range)	3.3±0.5 (2.9-3.6)		3.3±0.5 (2.4-3.6)	
Pathogens (log cfu/g) (range)	Count	Prevalence	Count	Prevalence
S. aureus	-		-	
Salmonella spp.	-		-	
Listeria spp.	-		-	
E. coli	-		-	

-None detected

	Mango Samples pH(3.08)		Melon Samples pH (5.94)	
Average total plate count (log cfu/g) (range)	3.4±0.6 (2.5-4.1)		4.7±0.2 (4.4-5.0)	
Average Enterobacteriaceae (log cfu/g) (range)	4.1±0.8 (2.5-4.9)		3.6±0.4 (3.1-4.5)	
Pathogens (log cfu/g) (range)	Count	Prevalence	Count	Prevalence
S. aureus	-		-	
Salmonella spp.	-		-	
Listeria spp.	-		-	
E. coli	-		-	

-None detected

	Mango Samples pH (3.43)		Melon Samples pH (5.98)	
Average total plate count (log cfu/g) (range)	3.8±0.51 (3.3-4.4)		5.7±0.6 (5.1-6.2)	
Average Enterobacteriaceae (log cfu/g) (range)	3.6±0.63 (2.6-4.6)		4.3±0.7 (3.9-5.4)	
Pathogens (log cfu/g) (range)	Count	Prevalence	Count	Prevalence
S. aureus	1.2±0.6 (0.5-1.2)	(33%)	1.7±1.5 (1.5-1.8)	(66%)
Salmonella spp.	-		-	
Listeria spp.	-		-	
E. coli	-		-	

-None detected

	Mango Samples pH (4.30)		Melon Samples pH (6.50)	
Average total plate count (log cfu/g) (range)	4.5±0.3 (4.2-4.7)		6.57±1.2 (5.00-7.51)	
Average Enterobacteriaceae (log cfu/g) (range)	4.3±0.5 (3.8-4.8)		5.48±1.6 (3.00-7.10)	
Pathogens (log cfu/g) (range)	Count	Prevalence	Count	Prevalence
S. aureus	1.6±0.13 (1.4-1.7)	(66%)	1.9±0.3 (1.5-2.1)	(66%)
Salmonella spp.	-		-	
Listeria spp.	-		-	
E. coli	3.3	(17%)	-	

-None detected

Figure 26: Summary of microbiological results obtained during the various stages of minimal processing and storage of fresh-cut mangoes and melons

E. coli, *S. aureus* and other Enterobacteriaceae species identified were probably transferred to the fruit during pre-and post-harvest practices (including processing). With regards to pre-harvest practices, the most important inputs of potential pathogen sources in the production environment for fresh produce include wildlife, livestock, human activity and wastes, irrigation water, soil and soil amendments, seeds, plant stocks and equipment (Gil *et al.*, 2013). It has been demonstrated that the use of microbiologically contaminated irrigation water can lead to the transfer of certain bacteria to produce (Ijabadeniyi *et al.*, 2011) and this is a significant problem in South Africa (Department of Water Affairs, 2012). Many of the obtained isolates may have come from irrigation water as some recent studies have shown their pre-dominant prevalence in South African irrigation waters. It was found that the Loskop canal has *E. coli*, *Enterobacter* spp, *Klebsiella* spp, *Enterococcus* spp and *Serratia* spp. The Skeerpoort river water contained *E. coli*, *Enterobacter* spp, *Klebsiella* spp, and *Raoutella* spp. In both waters, *E. coli* and *Enterobacter* spp, was found to be the most frequently isolated Aijuka (2013). In addition to the study by Aijuka 2013**,** other studies found similar results in South African irrigation waters (Zamxaka, Pironcheva, Muyima, 2004; Lin & Biyela, 2005; Ballot, Nana, Sriruttan, Cooper, 2012; Lötter, 2010).

The identified microorganisms could also have been transferred to the fruit during post-harvest practices in the processing plant from the hands of the minimally processing personnel and/or during the cutting procedure and/or from the processing environment and equipment. The total plate count obtained from swabs taken from the hands of the minimal processing operating personnel was ‘poor’ according to the hazard analyses and critical control points-total quality management (HACCP-TQM) (Lehto, Kuisma, Maatta, Kymalainen, Maki, 2011). Employee hygiene and particularly good hand hygiene is crucial in food contamination and the subsequent risk of food-borne illness (Rediers *et al.*, 2008). According to Barker, Vipond, Bloomfield (2004), hands that are contaminated with foodborne pathogens can transfer these to up to seven clean surfaces. *S. aureus* is found on the skin and in the nostrils of warm-blooded animals and the primary source of food contamination has been demonstrated to be the hands of food handlers (Le Loir, Baron, Gattier, 2003).The processing environment including containers, conveyor belts, cutting boards, knife blades and slicers could also have played a role in the transfer of microbes to the fresh-cut fruit and this has been demonstrated in other studies (Gibson *et al.* 1999; Gil *et al.*, 2013).

The microbial communities in the fresh-produce processing environment are diverse and can include bacteria that are capable of forming biofilms which can protect food spoilage and pathogenic bacteria allowing them to survive in the processing environment (Bridier *et al.*, 2011; Gibson *et al.*, 1999). *S. aureus* has been demonstrated to be endemic in the food processing environment (Borch, Neshaldsen, Christensen, 1996). Foodborne outbreaks associated with fresh-cut produce have been associated with the existence of biofilms in the fresh-cut produce processing facilities (Carmichael, Harper, Coventry, Taylor, Hickey, 1998). *K. pneumoniae*, *Enterobacter* spp, *S. aureus* and *E. coli*, all of which were identified in this study, have been demonstrated to form biofilms on equipment used in the fresh-cut processing plants (Gutierrez, Delgado, Vasquez-Sanchez, Martinez, Lopez Cabo, Rodriguez, Herrera, Garcia, 2012). It has been demonstrated that once *S. aureus* has formed a biofilm, it is very hard to remove, even after cleaning and disinfection (Brooks & Flint, 2008). Under certain conditions, microorganisms in the biofilm can detach and disperse (Midlet & Carpentier, 2002) and this could explain its presence on the fresh-cut mango and melons.

With regard to the identified pathogens which are a public health concern, further investigation is required to reveal the source of contamination. Furthermore, the effect of current process controls could be improved to lower their incidence. Despite the high microbial counts obtained for the melon samples and the presence of pathogenic bacteria in this study, it is important to note that none of the samples showed any signs of spoilage. Therefore, outward appearance may not be a good criterion for judging the microbiological safety and quality of fresh-cut fruit. The critical control point involving a chlorine wash at a concentration of 37-50 ppm with a pH of 7 at 20°C for a contact time of less than 5 minutes is clearly not sufficient. The effectivity of chlorine washes is dependent on various factors including the exposure time (<5min), chlorine concentrations (100-150 ppm), pH (6.5-7.5), temperature and characteristics of the produce surfaces (Pasha, Saeed, Tauseef-Sultan, Khan, Rohi, 2014). As a result of varying produce surface characteristics, optimization of these factors would be required to achieve better decontamination of these fruit surfaces. In addition to not completely decontaminating pathogens from fruit surfaces, the use of chlorine has other significant drawbacks. Chlorine vapour can cause irritation to the skin and respiratory tract of the handlers. Carcinogenic chlorinated compounds such as trihalomethanes can be produced when the chlorine contacts organic matter (Joshi, Mahendran, Alagusundaram, Norton, Tiwari, 2013).

Due to these drawbacks, the use of chlorine solutions to sanitize fruit and vegetables in the fresh-cut industry is forbidden in Switzerland and other countries of the European Union (Anonymous, 2004). There is an increasing demand for 'natural' and 'additive-free' products amongst consumers. Therefore, it would be desirable to preserve foods by natural means such as biocontrol (Kim, 1993). Some recent studies have revealed the potential of 'natural' means to prolong the shelf-life of fresh-cut mangoes and melons. Barbosa, de Araujo, Matos, Guitierrez, Carnelossi, de Castro (2013) demonstrated the potential of nisin-incorporated films to prolong the shelf-life of fresh-cut mangoes. Silveira *et al.* (2013) has shown that juices directly obtained from fresh fruit can prolong the shelf-life of fresh-cut sweet melons.

The change in the Enterobacteriaceae population over the various minimal processing stages was probably due to the change in intrinsic factors including pH, nutrient content, water activity and extrinsic factors including the gaseous headspace composition (oxygen (O_2) and carbon dioxide (CO_2) concentration), relative humidity, atmosphere composition and storage temperature of the melon ecosystem during each respective processing step (Oms-Oliu, Rojas-Garu, Gonzales, Varela, Soliva, Fortuny, Hernando Hernando, Munuera, Fiszman, Martin-Belloso, 2010). The gaseous headspace composition may have had a minimal influence on the Enterobacteriaceae population dynamics as completely permeable packaging material (polyethylene) is used by the fruit processing plant. These changing conditions may have selected for and resulted in the adaptation of certain Enterobacteriaceae species with certain genotypic and phenotypic characteristics to dominate at certain stages during minimal processing. For many bacterial species the combination of rapid growth rates, which was observed for the Enterobacteriaceae over the course of minimal processing of sweet melons, and large population sizes results in the introduction of many unique mutations giving rise to variants that are adapted to particular niches (Hibbing, Fuqua, Parsek, Peterson, 2010). Figure 27 shows the change in intrinsic and extrinsic factors over the course of minimal processing of sweet melons that may have selected for certain Enterobacteriaceae species to dominate at certain stages.

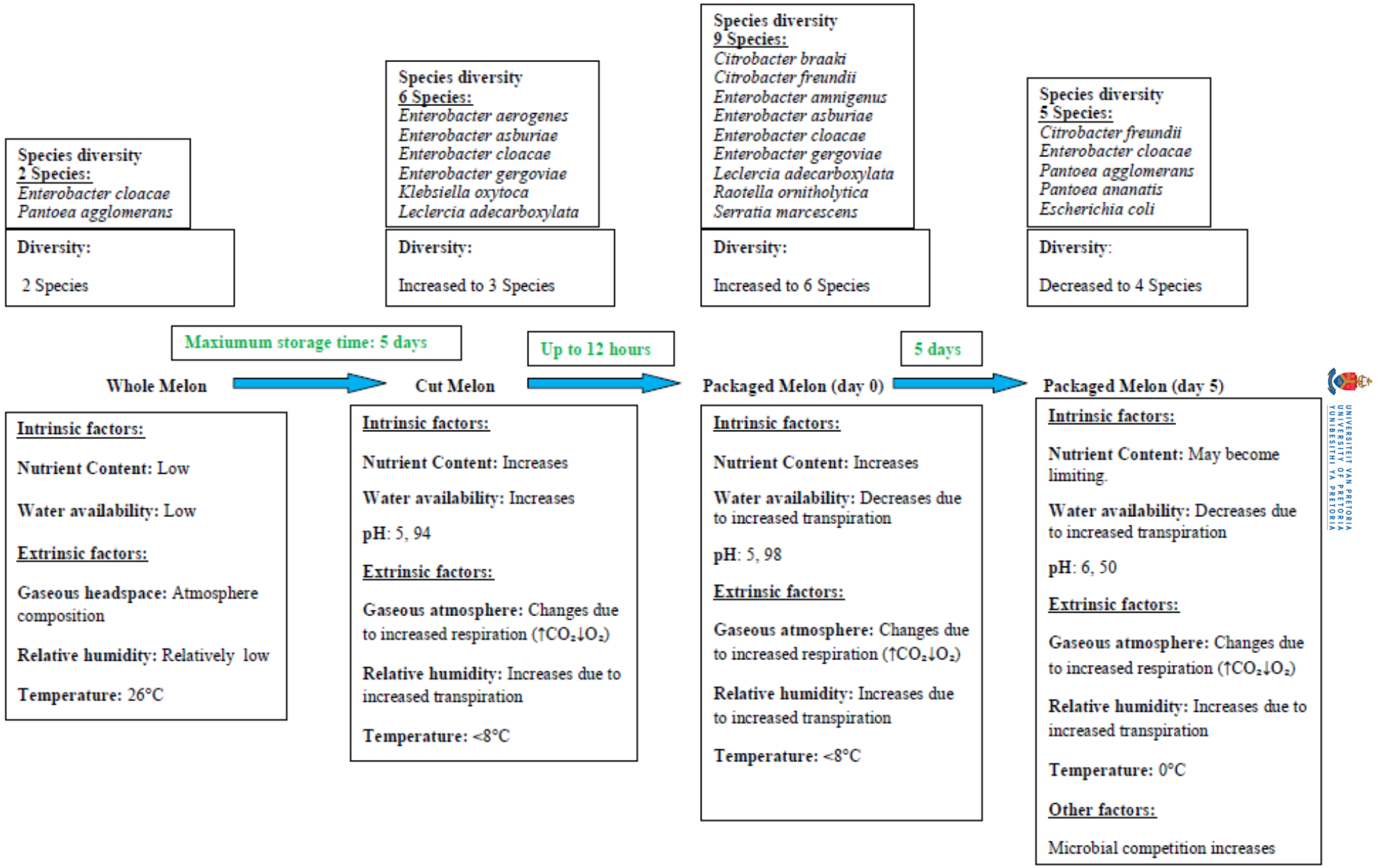


Figure 22: Influence of internal and external factors on Enterobacteriaceae species diversity over the course of minimal processing of sweet melons

The lowest diversity of the Enterobacteriaceae species was identified on the whole melon and is probably due to the hostile nature of the whole melon fruit surface which is not suited for microbial proliferation (Berger *et al.*, 2010). In addition to a lack of nutrients and water, the fruit surface is subject to extreme and often fluctuating environmental conditions (Berger *et al.*, 2010). It has been demonstrated that *Enterobacter* spp and *Pantoea* spp, the only two dominant Enterobacteriaceae species identified on the whole melon, are capable of survival on only water and minimal energy source conditions which persist on the outer fruit surface (Harris *et al.*, 2003) and this may explain their dominance. The Enterobacteriaceae species that were present on the whole melon must have been introduced during pre-harvest practices including fertilizers, irrigation water, containers, food handlers and soil (Berger *et al.*, 2010). This is very likely as *Enterobacter* spp and *Pantoea* spp are very abundant in the environment, including soil and water (Farmer *et al.*, 2007). Even though *Enterobacter* spp and *Pantoea* spp were identified as the dominant species on the whole melons, other Enterobacteriaceae species may have been present but were not dominant. The identification of *Pantoea* spp on the whole melon and then only again on the packaged melon at the end of shelf-life suggests that it must have been present at the other subsequent processing stages, but was not dominant.

The increase in diversification of two to three genera of Enterobacteriaceae from the whole to the cut melon could have been a result of the cutting operation performed during minimal processing as it is known that microbial growth is greater on fresh-cut products than on unprocessed fruit (Ayala-Zavala *et al.*, 2010a; Ayala-Zavala *et al.*, 2010b). Fruits consist of tissue that continues to be metabolically active even after harvest, during processing and storage (Siddiqui *et al.*, 2011). The slicing operation destroys protective membranes and barriers of living plants which leads to the release and intermixing of enzymes and substrates that can be used by native or exogenous microorganisms to grow on the product (Siddiqui *et al.*, 2011). Sweet melon is a rich source of nutrients including vitamin A, C, ß-carotene, carbohydrates, sugar, proteins and traces of vitamin K, B and niacin (USND, 2014b) to which microorganisms only have access after the cutting procedure. The Enterobacteriaceae species that were not identified as dominant species on the surface of the whole melon could therefore proliferate and dominate on the cut melon due to these changing conditions.

The dominant identified Enterobacteriaceae species could also have been introduced during the minimal processing procedure. Fresh-cut melon is metabolically active but has a higher respiration rate than whole fruit which causes physiological changes including increase in ethylene production, pH and total soluble solids content (Siddiqui *et al.*, 2011). Munira *et al.*, (2013) demonstrated that an increase in pH and soluble solids in fresh-cut melon leads to an increase in microbial growth. This may have been the reason why the greatest diversity of Enterobacteriaceae species was encountered on the packaged fresh-cut product. All the dominant species that were identified on the packaged melon product must have come from earlier processing stages as the product is sealed from the environment. The presence of *E. faecalis* as dominant species on the packaged melon suggests unhygienic practices from the minimally processing personnel as this species occurs only in the intestinal tract of animals and is therefore evident of faecal contamination (Upadhyaya *et al.*, 2009).

The packaged melon was stored at 0°C and this decrease in temperature may have caused the decrease in diversity of Enterobacteriaceae on the fresh-cut melon at the end of shelf-life. The majority of mesophilic microbes are still able to proliferate at 5°C but below this temperature growth is generally slow or completely inhibited (Huang *et al.*, 2011). The decrease in diversity may also have been due to competition as it is known that in a well-mixed environment, individuals with similar nutritional requirements, such as members of the same population, will be in competition for the acquisition of these nutrients as they become depleted by the growing population (Hibbing *et al.*, 2010). The identification of *Pantoea* spp corresponds to Jacxens, Deliegere, Ragaert, Vanneste, Debevere (2003) who identified *P. agglomerans* as the dominant species on fresh-cut fruit at the end of shelf-life.

The change in species composition of the Enterobacteriaceae over the various stages of minimal processing showed some findings that could potentially be beneficial to the minimal processing industry. Firstly, the prevalence of *E. coli* as predominant Enterobacteriaceae species at the end of shelf-life and no other preceding minimal processing stage shows the inefficiency of end-point testing to ensure food safety of a fresh-cut fruit product at the end of shelf-life (Figure 28). This demonstrates and confirms the advantage of hazard analysis and the critical control point based food safety management systems above the traditionally used end-point testing procedures (FAO, 2007). FSMS addresses food safety hazards that are 'reasonably expected to occur at different points of the food chain', or are difficult to monitor (CAC, 2003; FAO, 2007; Trienekens & Zuurbier, 2007).

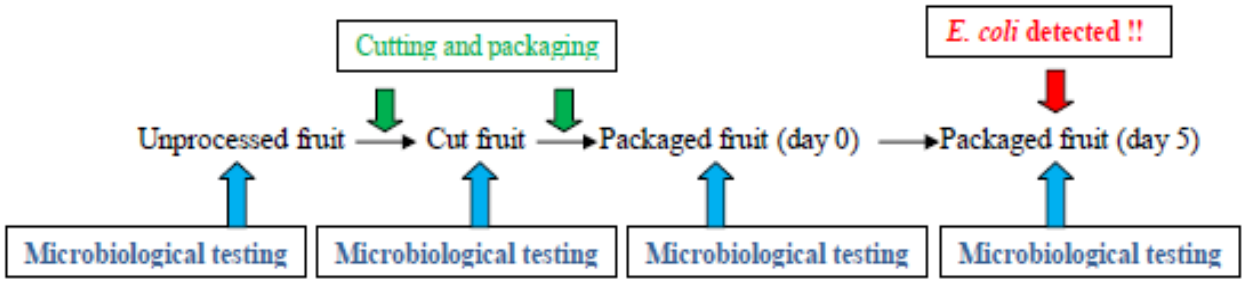


Figure 28: End-point testing does not ensure food safety at the end of shelf-life

Secondly, the presence of *E. coli* at the end of shelf-life suggests that it must have been present in preceding minimal processing stages, but was not detected. The pathogen may have been present in preceding minimal processing stages but was not predominant until the end of shelf-life. This demonstrates that if a microbiological test indicates the absence of a particular pathogen at a certain stage of minimal processing, it could actually be present but is not detected.

Thirdly, the consideration for *E. cloacae* as biocontrol agent could be beneficial to the fresh fruit minimal processing industry due to its predominance and uses for this purpose in the fresh-cut vegetable industry. The effectivity of an Enterobacteriaceae species that is dominant on fresh-cut apples as a biocontrol agent to control foodborne pathogens including *E. coli* O157:H7 has been demonstrated (Alegre *et al.*, 2012). Therefore, there may be potential to use this species as a biocontrol agent to reduce pathogen survival on fresh-cut fruit due to its dominance over the course of minimal processing.

There are limited methods to prolong the shelf-life of fresh-cut fruit. Biocontrol is considered to be health friendly by consumers and is expected to have a lower impact on nutritional and sensory properties as opposed to chemical or physiochemical treatments. Secondly, it could reduce costs while at the same time extending the product shelf-life period (Galvez, Abriouel, Benomar, Lucas, 2010).

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

The first hypotheses can be accepted. The higher levels of microbial spoilage indicators and higher prevalence of more foodborne pathogens on fresh-cut melon than fresh-cut mango suggest that fresh-cut melons are more susceptible to microbial spoilage and foodborne pathogens. Therefore, food safety management systems for fresh-cut melon from production to processing should be more stringent than for fresh-cut mango. The critical control points should be optimized for the respective fruit. In addition, more critical control points could be added in the production chain of fresh-cut sweet melons.

The second hypotheses can be accepted. The composition of the Enterobacteriaceae changed over the various stages of minimal processing and storage of fresh-cut melons. The diversity of species increased from the whole, fresh fruit to the packaged fresh-cut fruit product and then decreased again at the end of shelf-life. *E. cloacae* was identified to be the most predominant species throughout all stages of minimal processing and storage. *E. coli* was identified as a dominant species at the end of shelf-life.

This highlighted two findings that could be of importance to the minimal processing fruit industry. Firstly, it demonstrated the advantage of a hazard analysis critical control point (HACCP) food safety management system (FSMS) over end-point testing. Processors of fresh-cut mango and melon should incorporate process based testing such as HACCP rather than end point testing. Secondly, it showed the potential of *E. cloacae* to be used as a biocontrol agent in fresh-cut melon. The well adapted and pre-dominant *E. cloacae* should be further studied for potential utilization as biocontrol agent against foodborne pathogens on fresh-cut melons. Its use as a safe strain should then be studied as some strains within the species have been implicated with foodborne illness.

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