

Surface microbial ecology, food safety and horticulture production assessment of pear fruit (*Pyrus communis*)

By

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DECLARATION

I, Francois James Duvenage, declare that the thesis, which I hereby submit for the degree Master Science: Plant Pathology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Francois James Duvenage

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS	v
LIST OF TABLES	vi
LIST OF FIGURES	vii
AKNOWLEDGEMENTS	ix
ABSTRACT	1
CHAPTER 1	2
GENERAL INTRODUCTION	
REFERENCES	4
CHAPTER 2	6
POSTHARVEST BACTERIAL QUALITY AND SAFETY OF PEARS	
1. INTRODUCTION.....	6
2. MICROBIAL CONSIDERATIONS WITHIN PEAR PRODUCTION	7
2.1. POTENTIAL HAZARDS.....	9
2.2. FOODBORNE PATHOGENS	10
2.3. DETECTION OF FOODBORNE PATHOGENS.....	17
3. FOOD SAFETY	18
4. FOOD SAFETY MANAGEMENT SYSTEMS	19
5. CONCLUSION	22
6. REFERENCES	22
CHAPTER 3	33
COLONISATION AND SURVIVAL OF FOUR MAJOR BACTERIAL	
FOODBORNE PATHOGENS ON PEAR FRUIT (cv. FORELLE) SURFACES	
ABSTRACT	33
1. INTRODUCTION.....	34
2. MATERIALS AND METHODS	36
2.1. CULTURES.....	36
2.2. FRUIT	36
2.3. INOCULATION AND STORAGE OF FRUIT	37
2.4. METHODOLOGY FOR QUANTIFICATION OF MICROORGANISMS.....	37
2.5. SCANNING ELECTRON MICROSCOPY	37

2.6. STATISTICAL ANALYSIS	38
3. RESULTS	38
3.1. PEAR FRUIT SURFACE NEGATIVE CONTROL.....	38
3.2. <i>ESCHERICHIA COLI</i> O157:H7	41
3.3. <i>LISTERIA MONOCYTOGENES</i>	41
3.4. <i>SALMONELLA ENTERICA</i> SUBSP. <i>ENTERICA</i> SEROVAR TYPHIMURIUM.....	43
3.5. <i>STAPHYLOCOCCUS AUREUS</i>	43
4. DISCUSSION.....	43
5. CONCLUSION	47
6. REFERENCES	47
CHAPTER 4.....	53
BACTERIAL BIOMES AND DETECTION OF FOODBORNE PATHOGENS ON PEAR (cv PACKHAM'S TRIUMPH) FRUIT IN THE SUPPLY CHAIN	
ABSTRACT	53
1. INTRODUCTION	54
2. MATERIALS AND METHODS	55
2.1. SAMPLING STRATEGY	55
2.2. LABORATORY STRATEGY	57
3. RESULTS.....	60
3.1. PEAR SURFACE TEMPERATURES AT HARVEST	60
3.2. MICROBIAL QUALITY AND SAFETY OF PEARS	60
4. DISCUSSION.....	65
5. CONCLUSION	69
6. REFERENCES	70
CHAPTER 5.....	76
ASSESSMENT OF FOOD SAFETY MANAGEMENT SYSTEMS ON PEAR PRODUCTION FARMS IN THE WESTERN CAPE PROVINCE	
ABSTRACT	76
1. INTRODUCTION	77
2. METHODOLOGY	78
2.1. FARMER SURVEY AND DATA COLLECTION	78
2.2. FOOD SAFETY MANAGEMENT SYSTEM.....	78

2.3.	ASSESSMENT OF CONTEXT CHARACTERISTICS.....	79
2.4.	ASSESSMENT OF CORE SAFETY CONTROL ACTIVITIES	80
2.5.	ASSESSMENT CORE ASSURANCE ACTIVITIES	81
2.6.	ASSESSMENT OF FOOD SAFETY PERFORMANCE	82
3.	RESULTS AND DISCUSSION.....	82
3.1.	ASSESSMENT OF CONTEXT CHARACTERISTICS (PART I)	82
3.2.	ASSESSMENT OF CORE SAFETY CONTROL ACTIVITIES (PART II)	84
3.3.	ASSESSMENT OF CORE ASSURANCE ACTIVITIES (PART III)	88
3.4.	ASSESSMENT OF FOOD SAFETY PERFORMANCE (PART IV)	90
4.	CONCLUSION	94
5.	REFERENCES	95
CHAPTER 6	97
GENERAL DISCUSSION AND CONCLUSION		
REFERENCES	100
APPENDIX 1	103
APPENDIX 2	104
APPENDIX 3	105

LIST OF ABBREVIATIONS

CA.....	Controlled atmosphere
CDC.....	Centres for Disease Control and Prevention
CFU.....	Colony forming unit
CPC.....	Crop Protection Compendium
DAFF.....	Department of Agriculture, Forestry and Fisheries of South Africa
DoH.....	Department of Health
EU.....	European Union
FAO.....	Food and Agriculture Organisation
FDA.....	United States of America's Food and Drug Administration
FSART.....	Food Safety Rapid Response Team
FSI.....	Food Safety Initiative
FSMS.....	Food Safety Management System
GAP.....	Good Agricultural Practices
HACCP.....	Hazard Analysis Critical Control Point
HSMS.....	Horticulture Safety Management System
INFOSAN.....	International Network of Food Safety Authorities
ISO.....	International Standards Organization
MID.....	Minimum infectious dose
PPECB.....	Perishable Products Export Control Board
RASFF.....	Rapid Alert System for Food and Feed
SA.....	South Africa
SABS.....	South African Bureau of Standards
SEM.....	Scanning electron microscopy
USA.....	United States of America
USA EPA.....	United States Environmental Agency
WHO.....	World Health Organisation

LIST OF TABLES

TABLE 2.1. Top ten South African pear export destinations (2011-2013)	7
TABLE 2.2. Bacterial microorganisms typically associated with foodborne diseases	11
TABLE 2.3. Global food safety standards and management systems incorporated in the fresh food industries.....	20
TABLE 3.1. <i>Escherichia coli</i> O157:H7, <i>Listeria monocytogenes</i> , <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium and <i>Staphylococcus aureus</i> growth on pear surfaces over a period of seven days in cold storage conditions (5°C) following inoculation with a high and low concentration inoculum, respectively	40
TABLE 4.1. Primers for specific detection of human pathogens and identification through 16S.....	59
TABLE 5.1. The mean ratings of five farms on all assessments contributing to the mean food safety risk output	85

LIST OF FIGURES

FIGURE 3.1. <i>Escherichia coli</i> O157:H7, <i>Listeria monocytogenes</i> , <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium and <i>Staphylococcus aureus</i> growth trends on pear surfaces over a period of seven days in cold storage conditions (5°C) following inoculation with a high concentration inoculum	39
FIGURE 3.2. Electron micrograph of the pear surfaces (x4 500 magnification).....	40
FIGURE 3.3. Scanning electron micrographs of <i>Escherichia coli</i> O157:H7 at various time intervals.....	41
FIGURE 3.4. Scanning electron micrographs of <i>Listeria monocytogenes</i> at various time intervals.....	42
FIGURE 3.5. Scanning electron micrographs of <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium at various time intervals.....	42
FIGURE 3.6. Scanning electron micrographs of <i>Staphylococcus aureus</i> at various time intervals.....	44
FIGURE 4.1. Multiple approach workflow.....	56
FIGURE 4.2. Aerobic bacterial and coliform total viable counts on pear fruit surfaces, over two consecutive production seasons of four farms and central packhouse in the Western Cape Province (2013 – 2014)	61
FIGURE 4.3. Isolated dominant bacterial microorganisms identified from pear fruit surfaces	63
FIGURE 4.4. Phylogenetic tree based on dominant bacterial isolates' 16S rDNA sequences, constructed using the Neighbor-Joining methodology with allocated bootstraps.....	64
FIGURE 5.1. Assessment of product and production as well as organisation and chain characteristics from Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5 (E) The overall average assessment level of each farm and combined farms (F).....	86
FIGURE 5.2. Assessment of control activities design from Farm 1(A), Farm 2(B), Farm 3 (C), Farm 4 (D) and Farm 5 (E) The overall average assessment level of each farm and combined farms (F).....	87

FIGURE 5.3. Assessment of control activities operation of Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5(E). The overall average assessment level of each farm and combined farms (F).92

FIGURE 5.4. Assessment of food safety performance, external and internal food safety performance indicators of Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5 (E). The overall average assessment level of each farm and combined farms (F).93

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“Consult not your fears but your hopes and your dreams. Think not about your frustrations, but about your unfulfilled potential. Concern yourself not with what you tried and failed in, but with what it is still possible for you to do.” - Pope John XXIII

“If a man does not know to what port he is sailing, no wind is favorable.” - Seneca

ABSTRACT

Title: Surface microbial ecology, food safety and horticulture production assessment of pear fruit (*Pyrus communis*)

The World Health Organisation promotes increased consumption of fresh fruit and vegetables to address global health and nutritional challenges. Pome fruit are widely consumed and contribute to a healthy diet, therefore represent an important traded product. The South African pear export industry is ranked as one of the top ten international exporters of fresh fruit. The importance of retaining market access is thus important and compliance with international food safety requirements is essential. To the authors knowledge this is the first supply chain study that is focused on the microbial quality and safety of fresh pears in the postharvest environment. The findings in this study aid in a better understanding of the microbial dynamics of the fruit surface (carpoplane) and the microbial population shifts due to postharvest practices. Current national guidelines for ready-to-eat fresh produce place emphasis on bacterial loads and absence of selected foodborne pathogens. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* were demonstrated to attach, replicate and survive on the pear carpoplane. However, on market-ready pears these foodborne pathogens were not detected. Analysis of the carpoplane dominant bacterial populations reflected both harmful as well as beneficial residential bacteria that are known to either have the potential to affect human or plant health, respectively. The study provides an overview of the pear bacterial biome and this information can be used in future regulatory adjustments for food safety assurance. An assessment of the current industry wide food safety management practices reveal that not all aspects of the food safety assurance systems have been effectively implemented at an appropriate level.

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CHAPTER 1

GENERAL INTRODUCTION

South Africa export around 197 911 tons of pears annually [Perishable Products Export Control Board (PPECB), 2013] with 28% importing market share in the Netherlands (Department of Agriculture, Forestry and Fisheries, 2011). The South African pear export industry is considered the sixth largest in the world (PPECB, 2013). In order to retain the competitive edge in these international markets it is essential to assure full food safety compliance. A report by the Centres for Disease Control and Prevention (2013) stated that a proper risk analysis and microbial surveillance is needed, which includes determining the microbial load, profile and naturally occurring organisms, as well as determining the possible prevalence of human pathogens on fresh produce.

The number of foodborne disease outbreaks has been on the increase due to greater consumption and more extensive supply chains of fresh and raw fruit and vegetables (Beuchat, 2002; Brackett, 1999; Freshfel, 2013). South African Registry for the Food Safety Profession conducted a country-wide survey to determine the food safety health status of South African food. They found that in 2011 an estimated 16 million people were affected by foodborne illnesses and of these cases, 37 000 deaths were associated with diarrheal symptoms and waterborne pathogens (Petersen, 2011). Foodborne human pathogenic microorganisms accounted for contamination of fruit and vegetables globally causing outbreaks associated with *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and to a lesser extent *Staphylococcus aureus* (Batz *et al.*, 2012; Buck *et al.*, 2003; Nguyen, 2012; Olaimat & Holley, 2012).

Sources of foodborne pathogen contamination can occur at any point within the supply chain (Brackett, 1999; De Roever, 1998), therefore increasing the probability that consumers could be exposed to foodborne pathogenic microorganisms (Lammerding & Paoli, 1997). Supply chain management with proper food safety systems decreases the risk of foodborne pathogen exposure to the consumer. South Africa's pear export industry implemented internationally recognised good agricultural practices (GLOBAL-GAP) and has retained their certification status for most exporters. Good management practices include appropriate control measures to avoid contamination of the product by pathogenic (of plant and human origin) organisms along the supply chain (Hanning *et al.*, 2009).

The microbial balance on the fruit surface is characterised by a natural or residential bacterial, fungal and yeast population. These microbes are in constant interaction and contribute to the ecological stability of the micro environment (Leben, 1965). The constant competition provides a protective microbial barrier for the fruit surface (carpoplane) against foreign introduced pathogens with the potential to either cause foodborne disease outbreaks and illness or food spoilage (Barth *et al.*, 2010). This living carpoplane microflora (microbiome) is constantly evolving. Janisiewicz and Buyer (2010) concluded that it is therefore important to consider different sampling time intervals within the supply chain. Other factors that contributes to a population shift and favour disease development should also be kept in mind, such as favourable/unfavourable microbial environmental conditions and/or fruit surface wounding (Barth *et al.*, 2010).

Proper hazard assessment of fresh produce with on-farm production practices determines and drives the establishment of adequate food safety management system (FSMS) levels. Lunning *et al.* (2013) with adapted focus points by Kirezieva *et al.* (2013) developed a horticulture FSMS diagnostic tool. The functionality of the diagnostic tool determines the hazard risk levels in the overall production management, and indicate areas within the system that could be improved to decrease the overall risk of possible produce contamination by pathogenic microbial organisms.

The objective of this study was to define the status of a healthy pear carpoplane, through an in depth microbial food safety analysis, bacterial ecology assessment and an overall FSMS assessment. Firstly an inoculation experiment would determine the ability of the important and selected foodborne pathogen's to attachment and colonise the pear fruit surfaces. In addition, a cold chain simulation focusses on the effect of temperature on the growth rate and survival of the selected foodborne pathogens. A pear supply chain case study will be done to assess the food safety assurance status within the South African pear production industry and to determine presence or absence of foodborne pathogens within the system. To further fill the gap in knowledge, culturable dominant bacterial species on the healthy pear carpoplane was determined to propose the biota of a healthy fruit environment. Lastly the assessment of the FSMS further allowed recommendations to be made to producers allowing better implementation of food safety methods and to strengthen international/domestic pear trade within South Africa.

The outcome of this study seeks to add to the current knowledge base of the bacterial microflora status and safety of pears in the fruit chain.

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CHAPTER 2

POSTHARVEST BACTERIAL QUALITY AND SAFETY OF PEARS

1. INTRODUCTION

Fresh produce consumption has increased over the past 20 years due to a growing demand for healthy nutritious alternative food sources [Centres for Disease Control and Prevention (CDC), 2011a]. Due to this, complicated trade networks developed, which resulted in more effective and longer distribution systems. Pome fruit (apples and pears) are the most consumed fruit type in the European Union (EU) and second most important in the United States of America (USA) according to the World Apple and Pear Association (2014). Although pears are second to apples in quantity consumed, pears are highly versatile as the product can be consumed either raw, processed into juice, canned or dried.

Pears originated from eastern Europe and Asia. In South Africa (SA) the European pear (*Pyrus communis* L.) is the most favored pear species for commercial production. The pear industry is one of the most dynamic fruit sectors in the world with major producers including China (69%), USA (3%) and Italy (3%) [Food and Agriculture Organisation (FAO) Stat, 2015]. In 2013, South Africa was ranked as the seventh largest producer of pears in the world (343,203 tonnes) (FAO Stat, 2015). The SA pear industry accounts for R 1.4 billion (2009/2010) and is an important contributor to the total gross domestic product of the country [Department of Agriculture, Forestry and Fisheries (DAFF), 2011]. Favourable climatic and environmental conditions for the cultivation of pome fruit are found primarily in the southern parts of the country. The majority of producers are located in the Western Cape Province which is the centre of origin for 97% of all exported pears. The remaining 3% originate from seven of the nine provinces (DAFF, 2011). The total planted area is estimated to cover 12 690 ha (2012). Dominant pear cultivars in SA include Packham's Triumph (29%), Forelle (26%), Williams Bon Chretien (10%) and Early Bon Chretien (10%) (DAFF, 2011). The first two cultivars are not only the most planted but also most economically important in terms of exports (DAFF, 2011). South Africa exported 182,076 tons of pears from 2011 to 2013, with destination markets being the Netherlands, United Kingdom and the United Arab Emirates (Table 2.1) (Global Trade Atlas, 2014). The number of cartons (12kg) exported in 2013 were 3.1 million (20%) Forelle and 5.2 million (33%) Packham's Triumph, from January to August (Perishable Products Export Control Board, 2013).

Table 2.1. Top ten South African pear export destinations (2011-2013) (Global Trade Atlas, 2014)

Importing from South Africa	Trade Quantity (tons)		
	2011	2012	2013
Global Total	182,076	181,928	202,107
Netherlands	60,109	50,520	64,685
United Kingdom	20,102	17,785	20,258
United Arab Emirates	10,035	15,457	17,665
Russian Federation	13,271	16,180	15,486
Germany	9,945	7,770	10,794
France	5,980	6,575	8,705
Italy	4,670	5,241	7,032
Malaysia	7,237	8,020	5,714
Portugal	3,236	3,816	5,395
China, Hong Kong SAR	5,390	5,137	5,311

Although the harvesting season only spans over three months, the industry can store fruit for an extended period of time (up to nine months), ensuring consistent supply throughout the year.

In order for SA to maintain its market share, fruit quality and safety must be ensured. This requires expanding existing knowledge regarding the dynamic interactions between surface microflora and foodborne pathogens. In this context the bacterial quality of pears harvested and processed was investigated in this dissertation. The microbial flora (biota) on the pear fruit surface (carpoplane) and the impact that various interventions will have on the population stability is thus reviewed. In this chapter, the SA pear industry will be described in the context of potential hazards, food safety and quality assurance systems as well as the bacterial dynamics and quality assurance preventative control approaches.

2. MICROBIAL CONSIDERATIONS WITHIN PEAR PRODUCTION

In general, phyto-microbiomes are dynamic in composition when compared to the environments in which they proliferate (Lebeis, 2014). The plant microflora consists of a

variety of microorganisms coexisting within a characteristic habitat representing an ecological balance (Leben, 1965). Fruit surfaces harbor diverse natural residential microorganisms (Leff & Fierer, 2013), these include epiphytes, saprophytes, plant pathogens and human pathogens. The dominant phylogeny found on apples (pome fruit) have been identified as Microbacteriaceae and Sphingomonadaceae, as also described on peaches (Leff & Fierer, 2013). These authors described the impact of farming practices on the natural protection ability of the dominant microbial populations.

The naturally occurring microorganisms impact on the diversity and presence of plant and human pathogens (Abadias *et al.*, 2014; Alegre *et al.*, 2013; Alegre *et al.*, 2012), therefore influencing the exposure of the fruit and consumers to microorganisms possibly present. The epiphytic microorganisms provide a protective barrier against pathogenic microorganisms causing decay or food spoilage (Barth *et al.*, 2010), or contribute to food safety concerns. In an attempt to protect the fruit and the consumer from hazardous microorganisms, various biological; and other control measures have been developed and evaluated (Janisiewicz, 2012). In this context the carpoplane biome reflects the natural microflora with the potential to maintain an ecological balance and prevent pathogen multiplication, as illustrated by the biopreservation effect of *Pseudomonas* spp. on human pathogens possibly present in this sphere (Abadias *et al.*, 2014; Alegre *et al.*, 2013; Alegre *et al.*, 2012). This knowledge can be used to better understand the protective ability of the natural microbial community and the potential presence of pathogens. Focusing on the bacterial dynamics will give an insight into the likelihood of foodborne pathogens to establish, persist, compete, colonise and survive.

To date not much research has been done on the microbial populations of the pear carpoplane, specifically the bacterial populations with a focus on foodborne pathogens (Leff and Fierer, 2013). Therefore the investigation of the carpoplane microbiome at different postharvest stages will reflect on the impact of intervention strategies. Interventions such as washing and/or chemical application to the fruit surface disrupts the natural microbial balance, causing a population shift benefitting opportunistic organisms associated with food spoilage, decay or food safety (Corbo *et al.*, 2010).

2.1. POTENTIAL HAZARDS

Common practice in SA involves transporting harvested fruit for short distances in open trailers from orchards to packhouses, resulting in a period of open air exposure at room temperature. Upon arrival at the packhouse, fruit are drenched in horizontal drench baths containing 75ppm chlorine-water mixture, in order to disinfect fruit prior to controlled atmosphere (CA) storage, according to industry standards (South African Apple and Pear Association, 2013). The 75ppm chlorine-water mixture is managed according to Global Good Agricultural Practices (GAP), with the pH monitored daily. However, chlorine treatment (180ppm) has been shown not to significantly decrease titres of aerobic bacteria on cantaloupe (Fan *et al.*, 2009). Wei *et al.* (1995), have found that 100ppm chlorine solution failed to kill *Salmonella* Montevideo following a two minute treatment. In contrast, Iturriaga & Escatín (2010) demonstrated a 5 log reduction in *Salmonella* Montevideo following a 200mg/l chlorine treatment of tomatoes. Beuchat (1999) found that 200ppm chlorine killed or removed *E. coli* O157:H7 from lettuce at the same efficiency as deionised water. Rodgers *et al.* (2004) demonstrated 4.9 log reduction in *E. coli* O157:H7 and *L. monocytogenes* titres on fresh produce (apples, strawberries and cantaloupe) treated with chlorinated trisodium phosphate (100-200ppm chlorine). Following chlorine drenching fruit are moved to CA storage.

Postharvest practices differ between packhouses, some fruit are immediately packed and other fruit are stored under CA conditions (specific for the cultivar) prior to packing (Findlay & Combrink, 2013, Appendix 1). Fruit are stored in CA storage in order to extend shelf-life which can be up to nine months (Appendix 1). The extreme conditions of CA storage can cause a shift in the carpoplane population density and diversity (Corbo *et al.*, 2010). Farber (1991) reported that CO₂ has a significant and direct antimicrobial activity due to the alteration of the cell membrane. *Listeria monocytogenes* in particular is a facultative anaerobe and psychrotrophic organism, therefore the limited O₂ and cold temperature does not have an effect on this foodborne pathogen (Berrang *et al.*, 1989). In previous studies, it was found that CA storage had no effect on growth of *L. monocytogenes* (Berrang *et al.*, 1989), nor on the survival of *Salmonella* spp. (Daş *et al.*, 2006). Fruit are exposed to changing environmental conditions and treatments that can have an impact on the residual microflora. The exact effectiveness and influences that these combined production practises such as chlorine chemical drenching and CA storage have on the microbial population

dynamics and survivability of the bacterial organisms and in particular the major foodborne pathogen will be discussed in this dissertation.

2.2. FOODBORNE PATHOGENS

Demonstrating the presence of foodborne pathogens on fresh produce within the harvest environment is factual, but the collaborative hurdle effect of processing, storage conditions and the naturally occurring microflora place high pressure on the survivability of foodborne organisms (Abadias *et al.*, 2014; Alegre *et al.*, 2013; Alegre *et al.*, 2012). In the event that these foodborne pathogens are able to evade this hurdle (in the case of temperature mismanagement) these foodborne pathogens have increased potential of survival which could result in a foodborne outbreak. The detection of foodborne pathogens on fresh produce within the orchard is however not indicative of a looming outbreak. This is rather a sign that control strategies and a better food safety management systems (FSMS) are required in order to prevent foodborne disease causing organisms from establishing on the fruit. Food products that undergo processing steps prior to consumption cannot be assessed in the context of zero tolerance as is the case with ready-to-eat food.

Different foodborne pathogenic microorganisms have their own mode of infection and require infectious doses to cause disease in a broad spectrum of the population. The minimum infectious dose (MID) affects the quantitative level of risk. The concept that a single, viable, infectious pathogenic microbial cell is able to cause disease is considered a non-plausible argument and the MID response has been widely adopted. The MID is used as an indicator of risk of infection and are definite with an increased exposure to more pathogenic cells (Leggett *et al.*, 2012). Bacterial organisms more commonly associated with fresh produce disease outbreaks are illustrated in Table 2.2, including aspects such as the contamination level and food source specified. This study focused on three of the most important foodborne pathogens in terms of disease severity, impact from a health perspective, reported outbreaks and associated recalls. The most important pathogens in this context are discussed in the following sections.

ESCHERICHIA COLI

Escherichia coli have been identified as a potential pathogenic organism from the early 1885 (Feng *et al.*, 2002). Strains are grouped into different types, many of which are natural flora of the human intestine and are not considered harmful. These are noted to be environmental

Table 2.2. Bacterial microorganisms typically associated with foodborne diseases

Bacterial spp.	Type of disease symptom/s	Incubation period in humans	Disease symptom	MID (intake cells) or MTD (intake toxin)*	Food source reported on	Reference
<i>Bacillus cereus</i>	Infection or intoxication	1 to 24 hours	Diarrhea, nausea and vomiting	$> 10^5$ CFU	Rice dishes, sauces and soups, salads, fresh fruit	Kotiranta <i>et al.</i> , 2000
<i>Campylobacter jejuni</i>	Infection	2 to 5 days	Abdominal pain, diarrhea, enteritis, fever, and malaise	500 CFU	Contaminated water, poultry and unpasteurized milk, Fresh fruit and vegetables	Acheson & Allos, 2001
<i>Clostridium botulinum</i>	Intoxication	18 to 36 hours	Abdominal cramps, constipation, double and or blurred vision, dry mouth, muscle paralysis, nausea, vomiting, slurred speech, trouble breathing, and difficulty in swallowing.	LD50 = 0.03 ng/kg ("natural product")*	Fish products, honey (infant botulism), improperly canned foods, surfaces of fruits and vegetables	Arnon <i>et al.</i> , 2001
Diarrheagenic <i>Escherichia coli</i> (non-Shiga toxin-producing) (ETEC), (EPEC), (EIEC)	Intoxication or Infection	ETEC: 1 to 3 days. EPEC: 1 to 6 days. EIEC: 12 hours to 3 days.	Watery or bloody diarrhoea, abdominal cramps, with or without fever.	ETEC: 10^8 EPEC: 10^6 EIEC: 10^6 - 10^{10}	Warm blooded animal faeces contaminated food or water. Transmission from person-to-person may occur.	CDC, 2005; Nataro & Kaper, 1998; Public Health Agency of Canada, 2011
<i>Escherichia coli</i> (Shiga toxin-producing) (EHEC), e.g. O157	Infection	24 hours to 10 days	Abdominal cramps, acute hemorrhagic diarrhoea, hemolytic uremic syndrome (HUS) and vomiting	$10 - 10^2$	Contaminated water, raw fruit and vegetables, milk, juice, undercooked meat.	Hara-Kudo & Takatori, 2011; Nataro & Kaper, 1998

Table 2.2. continued

Bacterial spp.	Type of disease symptom/s	Incubation period in humans	Disease symptom	MID (intake cells) or MTD (intake toxin)*	Food source reported on	Reference
<i>Listeria monocytogenes</i>	Infection	12 hours	Confusion, fever, stiff neck, vomiting weakness, sometimes preceded by diarrhea	$< 10^6$	Meats, refrigerated spreads and seafood, raw vegetables and unpasteurized dairy products	Farber <i>et al.</i> , 1996
<i>Salmonella</i> spp.	Infection	12-72 hours	Abdominal cramps, diarrhea, fever and vomiting	$10 - 10^6$	Contaminated eggs, poultry, meat, unpasteurized milk or juice, cheese, contaminated raw fruits and vegetables (alfalfa sprouts, melons), spices, and nuts	Hara-Kudo & Takatori, 2011
<i>Shigella dysenteriae</i>	Infection	12 to 7 day	Central nervous system problems, colitis, malnutrition, rectal prolapse, reactive arthritis and tenesmus	<100	Contaminated water and food	Kurjak & Chervenak, 2006
<i>Staphylococcus aureus</i>	Intoxication	1 to 6 hours	Abdominal pain, diarrhea, erythematous rash, fever, hypotension, nausea, multiple organ failure, necrotizing pneumonia, shock, vomiting	1 – 25 μ g (toxin can be produced from 10^5 CFU/g)	Contaminated food source	Schmid-Hempel & Frank, 2007

*Toxins and Known LD50 Values; MID – Minimum Infectious Dose; MTD – Minimum Tolerated Dose. Table provided with minor changes from CDC (2001) and Department of Health (DoH) (2011).

E. coli strains, which could have beneficial effects (Kaper *et al.*, 2004; Rembacken *et al.*, 1999). *Escherichia coli* is typically divided into two groups, commensal *E. coli* and diarrhoeagenic *E. coli* (Omar & Barnard, 2010). Commensal *E. coli* can therefore be found in many different environments and are considered to be non-pathogenic. However, *E. coli* is used as an indicator of faecal contamination, due to the fact that the organism is a commensal inhabitant of warm blooded animals (Omar & Barnard, 2010). The spread of *E. coli* is mainly by means of contaminated faecal matter near a secondary source [World Health Organisation (WHO), 2011]. In SA, informal settlements along water catchment areas and rivers, ineffectively managed and overflowing sewage works as well as the contamination potential of livestock upstream have been associated with increased contamination of water sources with *E. coli* (Aijuka *et al.*, 2014; Du Plessis *et al.*, 2014; Olaniran *et al.*, 2009).

Diarrhoeagenic *E. coli* are disease causing strains and are divided into different patho-types including enterohaemorrhagic *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli* and enterotoxigenic *E. coli* (Nataro and Kaper, 1998). Enterohaemorrhagic *E. coli* is considered the most serious of the patho-types due to the severity of disease caused and are associated with most of the *E. coli* outbreaks. It is estimated that enterohaemorrhagic *E. coli* cause 173,000 illnesses and 21 deaths annually, of which *E. coli* O157 causes 63,000 illnesses and 20 deaths (CDC, 2015a). All other pathotypes cause 30,000 illnesses annually and no deaths (CDC, 2015a). Most studies focus on the presence of *E. coli* O157:H7, as this serotype is responsible for the most illnesses and deaths and can be considered a representative organism for enterohaemorrhagic *E. coli* (Abakpa *et al.*, 2015; Avila-Vega *et al.*, 2014; Feng *et al.*, 2015). *Escherichia coli* O157:H7 are able to spread between people coming into contact or through contaminated water sources (WHO, 2011). Detection of *E. coli* O157:H7 is challenging as the organism is found in low titres relative to other naturally occurring organisms (BioControl, 2015).

The CDC recorded the following fresh produce outbreaks due to various *E. coli* strains, from 2006-2013.

- *Escherichia coli* O157:H7 (EHEC) - Ready-to-eat salads, spinach and spring mix, Romaine lettuce, hazelnuts, fresh spinach and a melon outbreak resulting in several deaths.

- *Escherichia coli* O121 (EHEC) - Frozen food products, including quesadillas and other mini-snacks.
- *Escherichia coli* O45 (EHEC) - Shredded Romaine lettuce from a single processing facility.
- *Escherichia coli* O26 (EHEC) - Raw clover sprouts
- Shiga toxin-producing *E. coli* O104 (EHEC) - Sprouts produced in Germany and consumption of contaminated sprouts by travellers in Germany.

In a similar time frame, the Rapid Alert System for Food and Feed (RASFF) reported 27 *E. coli* alerts in the EU (RASFF, 2014). Infections were traced back to various sources which included; lentil-, bean-, soybean- and beetroot- sprouts, baby corn and baby spinach, lettuce, dates, cherry tomatoes, jackfruit, spinach, sugar peas and cucumbers.

Most recent studies of fresh produce at harvest revealed that *E. coli* O157 was detected from vegetables (Abakpa *et al.*, 2015), kiwi fruit (Feng *et al.*, 2015) and bell peppers (Avila-Vega *et al.*, 2014). All food recalls associated with *E. coli* contamination of produce were recalled due to the presence of enterohaemorrhagic *E. coli* [United States Food and Drug Administration (FDA), 2015]. A total of 12 recalls were associated with contaminated food and of those only six were associated with fresh produce (FDA, 2015). The only fruit associated with an *E. coli* recall was strawberries contaminated with *E. coli* O157:H7 (FDA, 2015). In the EU there were no *E. coli* - fresh produce related recalls (Food Recalls Europe, 2015).

LISTERIA MONOCYTOGENES

Listeria monocytogenes has been linked to food transmission since the early 1980s (Altekruse *et al.*, 1997). The organism causes listeriosis in humans consuming contaminated foods (Dieterich *et al.*, 2006), and the human pathogenic organism has a mortality rate of 20% of all infected people (BioControl, 2015). The annual estimated number of illnesses associated with *L. monocytogenes* in the USA is 1,600 and 250 deaths (CDC, 2015a). *Listeria monocytogenes* is commonly associated with uncooked meat and vegetables, dairy and dairy products and cooked and processed foods (CDC, 2012). From 1998 to 2008, *L. monocytogenes* was responsible for only 20 illnesses associated with plants, 52 with dairy, four with beef, three with pork and 127 with poultry (CDC, 2013). In the last three years there have been six major outbreaks of listeriosis in the USA, products included dairy

products (n=4) and fresh produce (n=2). In 2011, there was an outbreak of *L. monocytogenes* which was traced back to cantaloupe that resulted in 147 illnesses and 30 deaths (CDC, 2012), the original source of contamination is unknown. The RASFF reported five alerts in the EU of outbreaks of *L. monocytogenes* traced back to fresh produce in 2014 (RASFF, 2014). The most recent case of fresh produce associated *L. monocytogenes* outbreak in the USA was associated with pome fruit which resulted in seven deaths (CDC, 2015b). The source of the outbreak was pre-packaged caramel apples and the source of contamination was traced back to the company's apple packing facility, resulting in a voluntary recall of commercially produced and prepacked caramel apples (CDC, 2015b).

Listeria spp. are ubiquitous to the soil and water and are able to asymptotically survive in animal intestines (Ramaswamy *et al.*, 2006). Strawn *et al.* (2013) reported that prevalence of *L. monocytogenes* in terrestrial samples in production fields under study was 9.7% (51 of 526 samples collected). The presence of *L. monocytogenes* is therefore expected within the farming environment. However, in recent studies by Castro-Ibanez *et al.* (2015) and Avila-Vega *et al.* (2014), authors found no prevalence of *L. monocytogenes* from 144 samples (manure, soil, seed, water and baby spinach) and 528 bell peppers from the fresh produce production environment, respectively. Although this organism's prevalence within the fresh produce production environment is not fully described, *L. monocytogenes* is found on products ready for consumption as is evidenced by the number of annual recalls. *Listeria monocytogenes* contaminated fresh produce have resulted in 94 recalls in the USA all dated between 2011 and 2015 (FDA, 2015). Only 21 of those recalls were associated with *L. monocytogenes* contaminated fruit including recalls on contaminated apples and apple based products (n= 10), cantaloupe (n=7) and deciduous fruit mango (n=2) (FDA, 2015). In 2014, there were 11 recalls due to *L. monocytogenes* contaminated products, only one was associated with fresh produce (salad) (Food Recalls Europe, 2015).

SALMONELLA SPP.

Salmonella sp. was first described by Dr Salmon in 1885 and has been known to cause illness for over 125 years (CDC, 2015c). Only during the 1980s did it emerge as an important foodborne pathogen (Patrick *et al.*, 2004). Today, there are over two thousand known strains of the organism. Buzby and Roberts (2009) reported that 87% of all confirmed *Salmonella* spp. cases were confirmed to be foodborne. Today, *Salmonella* spp. are reported to cause the highest number of illnesses and deaths associated with food (CDC, 2015a). Annually,

one million illnesses and 380 deaths in the USA alone are associated with *Salmonella* spp. (CDC, 2015a). The human pathogenic *Salmonella* spp. are also responsible for the most diverse foodborne outbreaks covering a wide spectrum of products (CDC, 2015b; Heaton and Jones, 2008), including most commonly eggs, meat and poultry (Foodborne Illness, 2015). *Salmonella* spp. outbreaks are increasing annually with a 39% increase from 2012 to 2013 (CDC, 2015d). *Salmonella* spp. accounted for 41 multistate foodborne disease outbreaks in the USA over the past eight years (CDC, 2015b).

A few significant fresh produce related outbreaks accounted for these 41 multistate outbreaks, including the November 2014 outbreak, linked to the consumption of bean sprouts. The outbreak spread through 12 states in the USA, infecting 115 people (20% of the people hospitalised), no deaths were recorded and the causal agent was identified as *Salmonella* Enteritidis (CDC, 2015b). An earlier recorded outbreak was reported in Saintpaul in 2013 where contaminated cucumbers resulted in 84 illnesses with 17 hospitalisations and no deaths (CDC, 2013). Two major outbreaks were recorded in 2012 caused by *Salmonella* Braenderup, *Salmonella* Typhimurium and *Salmonella* Newport, resulting in 127 illnesses with 33 people hospitalised and 261 people affected resulting in the death of three. In this case contaminated foods were mangoes and cantaloupe, respectively (CDC, 2011). Sources of contamination were not specified and the pathogen could be traced back to the production companies. In the EU, RASFF was alerted to 39 illness cases of *Salmonella* spp. associated with fresh fruit and vegetables (RASFF, 2014).

Due to the frequency of *Salmonella* spp. outbreaks associated with food, the FDA recommends testing for the presence of *Salmonella* spp. and if detected the product should be recalled and destroyed (FDA, 2012). Of the 308 *Salmonella* spp. food recalls between 2009 and 2015 in the USA, 90 were associated with fresh produce (FDA, 2015). Fruit recalls included mangoes and mango products (n=8), cantaloupe (n=4), papayas (n=2) and other fruit (n= 14) with a total of 28 recalls associated with *Salmonella* spp. contaminated fruit (FDA, 2015). In 2014, there were eight recalls in the EU associated with *Salmonella* spp. contamination. Two were associated with fresh produce, one associated with contaminated sultanas and one with contaminated parsley and chives (Food Recalls Europe, 2015). However, recalls deal with the presence of *Salmonella* spp. on fruit ready for consumption and little is known about the presence of *Salmonella* spp. on fresh produce at the point of harvest and prior to retail sale. Three recent reports have described the presence of

Salmonella spp. within the production environment from basil and coriander (Delbeke *et al.*, 2015) and bell peppers (Avila-Vega *et al.*, 2014), however *Salmonella* spp. were not detected on kiwi fruit (n=193) (Feng *et al.*, 2014).

2.3. DETECTION OF FOODBORNE PATHOGENS

Detection of foodborne pathogens can be done through traditional methods such as viable plate counts and using indicator systems and using molecular tools such as PCR and sequencing (Law *et al.*, 2015). Both traditional and molecular methods have drawbacks and specific advantages in terms of accuracy, repeatability, sensitivity and timeliness. Viable plate techniques include the use of specific designed chromogenic media. The media functions to selectively allow for the growth of the targeted group of organisms. The viable count approach is based on non-selective and selective media. Eosin methylene blue agar specifically focuses on the suppression of Gram positive bacteria (Levine, 1918) to promote the growth of *E. coli*. Oxford-Listeria medium provides a combination of chemical substances to cause inhibition of organisms other than *Listeria* spp. (including *Listeria monocytogenes*). Chromogenic media such as Brilliance Salmonella agar contain inhibition properties that are able to lyse cell walls of non-targeted bacteria. The drawback of each of these chromogenic media detections are that possible high numbers of false positives are obtained as reported by Holfelder *et al.* (1998).

Multiplex PCR methodologies reported by Standing *et al.* (2013) uses a combination of primers targeting specific genes for each of the three foodborne pathogens. The primers (Chapter 4, Table 4.1) identifies, *E. coli* O157:H7 (Cebula *et al.*, 1995), *Listeria monocytogenes* (Thomas *et al.*, 1991) and *Salmonella* Typhimurium (Standing *et al.*, 2013). The biggest drawback on this methodology is that genomic level detection cannot distinguish between live and dead cells, only the presence and/or absence of targeted organisms. Therefore after positive identification of genomic level of detection, viable cultural methodologies should be focused on establishing whether the organism is alive in the matrix tested. Advanced levels of detection methodologies used are done through 3M Molecular Detection Systems where the technology uses isothermal DNA amplification and bioluminescence detection for accurate results.

3. FOOD SAFETY

The current increase in number of fresh produce associated outbreaks can directly be linked to increased consumption of raw fruits, vegetables and salads (Brackett, 1999). In the EU, 576 notifications were received by the EU RASFF in 2010, whereas 732 were received in 2014, also showing an increasing trend (RASFF, 2014). This has resulted in global food safety awareness and effective implementation.

Due to the increasing outbreaks associated with foodborne pathogens, countries have implemented set standards and guidelines with regard to presence of selected foodborne pathogens. The EU and United States Environmental Agency (US EPA) have a zero tolerance for *L. monocytogenes* and *Salmonella* spp. within a 25 gram sample size (European Commission, 2012), whereas it is required that *E. coli* be absent from one gram of food sample. Thermotolerant (faecal) coliforms should be lower than 10 CFU/g. Aerobic plate counts of food products is set to be lower than 10^5 CFU/g (European Commission, 2012). The SA Department of Health (DoH) also implemented a set of microbial food safety guidelines for the consumption of ready-to-eat raw fruit and vegetables to prevent possible foodborne disease outbreaks (DoH, 2006). The guideline directs the food industry towards safer food products at the ready-to-eat stage. This microbial guideline describes total viable limitations of microbial organisms on ready-to-eat foods including fresh produce consumed raw. There is currently no definitive criteria for aerobic bacteria on raw fruit and vegetables. For both *E. coli* and *L. monocytogenes* a zero tolerance level is set with food being considered safe if samples are free of viable colonies. Coliforms have to be less than 200 CFU/g and *Salmonella* spp. lower than 6.25 CFU/25g raw fruit and vegetables. In comparison to SA standards, the EU and US EPA are more stringent in terms of the presence of *Salmonella* spp. and thermotolerant (faecal) coliforms (DoH, 2006; European Commission, 2012). In order for discrepancies in standards to be addressed and to develop commodity specific standards, the natural population densities on fresh produce which needs to be reconsidered given the new body of evidence emerging regarding phytobiomes.

Set standards emphasise the growing importance of basic pre-requisite programmes such as GAP and effective hygiene and sanitation systems which have become the universally adopted minimum requirement for trade. Basic FSMS are thus not only being developed but also universally implemented through third party certification. All participants in the food system require some form of self-assessment going beyond self-regulatory systems. Safer

foods can only be assured through effective FSMS, regulatory compliance and a more advanced level of hazard analysis, risk assessment and preventative control. In essence a FSMS is based on ISO 22000:2005 and include more advanced systems such as Hazard Analysis Critical Control Point (HACCP), pre-requisite programmes and regulatory compliance. Alternative owner schemes have evolved over the past 20 years and include standards such as Global GAP, BRC, FSSC 22000 and FAMI-QS (Table 2.3). All major retailers require producer compliance to one of these systems and producers delivering products to more than one supplier will often have to be certified to the different standards or schemes. This despite the global push towards “once certified, accepted everywhere” (Global Food Safety Initiative, 2015).

4. FOOD SAFETY MANAGEMENT SYSTEMS

Current FSMS evolved on the basic principles of HACCP (The Registrar Company, 2015). The Codex Alimentarius Commission adopted the HACCP principles as a universal food safety system (Hulebak & Schlosser, 2002). During the 1990’s the first production assurance system was developed as EurepGAP (Global-GAP, 2015). Since then numerous other private certification systems were developed (Table 2.3) all having the same basic elements of management. As food safety systems evolve, the general criteria include additional parameters that cover microbial, chemical and physical hazard analysis, risk assessment, management systems, standard operational procedures, traceability and document control. Aspects like fair trade, worker health and safety and environmental protection can also form part of the FSMS. Clear documentation and transparency ensure food safety and retailer requirements are met. Despite certification, the proliferation of voluntary standards and government regulations, several high profile disease outbreaks have occurred and have been associated with certified farms. Thus indicating the need for improvement of the system (FDA, 2010) and following a more science based approach.

In 2013, in order to aid producers in continual improvement of their FSMS, a horticultural safety management system (HSMS) self-assessment tool was developed (Luning *et al.*, 2013) and adapted for the production systems (Kirezueva *et al.*, 2015). Therefore, some aspects in the production system (of fruit) does not apply to the current outline and/or design of the HSMS system (Kirezueva *et al.*, 2013). The value of the HSMS self-assessment tool is focused on the farmer/company and provides a better framework of understanding of effective FSMS implementation. This HSMS self-assessment tool is considered value-

Table 2.3. Global food safety standards and management systems incorporated in the fresh food industries

Title	Production Target	Criteria / Quality management / Principles	Date of est.	Ownership / Rights	Number of members/ certified firms / Auditors / Accreditation bodies	Food Safety Elements
Global GAP - Good Agricultural Practice (Global GAP IFA V5)	Food Safety and traceability	All relevant aspects on product safety, documentation, environmental impact and the health, safety and welfare of workers and animals	1997	Euro-Retailer Produce Working Group	50 Retail & Food Service 181 Producer & Supplier 149 Associate Members 140+ Certification bodies 5 Integrity Surveillance Committee members Across more than 100 Countries	Management responsibility, Food safety plan Documentation and record keeping, Worker education and training, Sampling and Testing Traceability, Recall program Corrective actions, Self-audits Worker hygiene, Agricultural chemicals and plant protection products
HACCP - Hazard Analysis and Critical Control Points	Food Safety	Conduct a Hazard Analysis Define Critical Control Points Establish: Critical Limits, Monitoring Procedures, Corrective Actions, Verification Procedures, Record-Keeping and Documentation Procedures	1963	None but accepted by World Health Organisation and adopted in Codex Alimentarius	Codex Alimentarius Commission: 186 Codex Members 185 Member Countries 1 Member Organization (EU) 225 Codex Observers - 52 IGOs, 157 NGOs, 16 UN.	Good Manufacturing Practices (GMPs) (relating to personnel and the food processing environment) and HACCP plans (prevent, eliminate or reduce potential hazards)
ISO 22000 - International Organisation for Standardization (ISO 22000:2005)	Food Safety management system	Quality Management System, Augment HACCP on issues related to food safety	2005	International Organisation for Standardisations	Member bodies (Counsel members) - 88 (15) Correspondent members - 46 Subscriber members - 14	Interactive communication System management Prerequisite programs HACCP principles

Title	Production Target	Criteria / Quality management / Principles	Date of est.	Ownership / Rights	Number of members/ certified firms / Auditors / Accreditation bodies	Food Safety Elements
SQF - Safe Quality Food standard	Food Safety and Quality	HACCP-based	2005	Administered by the Food Marketing Institute (FMI) (developed in Western Australia in 1990's)	30+ Certification Bodies	Company interrelationship, System management and adaptations, Information transfer to employees, Customer complaints, Production audit, Product analysis, Corrective actions and product withdrawal/recall.
IFS Food - International Food Standard	Food Safety and Quality	Establish standards with uniform evaluation systems, comparability and transparency throughout the entire supply chain, reduce costs and time for both manufacturers and retailers	2003	Hauptverband des Deutschen Einzelhandels (HDE) Fédération des Entreprises du Commerce et de la Distribution (FCD)	15 000+ Certificates (90+ countries) 800+ Auditors 100 Certification bodies	Control of critical control points; Involvement of management and employees; Tractability of product, production material and packaging; Implementation of corrective measures
BRC - British Retail Consortium	Food Safety and Quality	Evaluation of companies supplying retailer branded food provider of in-depth retail information	1992	BRC Trading Limited	22 000+ Certificated suppliers 123 Countries	Senior management commitment and Continual improvement, Food safety plan (HACCP) Food safety and Quality management system Production site standards, Product control Process control and documentation Personnel training
FSSC 22000 - Food Safety System Certification (FSSC 22000: 2015)	Food Safety and Quality	Conduct a hazard analysis establish: Critical limits, Monitoring procedures, Corrective actions, Verification procedures, Record-keeping and Documentation procedures	1996	National Board of Experts in HACCP	106 Certification Bodies 10 000+ Certified organisations	Process and manufacturing; Shelf life extension; Ingredient added management; Packaging management

adding for farmers/companies who have evolved a higher level of awareness (Kirezieva *et al.*, 2013). Moreover, Kirezieva *et al.* (2015) reported that in depth studies should accompany this tool to broaden the knowledge base and strengthen the tool to ensure improved management.

5. CONCLUSION

Current information published encourages consumption of fresh fruit and vegetables. Pears are a popular fruit consumed raw and often unprocessed. The pear carpoplane provides a dynamic environment for microbial populations which may include foodborne pathogens, these microorganisms thrive in a natural balance. Different production practices affect this natural balance by disrupting the pear carpoplane microbiome. Production practices allow fruit to be stored for extended periods of time, resulting in extensive supply chains with increased handling of fruit, therefore increasing the associated food safety risk. Currently, information on the presence of foodborne pathogens on pears at the point of harvest is limited, but the impact of one pathogen contamination event may cause extensive losses to the industry. The importance of the impact of production practices and its effects on the natural microbial populations has been shown in other studies and can ultimately influence the risk to the consumer. In order to manage food safety hazards in the food supply chain, FSMS have been designed and require the implementation of control points in order to reduce exposure of produce to various hazards. Although various standards and owner schemes exist foodborne disease outbreaks still occur reflecting the need for a more risk and science based approach.

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CHAPTER 3

COLONISATION AND SURVIVAL OF FOUR MAJOR BACTERIAL FOODBORNE PATHOGENS ON PEAR FRUIT (cv. FORELLE) SURFACES

ABSTRACT

Foodborne pathogens associated with fresh produce disease outbreaks include *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and to a lesser extent *Staphylococcus aureus*. Foodborne pathogens have been shown to contaminate fresh produce and survive on these surfaces for extended periods of time, therefore possibly leading to foodborne illnesses. In this study the ability of foodborne pathogens to survive on pear fruit surfaces was investigated. Freshly harvested pear fruit, cv. Forelle, were spot inoculated (10^5 cfu/ml, low and 10^7 cfu/ml, high concentration) and then stored under cold storage (5°C) conditions for one week to simulate direct, local, trading conditions. Viable counts on chromogenic media were used to determine the survival of the foodborne pathogens while scanning electron microscope was used to monitor the development of attachment structures, formation of microcolonies and colonisation. Viable counts of foodborne pathogens on fruit inoculated with high concentrations reflected a gradual increase in pathogen titres from immediately after inoculation to one hour later. Following cold storage conditions of 5°C after one day all microbial titres decreased significantly. No significant trend were observed following low concentration inoculation but there was an overall decrease in titres. Attachment structures from the pathogens on the fruit surfaces were visible within 30 seconds of exposure time with *E. coli* O157:H7 and *Salmonella* Typhimurium. All pathogens were able to colonise the pear surface by forming microcolonies following longer exposure times. High artificial inoculation studies indicated that only *Salmonella* Typhimurium survived at titres that would exceed the minimum infectious dose. In contrast, following low concentration inoculation studies, *E. coli* O157:H7 and *Salmonella* Typhimurium were able to survive at concentrations exceeding the minimum infectious dose. In conclusion, contaminated prior to a seven day storage period at refrigeration temperatures, only *E. coli* O157:H7 and *Salmonella* Typhimurium have the ability to survive on the fruit. However these conclusions are based on simulated laboratory conditions and artificial inoculum concentrations. Future studies should investigate other environmental factors, lower inoculation loads as well as host response to the interaction of foodborne pathogens, natural occurring biocontrol microorganisms, and host specific plant pathogenic and residential epiphytic microorganisms on the fruit surfaces.

1. INTRODUCTION

Bacterial foodborne pathogens on fresh produce have become a major topic in all the food safety related studies. The effect of human foodborne pathogen exposure and contamination of fresh produce could potentially be life threatening, and result in major disease outbreaks. In the United States of America (USA) alone it is estimated that 1 351 deaths occur and 55 961 people are hospitalised annually as a result of consumption of contaminated food products (Scallan *et al.*, 2011). The estimated number of sporadic foodborne diseases cases reported in the USA account for 9.4 million illnesses (Scallan *et al.*, 2011). Figures such as these are often considered as underestimations as not all incidences are recorded or reported (Mead *et al.*, 1999). In developing countries foodborne illness statistics are particularly underestimated because foodborne illness surveillance systems are not as effective and sometimes does not even exist.

The South African Registry for the Food Safety Profession concluded through a survey done in 2011 that an estimated 16 million people are affected by foodborne illnesses annually, of these cases 37 000 resulted in death with diarrhoeal symptoms (Petersen, 2011). No formal scientific article could support this information but it has been reported on in local newspaper. One example is for instance an episode of foodborne illnesses (the causal agent not released) that occurred in April 2013 which resulted in one fatality, and 27 hospitalisations (Mthethwa, 2013). Another example was in June 2014 through drinking of *Escherichia coli* contaminated river water which led to two mortalities (Mbangeni & Lee, 2014). The Human Rights Commission stated that 16 million South Africans do not have access to clean safe water (National Institute for Communicable Disease, 2013). A six month monitoring period recorded 423 cases of acute poisonings in South African hospitals, where 5.4% (n=23) were foodborne related (Malangu & Ogunbanjo, 2009).

Foodborne pathogens that can typically cause foodborne disease outbreaks on freshly consumed fruit and vegetables are *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and to lesser extent *Staphylococcus aureus* (Batz *et al.*, 2012; Buck *et al.*, 2003; Nguyen, 2012; Olaimat & Holley, 2012). In the USA from 2006 to 2014 a total of 66 foodborne outbreaks were investigated, 30% of the incidences were *E. coli*, 6% were cases of *L. monocytogenes* and 57% were cases of *Salmonella* spp. [Centres for Disease Control and Prevention (CDC), 2014a; Centre for Science in the Public Interest (CSPI), 2013]. Fresh produce were the source of the contamination in 15 *Salmonella* spp. outbreaks, these products

included cucumbers, mangoes, cantaloupe, nuts, papaya, alfalfa sprouts and tomatoes. Seven *E. coli* outbreaks were associated with fresh produce which included salad mix, clover sprouts, Romaine lettuce, hazelnuts and spinach. Only one *L. monocytogenes* outbreak was associated with fresh produce (cantaloupe). Currently a significant number of outbreaks of foodborne diseases have been linked to the increase in consumption of fresh fruits, vegetables and salads [Brackett, 1999; Beuchat, 2002; CDC, 2015a; CDC, 2015b; CDC, 2015c; CDC, 2014b; CDC, 2013; CDC, 2012; Freshfel, 2013)].

The increase in number of foodborne pathogen outbreaks yearly are attributed to a number of factors. These include the increase in consumption of fresh produce, increased surveillance and consumer awareness of foodborne illnesses as well as an increase proportion of consumers being immunocompromised. The likelihood that fresh produce can become contaminated has lead producers, exporters and retailers to increase food safety assurance measures. Food safety assurance can be achieved through voluntary and trade enforced food safety management practices that ensure all the appropriate controls are in place to avoid contamination of the product along the supply chain (Hanning *et al.*, 2009).

Bacterial contamination of fresh produce can occur at any point along the supply chain (Brackett, 1999; De Roever, 1998). Foodborne pathogens can be introduced to the fruit surface and contaminate the product through the use of contaminated irrigation and pesticide water, unpasteurised manure, the unsanitary handling of fruit, contaminated fruit wash water, ineffective sanitation systems as well as contaminated equipment (Althaus *et al.*, 2012, Brackett, 1999; De Roever, 1998; James, 2006; Koo *et al.*, 2014; Lambertz *et al.*, 2013). A number of previous studies have demonstrated that *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *S. aureus* are able to attach, colonise and form biofilms on the surfaces of plant tissue (Annous *et al.*, 2005; Bae *et al.* 2013; Barak *et al.* 2002; Brandl & Mandrell, 2002; Charkowski *et al.*, 2002; Cooley *et al.*, 2003; Collignon & Korsten, 2010; Han *et al.*, 2000; Palumbo *et al.*, 2005; Patel & Sharma, 2012; Richards *et al.*, 2004; Sirinutsomboon *et al.*, 2011; Solomon & Matthews, 2006; Tang *et al.*, 2012). To the authors' knowledge no studies have investigated the attachment, colonisation and survival of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium and *S. aureus* on pear fruit surfaces.

The aim of the study was therefore to determine the ability of these foodborne pathogens to attach, colonise and survive on pear fruit (*Pyrus communis* cv. Forelle) surfaces under simulated cold chain conditions for direct marketing of fruit.

2. MATERIALS AND METHODS

2.1. CULTURES

American Type Culture Collection (ATCC) cultures of *E. coli* O157:H7 (ATCC 35150), *L. monocytogenes* (ATCC 19115), *S. enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), and *S. aureus* (ATCC 12600) were used. All pure cultures were maintained freeze dried and stored at -70°C. Subcultures were grown on Baird-Parker agar (BP) for *S. aureus*, Oxford Listeria selective agar (OL) for *L. monocytogenes*, Levine eosin–methyl blue agar (EMB) for *E. coli* O157:H7, and xylose lysine deoxycholate agar (XLD) for *Salmonella* Typhimurium (All media were purchased from Merck, Johannesburg, South Africa) prepared 24 hours before use and culture preparations for inoculation done according to Collignon and Korsten (2010). The final inoculum concentrations were at 7 log CFU/ml (high) and 5 log CFU/ml (low). Titres were confirmed by serial dilution and subsequent plating in duplicate.

2.2. FRUIT

Freshly harvested physiological mature pear fruit (*Pyrus communis* cv. Forelle) of similar morphological characteristics (size and weight) with no pest, disease or mechanical damage were sourced from a commercial farm in the Western Cape Province of South Africa. Fruit collected were placed in paper bags, transported in cooler boxes and stored at 4°C (approximately 12 to 15 h) before processing. The cultivar was used because it is one of the most important export cultivars (20.07 %; PPECB, 2014) and has a soft skin that is easily perishable.

Eighty pears were inoculated for pathogen quantification and 20 fruit for scanning electron microscopy (SEM) analysis. The fruit were divided into three sets. Set 1 and Set 2 were used to quantify the pathogen titre. Set 1 fruit were used for high-inoculum inoculation and consisted of 50 pears (5 replicates for 9 time intervals selected plus 5 negative controls). Set 2 were used for low-inoculum inoculation and consisted of 30 pears (5 replicates for 5 time intervals selected, plus 5 negative controls). Set 3 was used for SEM analysis and consisted of 20 pears (9 time intervals with 4 pathogens per fruit and 1 negative control per time interval). Fruit for SEM studies were surface sterilised using a 30 seconds dip treatment in 70% ethanol (Spurr, 1979) followed by air drying in the laminar flow cabinet. Fruit from Set 1 and Set 2 were washed with 0.05% (vol/vol) sodium hypochlorite for 30 seconds, rinsed twice with sterile distilled water, and allowed to air dry in the laminar flow cabinet.

2.3. INOCULATION AND STORAGE OF FRUIT

Spot inoculation of high, low concentration and SEM sample preparation was conducted as outlined by Collignon and Korsten (2010) with the following time intervals for the SEM and high concentration: 0, 30 and 60 seconds, 15 minutes, 1 hour, 1, 2, 5 and 7 days. The low concentration inoculated fruit were analysed at the following time intervals: 0 seconds, 1, 2, 5 and 7 days. Inoculated fruit were stored at 5°C immediately after inoculation until the following time intervals: 1, 2, 5 and 7 days. Cold storage up to a week was used to simulate the local pear distribution system to the point of sale.

Following spot inoculation, the concentrations of all cultures were confirmed by serial dilution and plating onto selective media [BP (Merck) for *S. aureus*, OL (Merck) for *L. monocytogenes*, EMB (Merck) for *E. coli O157:H7*, and XLD (Merck) for *Salmonella* Typhimurium].

2.4. METHODOLOGY FOR QUANTIFICATION OF MICROORGANISMS

Following outlined time intervals, fruit were washed and bacterial pathogen titres were quantified (Collignon and Korsten, 2010). Volume displacement (vd) was also recorded for each fruit and converted to area (cm²) (De Jager, 1999) to determine a CFU/cm²:

$$A = 4.84 [(vd)^{1/3}]^2$$

Counts were converted to CFU/cm² and transformed to log (x+1) CFU/cm².

2.5. SCANNING ELECTRON MICROSCOPY

Set of three fruit were used for the SEM evaluation and uninoculated fruit was used as a negative control. The excised sections were stored, critical point dried, mounted and viewed according to Coetzee and van der Merwe (1994).

Sample analysis under the SEM started with the negative control to ensure no contaminating microorganisms were present on the fruit surface and to become familiar with the carpoplane. A positive control was viewed to familiarise the viewer with actual bacterial shapes and sizes. Thereafter the later time intervals were viewed to ensure recognition of pathogenic microorganisms followed by the rest of the samples. Viewing constituted of 15 randomly selected areas, with a magnification set at 3000x, authors ascertain glycocalyx formation, replication and biofilm formation based on visual observations and comparisons with previous publications described by Yaron and Römling (2014).

2.6. STATISTICAL ANALYSIS

Five replicates were used throughout and the experiment was repeated once. Statistical analysis was performed on log CFU/cm² and log cells/cm². Data were analysed using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC). A one-way analysis of variance was used to determine the difference in pathogen titres on fruit surfaces. At a 5% level of significance and by using least significant differences (using the Fisher test), means were able to be determined.

3. RESULTS

The results reflect viable counts of the bacterial pathogens over a predetermined timeframe. The growth trend on fruit inoculated with a high concentration show a common peak at one hour by all four pathogens tested, thereafter all pathogen titres decreased significantly (Figure 3.1 and Table 3.1). Growth of pathogens on fruit inoculated with a low concentration inoculum had varying results. All individual results are further outlined per pathogen group.

Scanning electron microscope image results depicting survival are supported through the total viable count data (Figure 3.3, Figure 3.4, Figure 3.5 and Figure 3.6). The uninoculated pear surface characteristics had no epiphytic or other residential microbes present (Figure 3.2). Attachment and colonisation could be confirmed through comparative description of similar referenced articles as well as SEM imaging and expert assessment.

3.1. PEAR FRUIT SURFACE NEGATIVE CONTROL

Negative controls under the SEM showed that the surface was free of contamination, therefore demonstrating that the surface disinfection was effective (Figure 3.2). Surfaces were considered to be smooth with no trichomes and or other structures. The pear fruit surfaces were covered with micro cracks and the occasional minor microscopic mechanical damage i.e. ruptures of the pear skin.

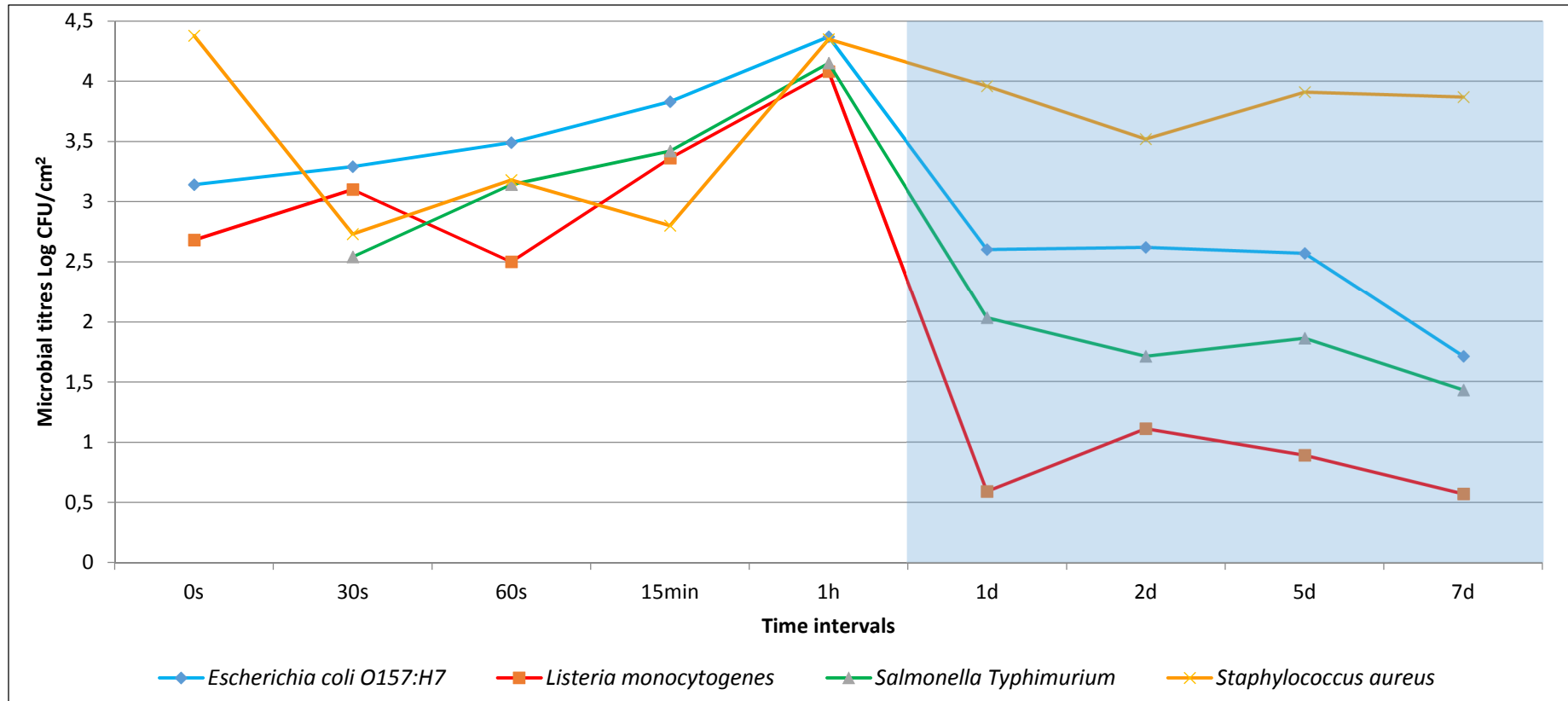


Figure 3.1. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* growth trends on pear surfaces over a period of seven days in cold storage conditions (5°C) following inoculation with a high concentration inoculum.

Area and time intervals shaded in blue indicate when fruit were stored at refrigeration temperatures (5°C).

Table 3.1. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* growth on pear surfaces over a period of seven days in cold storage conditions (5°C) following inoculation with a high and low concentration inoculum, respectively

	Time Interval	<i>Escherichia coli</i> O157:H7	<i>Listeria monocytogenes</i>	<i>Salmonella</i> Typhimurium	<i>Staphylococcus aureus</i>
High Inoculum concentration (7 log CFU/ml)	0s	3.14 bc	2.68 b	*	4.38 a
	30s	3.29 bc	3.10 ab	2.54 de	2.73 c
	60s	3.49 abc	2.50 b	3.14 bc	3.18 bc
	15min	3.83 ab	3.36 ab	3.42 b	2.80 c
	1h	4.37 a	4.08 a	4.15 a	4.35 a
	1d	2.60 cd	0.59 c	2.03 ef	3.96 ab
	2d	2.62 cd	1.11 c	1.71 fg	3.52 abc
	5d	2.57 cd	0.89 c	1.86 fg	3.91 ab
	7d	1.71 d	0.57 c	1.43 g	3.87 ab
Low Inoculum concentration (5 log CFU/ml)	0s	2.71 a	0.85 b	1.52 a	2.45 a
	1d	2.41 a	1.60 a	1.78 a	1.96 ab
	2d	1.90 a	0.06 c	1.44 a	1.26 c
	5d	2.26 a	0.02 c	1.70 a	1.68 bc
	7d	2.08 a	0.46 bc	1.29 a	1.64 bc

Bolded small caps represent the least significant difference according to the Fischer Test (P<0.05). Values shaded in blue indicate when fruit were stored at refrigeration temperatures (5°C).

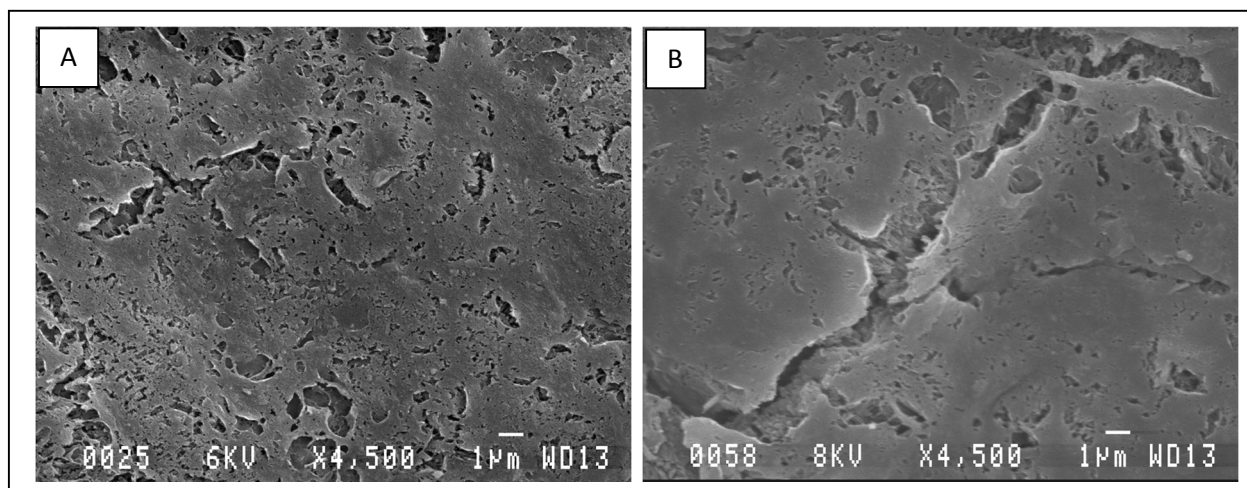


Figure 3.2. Electron micrograph of the pear surfaces (x4 500 magnification).

(A) The pear surfaces have numerous micro cracks. (B) The rupture in the surface indicates a possible niche area with possible flow of nutrients that may occur here, but is clear of microorganism contamination.

3.2. *ESCHERICHIA COLI* O157:H7

Escherichia coli O157:H7 titres on fruit inoculated with low concentration inoculum were not significantly different from fruit viewed immediately to seven days after inoculation, following storage at refrigeration temperatures (Table 3.1). Titres on fruit inoculated with high concentrations showed an overall gradual decrease from immediately to seven days after inoculation (Figure 3.1 and Table 3.1). Titres were the highest after 60 seconds, 15 minutes and 1 hour, thereafter titres were significantly lower at 1 day after storage at 5°C.

Attachment structures were observed following 60 seconds (Figure 3.3 A) and colonisation was evident with the observation of replication at 2 days (Figure 3.3 B).

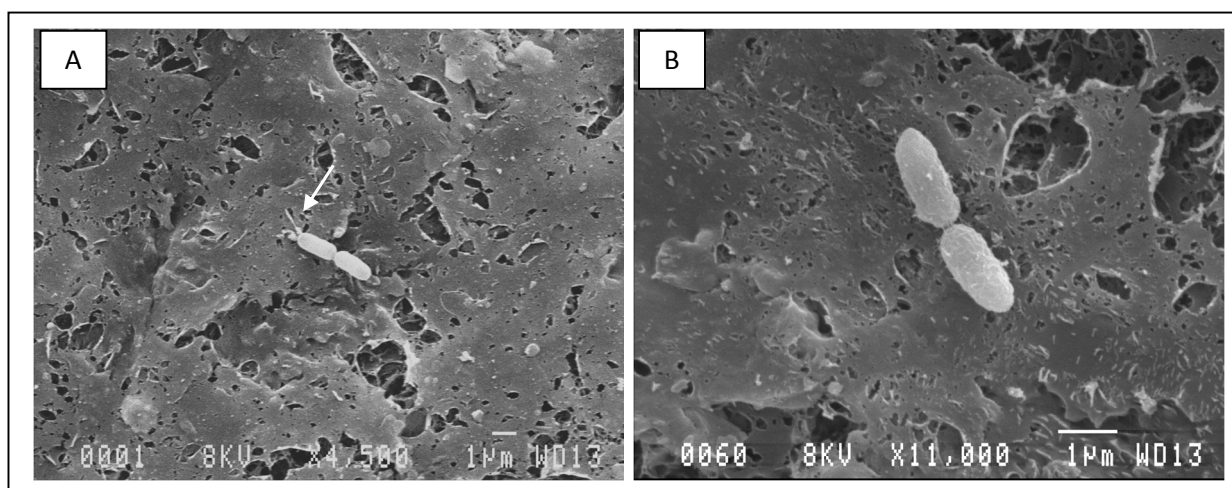


Figure 3.3. Scanning electron micrographs of *Escherichia coli* O157:H7 at various time intervals.

Escherichia coli O157:H7 on pear fruit surfaces at 1 minute (A) and 2 days (B) where replication was observed (A and B) taken at magnification of 4,500x and 11,000x.

3.3. *LISTERIA MONOCYTOGENES*

Listeria monocytogenes titres on fruit following low concentration inoculation were the highest following one day storage at refrigeration temperatures (Table 3.1). Following one day storage at 5°C the titres decreased significantly and remained unchanged to the end of the cold chain. In contrast titres on fruit following high concentration inoculation remained not significantly different from immediately after inoculation to one hour followed by a significant decrease in titres were observed following one day cold storage (Figure 3.1 and Table 3.1). Titres remained

unchanged from 1 to 7 days cold storage. Replication was observed as early as 60 seconds and further colonisation was observed following two days (Figure 3.4).

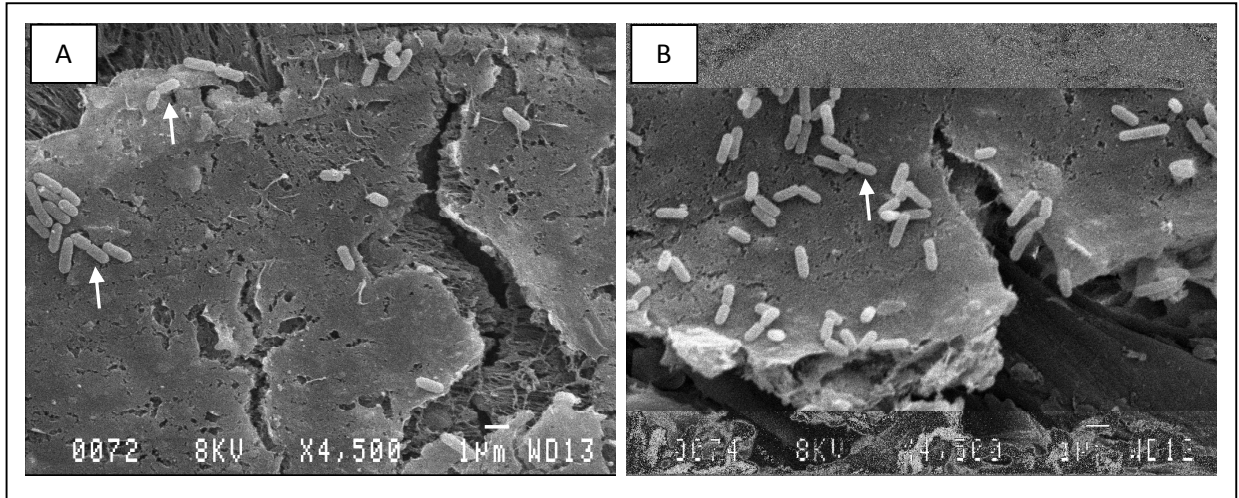


Figure 3.4. Scanning electron micrographs of *Listeria monocytogenes* at various time intervals *Listeria monocytogenes* on pear fruit surfaces at 1 minute (A) and 1 day (B) where replication can be seen. Both were taken at 4,500x magnification.

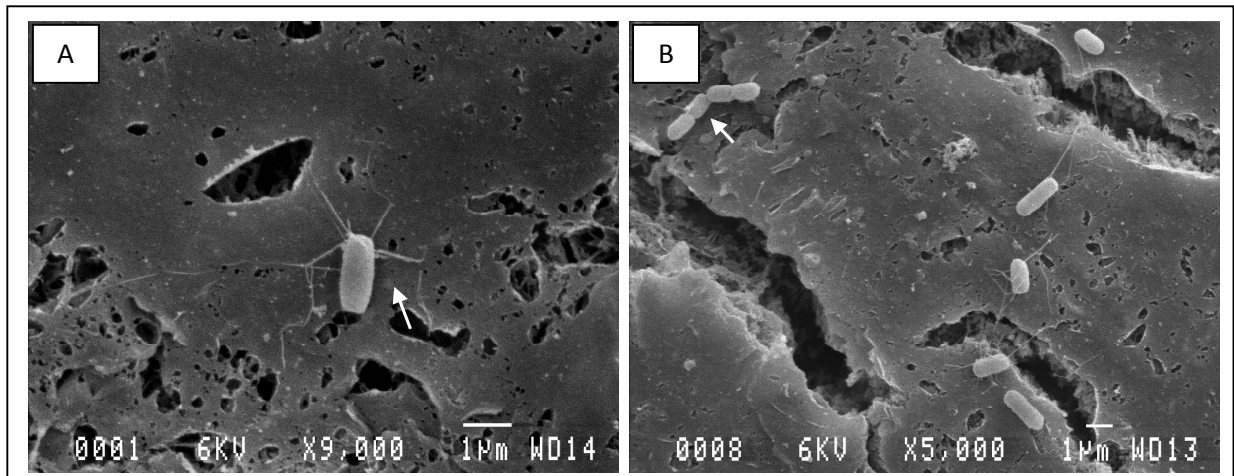


Figure 3.5. Scanning electron micrographs of *Salmonella enterica* subsp. *enterica* serovar Typhimurium at various time intervals.

Salmonella enterica subsp. *enterica* serovar Typhimurium on pear fruit surfaces at 1 minute (A) and 15 minutes (B), where attachment (A) structures and replication (B) were observed. Figure A was taken at a magnification of 9,000x and B were taken at 5,000x magnification.

3.4. *SALMONELLA ENTERICA* SUBSP. *ENTERICA* SEROVAR TYPHIMURIUM

Analysis of the low concentration *Salmonella* Typhimurium had no significant difference from immediately post-inoculation to the end of the simulated cold chain (Table 3.1). High concentration inoculation demonstrated that *Salmonella* Typhimurium titres had a constant significant increase peaking after 1 hour (Figure 3.1 and Table 3.1). A further decrease in titre was observed from 1 hour to the end of the cold storage period.

Extensive attachment structures were observed under the SEM as early as 1 minute (Figure 3.5 A) and replication was observed as early as 30 seconds (data not shown). At 15 minutes (Figure 3.5 B) colonisation starts taking place with attachment structures beginning to form more expansive structures and replication was observed to be active.

3.5. *STAPHYLOCOCCUS AUREUS*

There was a significant decrease in low inoculum inoculation titres after 48 hours storage at refrigeration temperatures, and there after populations remained consistent showing no further growth or significant increase in numbers (Table 3.1). *Staphylococcus aureus* titres regained original titres as were observed immediately after inoculation at 1 hour and thereafter did not change significantly until the completion of the cold storage period after inoculation with high concentration inoculums (Figure 3.6 and Table 3.1). Prior to 1 hour titres fluctuated in the following manner: from immediately after inoculation to 30 seconds there was a significant decrease, from 30 seconds to 15 minutes there was no significant difference and from 15 minutes to 1 hour there was a significant increase. Colonisation was observed through cell replication and colony formation as early as 1 minute and 10 minutes, respectively (Figure 3.6).

4. DISCUSSION

Human pathogens may be introduced into the food chain from the production environment and can survive up to the point of consumption by the consumer possibly resulting in foodborne illness outbreaks (Todd *et al.*, 2009). In this study the effect of a simulated cold chain and the response of foodborne pathogenic microorganisms after high and low inoculum exposure on pear fruit surfaces has been the main focus area. This aspect of food safety has to the authors knowledge not yet been investigated and is important to provide a better understanding of contact time, cross contamination potential and ability to survive under cold storage conditions.

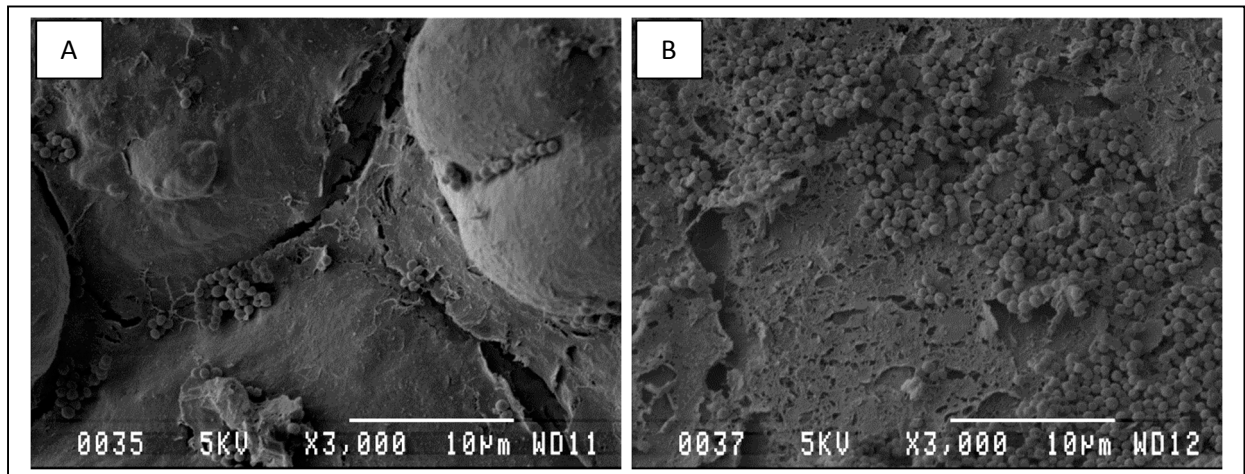


Figure 3.6: Scanning electron micrographs of *Staphylococcus aureus* at various time intervals. *Staphylococcus aureus* on pear fruit surfaces at 1 minute (A) and 10 minutes (B), where replication and microcolony formation/ colonisation (A and B) can be seen. Both were taken at 3,000x magnification.

These microorganisms are capable to survive and this is mainly due to the organisms' ability to resist cold storage environmental conditions in combination with rapid recognition of suitable surfaces for attachment, followed by colonisation (Collignon & Korsten, 2010; Sapers *et al.*, 2005). Solomon and Matthews (2006) reported that not only survival of foodborne pathogens is of importance but also attachment as related to fresh produce to determine contamination potential. Plant surfaces and bacteria are both negatively charged and therefore a repulsive force will naturally push the two apart (Van Loorsdrecht *et al.*, 1990). However, Solomon and Matthews (2006) found that heat-killed bacteria could adhere to lettuce leaves, demonstrating that no physiological activity was required for adhesion. Adhesion and attachment of foodborne pathogens is required for survival on fresh produce surfaces. Adhered cells become attached to the plant surface through exopolysaccharides.

We found in this study that *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium demonstrated the ability to attach at various time intervals. Once foodborne pathogens have attached, a microcolony can form demonstrating colonisation and subsequent survival (Van Loorsdrecht *et al.*, 1990). This study demonstrated for the first time that foodborne pathogens were able to produce attachment structures on the surface of pear fruit and therefore can establish on the pear fruit surface. In this study assumed attachment structures were noted within 30 seconds for *E. coli* O157:H7 and 15 minutes for *Salmonella* Typhimurium. Collignon and Korsten (2010) found similar findings on peaches and plums, they investigated the

attachment, colonisation and survival of these four pathogens on plum and peach surfaces. The authors found that *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium were able to produce exopolysaccharides on the surfaces and all four pathogens were able to produce microcolonies. Furthermore the host–pathogen interactions of foodborne pathogens were not only observed in this study but also on other fruit and vegetables (Berger *et al.*, 2010; Collignon & Korsten, 2010; Ziuzina *et al.*, 2014). Future studies should confirm the production of attachment structures using confocal microscopy.

Low *E. coli* attachment levels observed by Ziuzina *et al.* (2014) in a study done on cherry tomatoes and strawberries were focussed on determining the residential epiphytic bacteria. Therefore both in this study and that of Collignon and Korsten (2010) surface sterilisation were applied in order to determine the full potential of the pathogenic bacteria without external factors such as epiphytic microorganisms. External factors that could also influence attachment levels (Ziuzina *et al.*, 2014) include, fruit and vegetable surface morphology, epithelial chemical excretions and microbe surface recognition (Keeratipibul *et al.*, 2011). In this study the pear surface was found to be non-corrugated, smooth surfaced with wax scales and without trichomes making it a relative easy surface to colonise.

All foodborne pathogens in this study demonstrated the ability to form microcolonies. Collignon and Korsten (2010) also observed that *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium and *S. aureus* were able to produce microcolonies on peach and plum surfaces. Brandl and Matthews (2002) demonstrated that *Salmonella enterica* was able to colonise the surface of cilantro leaves. Annous *et al.* (2005), Brandl and Mandrell (2002), Charkowski *et al.* (2002) and Collignon and Korsten (2010) all similarly demonstrated that foodborne pathogens are able to form microcolonies on plant tissues.

Staphylococcus aureus are naturally well known to survive nutrient poor surfaces for long periods of time (Neely & Maley, 2000). Results reported by Neely and Maley (2000) show that *S. aureus* are able to survive in hospital settings on plastics and fabrics exceeding 90 days. *Staphylococcus aureus* have survived up to seven day cold storage under adverse cold storage conditions at 5°C. Similar results were found after a 10 day simulation of temperature fluctuations imitating consumer handling of cold cuts (Røssvoll *et al.*, 2014). A common growth trend for all pathogens was observed in this study, with the growth peaking after one hour at room temperature. Room temperature is close to the optimal temperature for all four pathogens. However *E. coli* O157:H7, *Salmonella* Typhimurium and *L. monocytogenes* when

inoculated with high concentration showed a significant decrease after the beginning of cold storage. Interestingly, titres of *L. monocytogenes* a known psychrotroph showed a significant increase when inoculated using the low concentration inoculum but the same was not observed for the high concentration inoculum. Low inoculation concentrations of *Salmonella* Typhimurium indicated the highest survival and concentration rate followed by *E. coli* O157:H7, *S. aureus* with *L. monocytogenes* surviving at the lowest titres. *Listeria monocytogenes* is a psychrotrophic organism that is not only able to survive at refrigeration temperatures but can proliferate. *Listeria monocytogenes* was not able to proliferate on the surface of pears under cold conditions within seven day study period. In contrast a study done by Beuchat and Brackett (1990) after eight days showed that *Listeria* significantly increase at refrigerated temperatures on lettuce.

All four foodborne pathogens are capable of contaminating different food sources (CDC, 2014). Therefore it is important to determine how the bacterial foodborne pathogens interact and survive on fruit and vegetable surfaces in order to implement the appropriate control steps required within the production and transport environments. This is the first report of the survival of foodborne pathogens on pear surfaces. Following the cold storage of pears that were artificially contaminated with unrealistically high concentrations of foodborne pathogens, only *Salmonella* Typhimurium was able to survive at titres that could potentially lead to foodborne illnesses since the minimal infectious dose of *Salmonella* is 10^1 cells [USA Food and Drug Administration (FDA), 2009]. If fruit were contaminated with high concentration of foodborne pathogens and consumed seven days after cold storage it may represent a hazard, provided the organisms' growth followed the same trend. The fruit inoculated with the low concentration of *E. coli* O157:H7 and *Salmonella* Typhimurium could also affect consumer health even if consumed within a week after cold storage since final titres exceeded the minimum infectious dose (FDA, 2009). *Staphylococcus aureus* was the organism which best survived cold storage conditions. However the toxin producing minimum titre of *S. aureus* is log 5 and in this study that value was not achieved under these cold storage conditions (FDA, 2009).

Even though these foodborne pathogens have the capability to rapidly attach, multiply and colonise on the pear fruit surface, the detection of these organisms are limited due to sample size and the hurdle technologies within the supply chain system (Chapter 4). Foodborne pathogen detection in the supply chain of four pear production farms over two consecutive seasons showed that no *E. coli* O157:H7 were found throughout the study but *Salmonella*

Typhimurium and *L. monocytogenes* were detected on orchard pear samples (Chapter 4). Therefore consumers of fresh pear fruit have a low probability to be exposed to foodborne pathogens due to production storage and packing practices (Chapter 4).

5. CONCLUSION

Under more realistic conditions (inoculum load of 10^4 CFU/fruit), *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium did not change significantly in concentration throughout the simulated cold chain. *Staphylococcus aureus* had a significant overall decrease in titres from point of inoculation to the simulated local market point of sale. It was observed that all of the pathogens were capable of colonising and surviving on the pear fruit surfaces. *Escherichia coli* O157:H7 and *Salmonella* Typhimurium were able to survive at a high enough titre that exceeded the pathogens theoretical infectious dose. Survival of a pathogen at such a threshold level could potentially lead to food poisoning. Foodborne pathogens were detected early in the pear supply chain but following postharvest handling of chemical drenching and controlled atmosphere storage none of the above mentioned foodborne pathogenic microbial bacteria detected (Chapter 4). Therefore effective food safety management practices should ensure that contamination must be prevented, and survival of foodborne pathogens are impaired. Future studies should investigate other environmental factors that influence the survival of foodborne pathogens on pear surfaces as well as lower inoculation loads and longer cold chain conditions. Other aspects such as host response to the interaction of foodborne pathogens, natural occurring biocontrol microorganisms, host specific plant pathogenic and residential epiphytic microorganisms on the fruit surfaces can contribute to a better understanding of plant health.

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CHAPTER 4

BACTERIAL BIOMES AND DETECTION OF FOODBORNE PATHOGENS ON PEAR (cv PACKHAM'S TRIUMPH) FRUIT IN THE SUPPLY CHAIN

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ABSTRACT

The increased global demand for seasonal fruit to be available all year round has resulted in an upscale in production, distribution and advanced technologies to extend shelf life for extended trade. Safe pear fruit is essential to ensure market access. This chapter is focused on the South African pear industry to determine the natural bacterial profile of pear fruit surfaces (carpoplane) using the commercial export cultivar Packham's Triumph. In this study the bacterial load and more specifically the presence or absence of foodborne pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium) on export fruit were determined. In support of this an industry wide food safety compliance assessment was done to determine the level of understanding and implementation. The healthy viable microbial load was determined through serial dilutions and subsequent plating onto standard 1 agar and 16S DNA identification to determine dominant bacteria. Presence or absence of foodborne pathogens was done using standard selective chromogenic media and multiplex PCR with pathogen specific primers. High false presumptive positives were found with the selective media, whereas DNA molecular methods confirmed pathogenic status and were accurate, reliable and less time consuming. The microbial load indicated a decreasing trend in the first season for three of the farms, as pears went through a drenching step and were stored under controlled atmosphere (CA) conditions. The variety of bacterial spp. isolates were lower after CA storage with predominant organisms such as *Bacillus* spp., *Curtobacterium flaccumfaciens*, *Erwinia billingiae*, *Pantoea* sp. and *Pseudomonas* spp. No foodborne pathogens were detected after CA storage conditions. A defining statement on the status of a healthy fruit bacterial population and absence of foodborne pathogens reflect safety assurance.

1. INTRODUCTION

South Africa is the sixth largest pear exporter in the world, with 197,911 tones being shipped per annum [Perishable Products Export Control Board (PPECB), 2013]. Retaining a dominant profile on the export market requires a sound knowledge and effective food safety assurance systems. To date not much research has been done on natural pear fruit surfaces and the bacterial populations present and more specifically the component associated with known foodborne pathogens (Leff & Fierer, 2013). Current microbial guidelines for raw fruit and vegetables [Department of Health (DoH), 2006] stipulate the absence of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp. at ready-to-eat products (DoH, 2006). Coliform levels should also not exceed 200 CFU per gram on ready-to-eat fruit and vegetables. No scientific data of healthy natural microbial loads could be found in regulatory guidelines. Fruit that are consumed raw, like pears, pose a potential risk to consumers if not produced within a food safety assurance framework. In order to manage the risks, proper microbial surveillance is needed, which includes determining the microbial load and profile and assuring the absence of human pathogens on fresh produce [Centres for Disease Control and Prevention (CDC), 2013].

Residential surface bacteria, fungi and yeast or yeast-like organisms are all part of the biological community that contributes to an ecological balance on the living fruit surface (Leben, 1965). The epiphytic microorganisms provide a protective barrier against pathogenic microorganisms causing food spoilage (Barth *et al.*, 2010), decay and food safety concerns. Interventions such as washing and/or chemical applications to the fruit surface disrupts the natural microbial balance, causing a population shift benefitting opportunistic organisms associated with food spoilage, decay or food safety (Corbo *et al.*, 2010). Foodborne pathogens such as *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aureus* are able to attach and colonise the pear fruit surface (Chapter 3). Population shifts due to more favourable environmental conditions and/or wounding of fruit skin favour postharvest disease development (Barth *et al.*, 2010). Wounded fruit have also been shown to retain *E. coli* O157:H7 (Mathews *et al.*, 2014). In addition to production driven change can be focused by production steps while fruit move through the supply chain (Janisiewicz & Buyer, 2010).

Traditional fruit epiphytes such as, *Curtobacterium* spp. (Shreedhar *et al.*, 2014), *Pantoea agglomerans* (Kapetas *et al.*, 2011), *Serratia marcescens* (Kumar *et al.*, 2011) are able to survive in acidic (low pH) conditions. In addition it has been found that *E. coli* O157:H7 is

able to survive at pH 3 (Jordan *et al.*, 1999), in a similar study it was found that viable *L. monocytogenes* were detected after seventh day at a low pH of 3.44 (Budzińska & Wroński, 2008).

The aim of the research was to fill the gap in knowledge on the culturable bacterial load and dominant bacterial species as well as presence or absence of foodborne pathogens on the pear fruit surfaces. This information will contribute to a better understanding of healthy safe pears and the potential for cross contamination while the fruit is moving through the supply chain. The study focuses on three critical aspects within the pear production chain: 1) the identification of dominant residential bacterial spp. on pear fruit surfaces at harvest, up to and after controlled atmosphere (CA) storage; 2) the microbial status of healthy pears; and 3) determining if human foodborne pathogens were present on the fruit surfaces by using different detection methods.

2. MATERIALS AND METHODS

Please refer to Figure 4.1 in order to have an overview of the combined technology approach, which is discussed in detail below.

2.1. SAMPLING STRATEGY

Site Description

Samples were collected from four farms in the Western Cape, South Africa, within a 30km radius with the communal packhouse situated central to all the farms. A single chemical drenching system (chlorine as active ingredient, 75 ppm) was applied before the pears were stored in the CA facility set at industry specifications conditions (1.5% O₂, 1.5% CO₂ and at -0.5°C).

Sample Collection

Two consecutive seasons of pears *Pyrus communis* L. cv. Packham's Triumph pears were collected at harvest, before and after CA storage for each of the four farms. The cultivar was selected as it is the main cultivar planted in SA with 3 325ha which accounts for 29% of pears (National Department of Agriculture, Forestry and Fisheries, 2012) and the most important export cultivars with 34.22% exported annually (PPECB, 2014). All farm's orchard blocks were commercially harvested on the same sampling day. One orchard block was selected from each farm. A total of 20 pear fruit per orchard block were sampled from four random rows,

from randomly targeted trees within each row in each orchard, therefore equating to a sample size at each sampling stage of four replicates of five pears each.

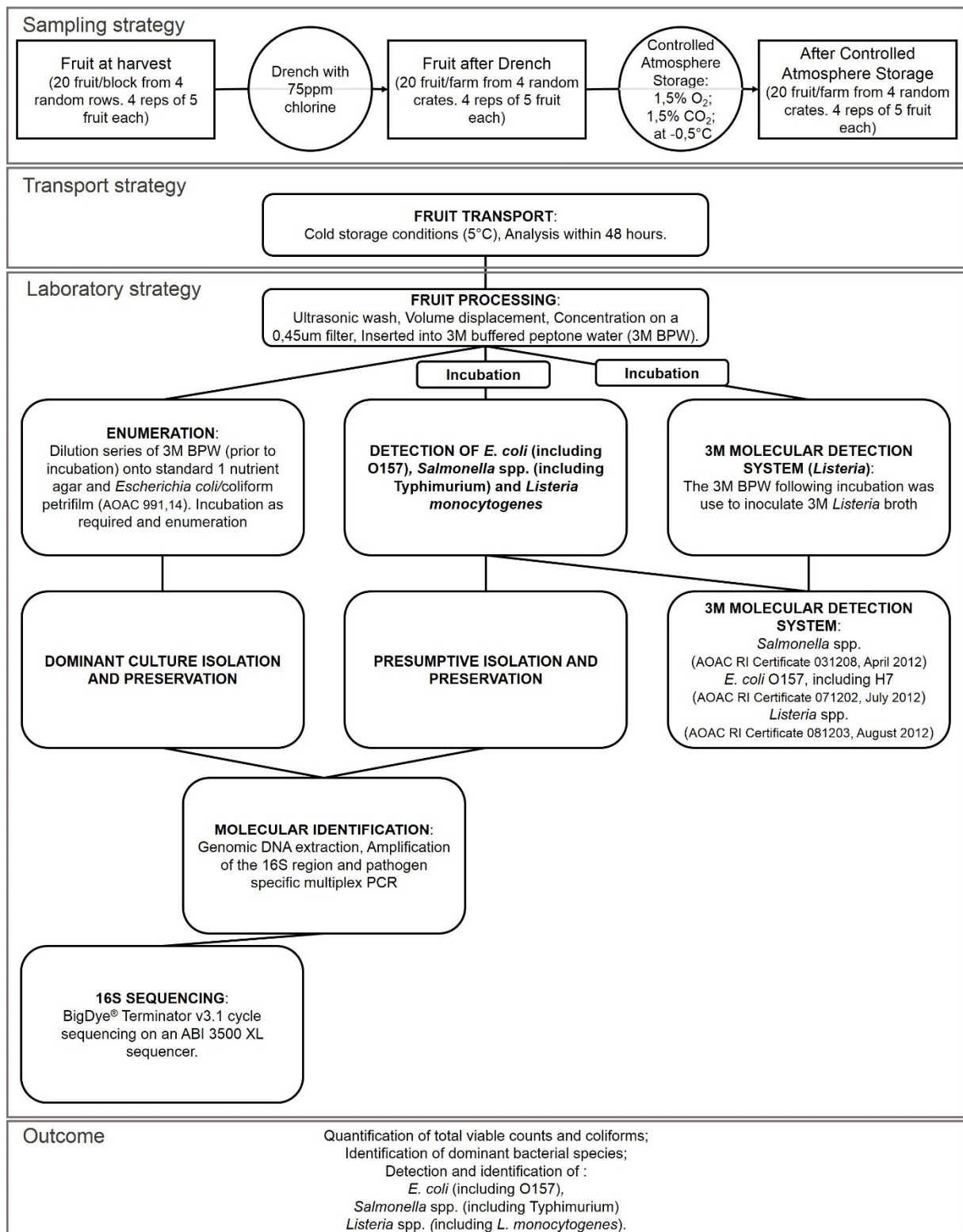


Figure 4.1. Multiple approach workflow

After drenching, a total of 20 fruit were collected, five fruit each from four random crates originating from the same four farms sampled earlier that day. The same sample practises were used for pears after CA storage which were collected twelve weeks later. The total number of pears analysed were 240 in total, consisting of 12 replicates, per season. At each sampling stage the pears were placed in labelled brown paper bags kept in cold storage (± 5 °C) until laboratory analysis within 48 hours. Pear surface temperatures of all samples were datalogged as they were collected at each sampling stage (Extech Instruments, IR Thermometer, Part # 42580).

2.2. LABORATORY STRATEGY

Sample Processing

Quarter strength Ringer's (Merck, Johannesburg, South Africa) solution (500 ml) amended with 0.02% Tween 80 (Sigma, Johannesburg) was added to each sample (one fruit at a time) and volume displacement was recorded. Samples were sonicated in the Ringer's-Tween solution in an ultrasonic bath (Labotec, Johannesburg) for 5 minutes and filtered through a sterile 0.45 μm (Sartorius Stedim, Biotech, Germany) pore size nitrocellulose membrane. This method was optimised prior to doing the full trial (data not shown).

Microbial Analysis

After filtering the pear washwater, filters were placed into 9ml 3M Buffered Peptone Water (BPW) (3M Food Safety, Minnesota, USA) and vortexed vigorously. Total viable bacterial and coliform/*E. coli* counts were determined by plating a tenfold dilution series of each of the samples onto Standard 1 (STD1) media (Merck) and *Escherichia coli* 3M petrifilm (3M Food Safety, USA). Plates and film were incubated for three to five days at 25°C and 37°C for 24 hours, respectively (SABS ISO 4833, 1991). Counts were recorded and data was converted to $\log_{10}(x+1)$ CFU cm^{-2} (as described in section 2.4).

Selective Enrichment

Following dilution series, each of the BPW sample solutions were enriched to determine the presence of *E. coli* (including *E. coli* O157:H7) and *Salmonella* spp. Samples were incubated in 9 ml 3M PBW (3M Food Safety) containing the filter membranes at 37°C for 24 hours. Additionally, a millilitre of the 3M PBW broth was transferred into a 9 ml of 3M *Listeria* selective broth (3M Food Safety, USA) for enrichment purposes and incubated at 37°C for 24 hours. Subsequently, a loopful of each of the pear enrichment samples (3M PBW enriched)

were streaked onto the following: eosin methylene blue differential medium (EMB) (Merck) for detection of typical *E. coli* colonies; *Salmonella* brilliance medium (Oxoid, Johannesburg) for detection of typical *Salmonella* spp. colonies; and *Listeria* selective medium for detection of typical *Listeria* spp. colonies.

Dominant bacterial culture were selected according to colony morphology and needed to be present on at least 33% of the plate to be classified as dominant. Dominant cultures as well as presumptive foodborne pathogen colonies (based on typical colony morphology) were selected from the STD1 and selective agar plates, respectively and restreaked for purification. Pure cultures were stored in glycerol (32.5%) at -70°C for later processing.

Molecular Detection System Analysis

The 24h 3M PBW and 3M *Listeria* specific enrichment broths were used in addition to determine the presence/absence of *E. coli*, *Salmonella* spp. and *Listeria* spp. using the respective 3M MDS kits according to the manufactures specifications: 3M Molecular Detection Assay *Salmonella* (AOAC RI Certificate 031208, April 2012), 3M Molecular Detection Assay *E. coli* O157, including H7, (AOAC RI Certificate 071202, July 2012) and 3M Molecular Detection Assay *Listeria* (AOAC RI Certificate 081203, August 2012).

16S rRNA DNA Extraction and Polymerase Chain Reaction of Microbial Isolates

Purified dominant single colonies and presumptive positive foodborne pathogens were cultured aerobically in Tryptone soy broth (Biolab, Johannesburg) for 24 hours at 37°C. The cells were pelleted by centrifugation at 10 000x g for 5 minutes at 4°C, followed by the extraction of the bacterial DNA using a Quick-gDNA miniprep kit (Zymo Research, California, USA). The DNA concentration of each isolate was determined with the Qubit 2.0 Fluorometer (Lifescience Technology, Johannesburg).

Universal 16S primers (Brosius *et al.*, 1978) were used in identification of isolated dominant microbial organisms (Table 4.1). The identity of each presumptive colony was further tested using a multiplex PCR amplifying *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Typhimurium as described by Standing *et al.* (2013). Details of the primers used are shown in Table 4.1. PCR analysis conditions were similar for both 16S and pathogen detection using a T100™ Thermal Cycler (BioRad Laboratories Ltd, Johannesburg). The PCR mixtures contained 25 ng of genomic DNA, 200 µM of each deoxynucleotidetriphosphate, 0.5

μM of each primer, and 1 U My Taq (Bioline, Taunton, Massachusetts, USA) in a total reaction volume of 25 μl . The PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 90 seconds, with a final extension at 72°C for 5 min. The amplified PCR products were purified from a 2% (w/v) agarose gel using a GeneClean kit (Zymo Research, California) and the PCR products sequenced using BigDye Terminator v3.1 on an ABI 3500XL sequencer in forward and reverse direction (InquabaBiotec, Johannesburg).

Table 4.1. Primers for specific detection of human pathogens and identification through 16S

Primer	Specificity	Sequence 5' - 3'	Size (bp)	Reference
UidAa (30 pmol)	<i>Escherichia coli</i> O157:H7	GCGAAAACACTGTGGAATTGGG	252	Cebula <i>et al.</i> , 1995
UidAb (30 pmol)	<i>Escherichia coli</i> O157:H7	CGCTTTTGACACCTTAACCC	252	Cebula <i>et al.</i> , 1995
LMFP (20 pmol)	<i>Listeria monocytogenes</i>	AGCTCTTAGCTCCATGAGTT	450	Thomas <i>et al.</i> , 1991
LMRP (20 pmol)	<i>Listeria monocytogenes</i>	TCGAGAATCGAGGTACTCAA	450	Thomas <i>et al.</i> , 1991
SLDF (50 pmol)	<i>Salmonella</i> Typhimurium	CCTGTGAATGCCCTGATGAT	787	Standing <i>et al</i> 2013
SLDR (50 pmol)	<i>Salmonella</i> Typhimurium	GGACACTTACGGGACTACTA	787	Standing <i>et al</i> 2013
F 27	16 S rDNA	AGAGTTTGATCMTGGCTCAG	1465	Brosius <i>et al.</i> , 1978
R 1492	16 S rDNA	CGGTTACCTTGTTACGACTT	1465	Brosius <i>et al.</i> , 1978

A high number of false positives obtained in 2013 from the selective chromogenic media resulted in an adaptive approach by using a DNA screening first before continuing on the chromogenic media during the second season. Samples positively detected for foodborne pathogens were subjected to traditional viable plating on selective chromogenic media, where after single colonies were stored in glycerol (32.5%) at -70°C as described for further analysis.

Statistical and Phylogenetic Analysis

Pear sampling was conducted across two consecutive production seasons (2013 and 2014). Statistical analysis was performed on log CFU cm⁻². Data were analysed using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC). A one-way analysis of variance was used to determine the difference in microbial load on fruit surfaces. Means were analysed using the least significant difference (using the Fisher test) at a 5% level of significance. The statistical examination focused on each farm separately over two seasons through all sample stages.

Sequences were analysed through BLAST nucleotide identification. Phylogenetic alignment analyses were conducted using MEGA version 6 (Tamura *et al.*, 2013). Microbial phylogenetic trees were created with MEGA using the distance Neighbour Joining statistical algorithm

(Saitou and Nei, 1987). Corrected nucleotide substitutions were calculated using the Tamura-Nei model.

3. RESULTS

3.1. PEAR SURFACE TEMPERATURES AT HARVEST

In the 2013 season temperatures of the pear fruit surfaces ranged from an average of 12.5°C at 9:00 am taken on farm 1, up to an average of 25.3°C at 13:20 pm on farm 4. The lowest recorded pear surface temperature was 11.2°C and the highest recorded temperature was 27°C (Appendix 2, Table 1). In the 2014 season temperatures of the pear surfaces ranged from an average of 18°C on farm 2 at 11:40 am up to 23.5°C on farm 1 at 10:05 am. The lowest recorded pear surface temperature was 17.2°C and the highest recorded temperature was 29.5°C (Appendix 2, Table 1). The atmospheric temperatures of season 2 were considerably lower than season 1 but reaches a peak temperature similar to that of season 1. The humidity throughout the morning were between 10-20% less in season 2 than season 1 (Appendix 2, Figure 1).

3.2. MICROBIAL QUALITY AND SAFETY OF PEARS

Aerobic Bacterial Plate Counts

The total viable counts ranged from a minimum of 1.23 log (CFU+1)/cm² in the orchard to a maximum of 4.14 log (CFU+1)/cm² before CA storages (Figure 4.2). The total viable counts after drenching (before CA) were significantly higher than after harvest and after CA storage (Figure 4.2). During the 2014 season on Farm 2 and Farm 3 a similar pattern was observed. However during the 2013 the total viable counts on fruit collected from Farm 2, 3 and 4 showed a gradual decrease in overall populations (Figure 4.2). During the 2014 season there was no significant difference after drenching but after CA storage there was a significant increase in total viable counts (Figure 4.2). Comparing values between the two seasons, Farm 1 (except for 2013 season), 2, 3 and 4 (except for 2014 season) decreased at after CA storage.

Coliform Petrifilm Plate Counts

Counts ranged on average around log 2.3 (CFU+1)/cm² (Figure 4.2). Farm 2 (season 2), Farm 3 (season 1) and Farm 4 (season 1) showed significant decreases in titres through the production stages. A single significant increase was observed at Farm 4 during season 2.

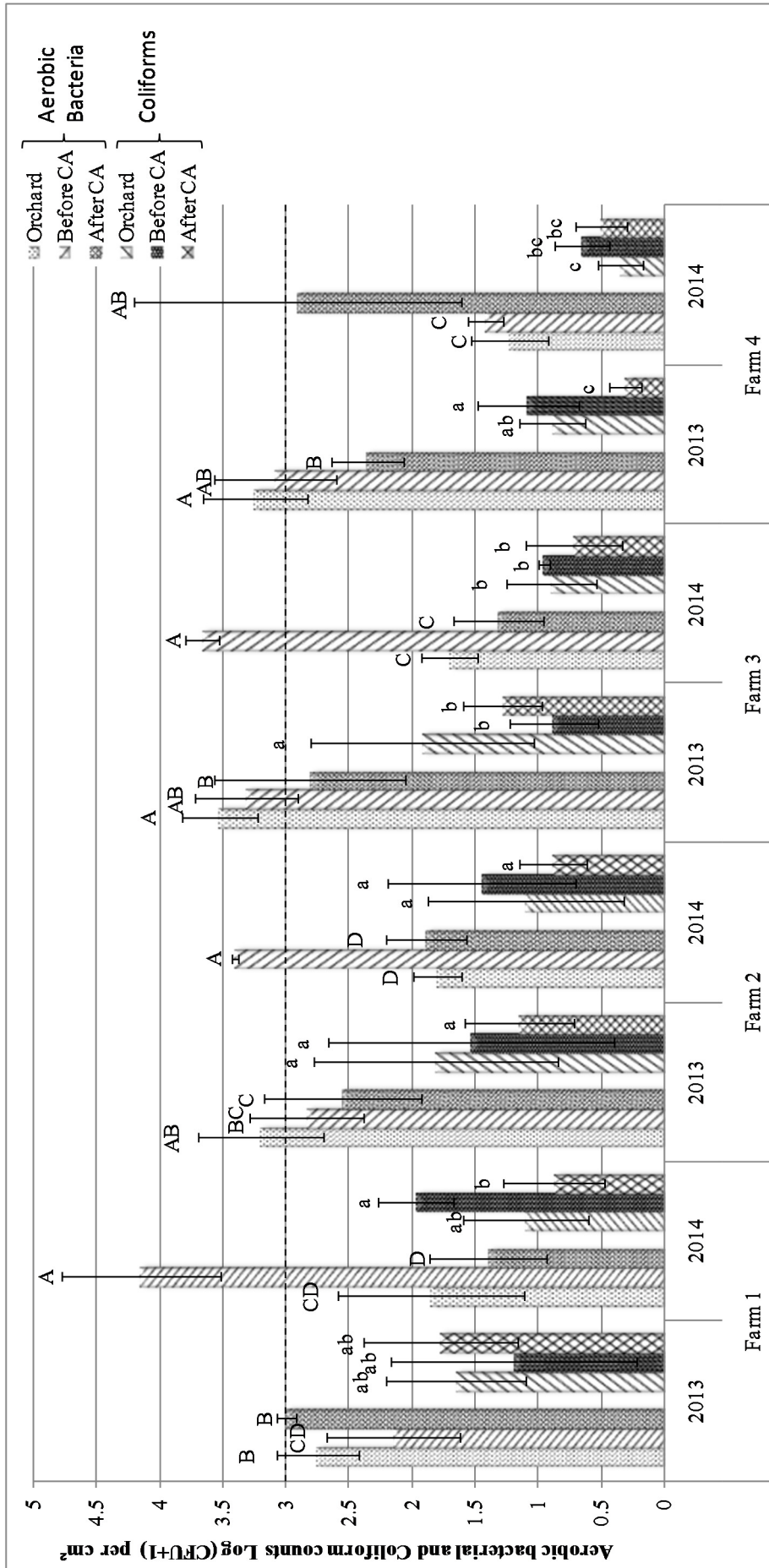


Figure 4.2. Aerobic bacterial and coliform total viable counts on pear fruit surfaces, over two consecutive production seasons of four farms and central packhouse in the Western Cape Province (2013 – 2014).

Note: Capital letters indicate the aerobic bacterial (small letters: coliforms) least significant differences of individual farms across different sampling stages over two seasons.

Detection of *Escherichia coli* (including O157:H7), *Listeria monocytogenes* and *Salmonella* spp. on Pear Fruit Surfaces

Initial testing during 2013 on chromogenic selective media, 17 presumptive *Escherichia coli*, 33 presumptive *Listeria* spp. and 4 presumptive *Salmonella* spp. were detected. Of these only three were confirmed positive for *Salmonella* Typhimurium and two for *L. monocytogenes* from fruit collected in the orchard of Farm 2, 3 and 4 respectively. Using the 3M MDS, only one of the enriched PBW from the fruit tested positively for a *Listeria* sp. after CA storage from Farm 4. However this was confirmed to be a non-human pathogenic *Listeria* spp. *Escherichia coli* (including O157:H7) was not detected in any of the samples analysed and no foodborne pathogens were detected on pears sampled during season 2.

Identification and Relevance of Dominant Microbes

Dominant bacterial isolates identified by 16s rRNA sequencing are summarised in Figure 4.3. The dominant microbial diversities on pear surfaces before CA storage stages were greater than that of the pears after CA storage. Several bacterial isolates were identified as potential non-host specific plant pathogenic microorganisms including *Curtobacterium* spp., *Pantoea* spp., *Pseudomonas* spp. and *Serratia marcescens*. *Bacillus* spp. was also detected as well as, *Erwinia billingiae* and *Pseudomonas putida*.

Clustering of other phylogeny groups included 13% Bacillaceae, 26% Enterobacteriaceae, 9% Pseudomonadaceae and 6% other families. *Lactobacillus plantarum* (Lactobacillaceae), *Streptomyces thermocarboxydus* (Streptomycetaceae) and *Chromobacterium* sp. (Neisseriaceae) clustered separately. *Lactobacillus plantarum* aligned under Firmicutes grouping, closely related to *Streptomyces thermocarboxydus* that allocated under Actinobacteria, whereas *Serratia marcescens* and *Chromobacterium* sp. aligned under the Proteobacteria grouping.

Phylogenetic Analysis of Dominant Cultures

A Neighbour-Joining phylogenetic tree was constructed, based on CLUSTAL W multiple sequence alignment from the forward and backward 16S sequences. Figure 4.4 consisted of 74 dominant bacterial isolates, the phylums separated at a single node into Actinobacteria (including Firmicutes) (62% of dominant organisms) and Proteobacteria (38% of dominant organisms). Multiple similar genera have been found before as well as after CA.

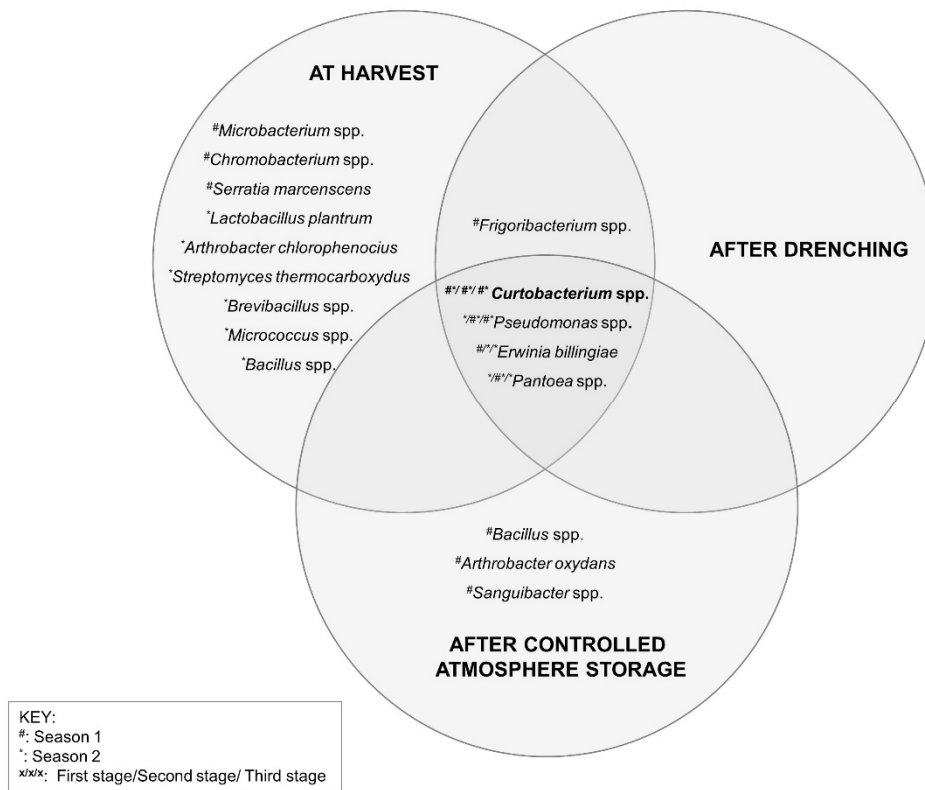


Figure 4.3. Dominant bacterial species grouped per stage (at harvest, after drenching and after controlled atmosphere storage).

Species prefixed with # indicates presence in season one, species prefixed with * indicated presence in season two. Species prefixed with ^{x/x/x} indicates presence in all three stages in different seasons.

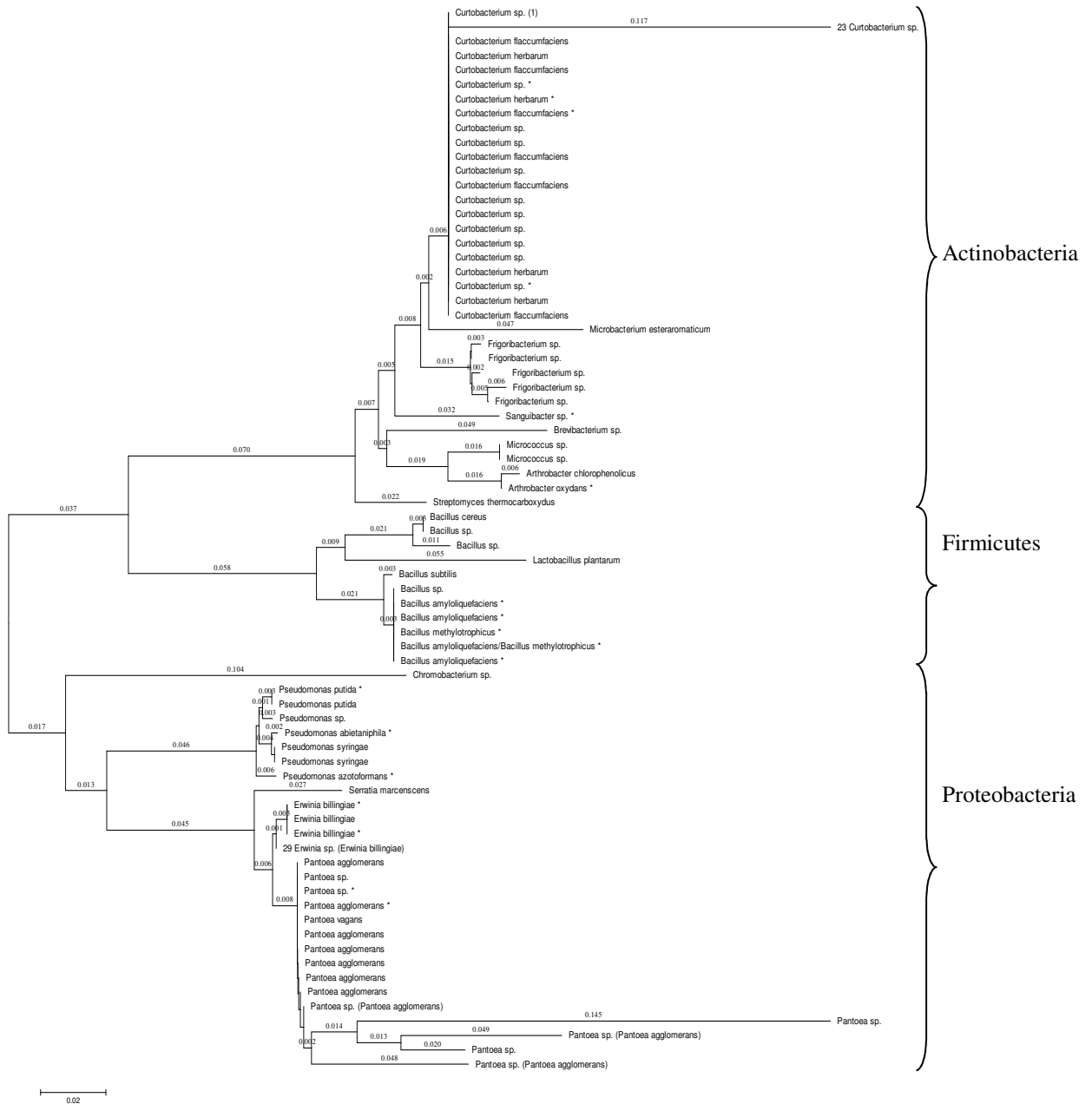


Figure 4.4. Phylogenetic tree based on dominant bacterial isolates' 16S rDNA sequences, constructed using the Neighbour-Joining methodology with allocated bootstraps. Identifications labelled with an asterisk (*) indicates dominant microbes isolated after CA storages (twelve weeks at 1.5% O₂; 1.5% CO₂ and -0.5°C).

4. DISCUSSION

To the authors' knowledge this is the first study investigating the bacterial load and selected foodborne pathogen presence or absence on pear surfaces through a supply chain approach. Other work focussed on culture-independent methodologies to identify epiphytic microbial organisms on other fruits such as pome fruit, apples and grapes (Leff & Fierer, 2013). Throughout the processing stages (2013 and 2014 season) the pear fruit went through bacterial population fluctuations. Uncontrollable factors like orchard climate changes and weather patterns as well as controllable factors such as chemical drenching, atmosphere modifications within the CA storage facility and lastly removal from the CA storage can all contribute to bacterial load fluctuations on the carpoplane.

Optimum growth temperatures are unique to each individual microbial species, therefore different environments can influence growth (Nedwell, 1999). Temperatures measured on fruit surfaces reached an average maximum of 29°C at midmorning, the recorded temperature fell in the optimum growth range for the dominant bacterial spp. such as *Curtobacterium flaccumfaciens* (Funke *et al.*, 2005), *Pantoea agglomerans* (Jung *et al.*, 2002), *Pseudomonas azotoformans* (Iizuka & Komagata, 1963), *P. syringae* (Young *et al.*, 1977) and *Serratia marcescens* (Hejazi & Falkiner, 1997). Microbial presence were significantly lower in season 2 (2014) when compared to season 1 (2013), this could be attributed amongst other differences to the recorded atmospheric temperature and relative humidity that were also lower in season 2. Even though the temperatures recorded during this study do not favour the optimal growth temperature of human pathogenic microorganisms, it is still suitable for growth (Beuchat & Bracket, 1990). Both *Salmonella* Typhimurium and *Listeria monocytogenes* were however detected during season 1 (averages of 21.5°C, relative humidity of 77.5%) when atmospheric temperatures and humidity were higher than in season 2 (averages of 15.5°C, relative humidity of 61.3%).

Pear fruit surfaces as well as ciders and juices all have low pHs and therefore in order for microorganisms to persist in these environments they need to be able to adapt and survive in these more acidic conditions. A list provided by Corbo *et al.* (2010) showed pH of fruit (including pome fruit) to be between 2.9 and 3.9. The microbial load fluctuations may also vary due to the changes in environmental conditions associated with bacterial specific growth characteristics. The pear fruit industry in South Africa use commercial CA storage to extend shelf life and obtain market access over extended periods of time (Gunes & Lee,

1997). Packham's Triumph pears can be stored for up to 9 months at -0.5°C , with a CO_2 and O_2 concentration at or below 2% to retain quality. Extreme environmental conditions can cause a shift in population density and species diversity (Corbo *et al.*, 2010). In this study the total viable bacterial and coliform counts decreased after CA storage. After controlled atmosphere storage 47% less diverse dominant microorganism were found. No previous study have described the effect of CA storage on the pear carpoplane.

The clustering of the associated bacterial species resulted in three dominant phyla, these include Firmicutes, Actinobacteria and Proteobacteria (divided into the Beta- and Gamaproteobacteria classes). Dominant Microbacteriaceae account to 46% of dominant species identified, in a study done by Leff and Fierer (2013) on apple and peach surfaces similar results with Microbacteriaceae were found. *Curtobacterium* spp., *Pantoea agglomerans* and *Pseudomonas putida* were some of the significant bacterial microorganisms that survived from the same individual farm from before CA storages (after drenching) up to and after CA storages stages, with *Curtobacterium* spp. being the only isolate from harvested pears. These bacterial species are known to have plant and non-plant specific pathogenic properties (Braun-Kiewnick *et al.*, 2000; Janisiewicz & Buyer, 2010; Patten & Glick, 2002).

A known human pathogen, *Serratia marcescens*, was detected on orchard pears, the pathogen is known to infect the urinary tract in humans and open wounds (Hejazi & Falkiner, 1997). *Serratia marcescens* not only affects humans but also vegetables, fruit and herbs causing; corn whorl rot (Wang *et al.*, 2015), bell pepper soft rot (Gillis *et al.*, 2014), and crown rots in alfalfa (Lukezic *et al.*, 1982). Not all dominant isolates are considered harmful. *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 has been found to be an excellent biofungicide and has been registered and approved by the European Union for commercial use against *P. syringae* pv. *actinidiae* (Agropages, 2014; CABI, 2014). *Erwinia billingiae* and *Pseudomonas putida* have known beneficial characteristics in development and survival of plants. *Erwinia billingiae* can be applied as a biological antagonist known to compete for nutrients and space against *E. amylovora* (Jakovljevic *et al.*, 2008). Further research on the antagonistic properties of dominant microorganisms isolated could be significant in the development of organic production systems through biocontrol of potential post-harvest and foodborne pathogens.

Food safety on raw consumed fresh fruit and vegetables has become a concern as an increase number of outbreaks are recorded (CDC, 2014). The inoculation study done in Chapter 3 as well as the attachment study conducted by Collignon and Korsten (2010) showed that *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* Typhimurium, are able to attach within 60 seconds (Collignon & Korsten, 2010). Badosa *et al.* (2008) tested fresh fruit, sampled at the market, which included apples and pears for the detection for *Salmonella* spp., *Listeria* spp. and *Listeria monocytogenes* through PCR detection methods. Their study resulted in no positive detections of any of the named organisms on freshly harvested or market fruit. In this study foodborne pathogens were detected on fruit in the orchards but no foodborne pathogens were present following CA storage.

The effect of controlled atmospheric storage on *L. monocytogenes* were reported by Berrang *et al.*, (1989) to have no effect on the growth rate even though populations were found to increase. Similarly, typical CA storage conditions do not have an inhibition effect on the survival of *Salmonella* spp. (Daş *et al.*, 2006). Detections of the foodborne pathogens also resulted in no positive detections over two seasons, after controlled atmosphere storage in this study. Upon investigation of the uniqueness of the dominant species in season one at harvest it was found that *Pseudomonas* spp. were not present at this stage, but detectable after drenching and after CA storage and throughout season two. *Pseudomonas syringae* was found to prevent *E. coli* O157:H7 growth in apple wounds (Janisiewicz *et al.*, 1999). *Pseudomonas graminis* (CPA-7) has also been shown to reduce titres of *Salmonella* spp. and *L. monocytogenes* in fresh cut melons (Abadias *et al.*, 2014) and apples (Alegre *et al.*, 2013a) and reduce titres of *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* on fresh cut apples and peaches (Alegre *et al.*, 2013b). Presence of these species could reflect a natural protective barrier for food safety systems. Future studies need to focus on the correlation between presence of *Pseudomonas* spp. and the presence or absence of foodborne pathogens. These findings show that even though the pathogens are able to adhere to the fruit surfaces (Chapter 3) and *Salmonella* Typhimurium and *L. monocytogenes* were present on fruit at harvest, the competition of possible natural antagonists, non-targeted impact of chemical drenching, CA storage conditions and extended fruit storage periods at low temperatures cause high pressure on foodborne pathogens and their ability to survive a range of extreme conditions.

This study provides some insight into microbial load and on the presence of microorganisms on fruit surfaces. This information can be used to develop a health and safety index for fresh produce. The aerobic bacterial population on the pear fruit surface expressed as colony forming units per square centimetre can provide an indication of the actual true bacterial load on healthy fruit surfaces. The DoH does not have a standard for an acceptable level of aerobic bacterial on raw ready- to- consume fruit and vegetable surfaces. Currently data on fungal, yeast, coliforms and foodborne pathogens (including *E. coli*, *Salmonella* spp. and *L. monocytogenes*) titres are expressed as CFU/g, which is not surface specific representation (DoH, 2006). Microbial guidelines for fresh produce need to be developed to reflect surface contamination and actual microbial loads.

Beuchat *et al.* (2003) has previously shown the variability between results presented as CFU/g and CFU/cm² as a result of the differences in ration variance of weight vs. surfaces of different products. The complexities in converting data from CFU/g to CFU/cm² is difficult and nearly impossible to do. The review highlights this with an example by comparing the decontamination processes between tomato and lettuce leaves. Vast differences are seen as described by Beuchat *et al.* (2003). A 3-log decontamination in CFU/g of lettuce leaves would result 0.114 log reduction in CFU/cm², whereas the same log decontamination expressed as CFU/cm² results in approximately 78.9 log reductions in CFU/g. Tomatoes resulted in the opposite, as a 3-log reduction in CFU/g result 18 log reductions in CFU/cm² whereas the 3 log decontamination in CFU/cm² resulted in approximately 0.5 log reductions in CFU/g. This study highlights the importance of proper microbial surface microflora and microbial loads for food fit-for-purpose to represent a healthy safe food portion. Therefore a natural aerobic bacterial load for pears can be considered to be as high as 5 log CFU/cm² based on this study.

The required absence of foodborne pathogens (*L. monocytogenes*, *Salmonella* spp.) indicator organisms (*E. coli*, coliforms) on ready-to-eat fresh fruit is not applicable to field harvested fruit. This study has shown that these foodborne pathogens may be present in the field but could not be detected post controlled atmosphere storage conditions. Commercial intervention steps are thus found to be significant to prevent foodborne pathogen introduction into the consumer's end product. Food products that undergo processing steps prior to consumption are not assessed in the same manner as ready-to-eat food. In the absence of appropriate guidelines retailers often refer to guidelines or standards developed

in the food industry. Fresh produce microbial criteria should therefore be developed that are more realistic and scientifically justifiable for food safety assurance in primary products. In food safety systems raw material used in food processing or prepared must also be acceptable and not introduce a potential hazard in the food chain.

In the detection of foodborne pathogens, chromogenic media resulted in a high number of presumptive positives. Further analysis by multiplex PCR and 3M MDS of the presumptive positives identified on chromogenic selective media were in fact false positives. Subsequently we concluded that our results show high variability and poor sensitivity when only a selective media approach was used as reported by Holfelder *et al.* (1998). The unreliability of results obtained from selective media was similar to the findings of Alvarez *et al.* (2012). Arroyo and Arroyo (1995) stated that the enumeration step results in selection against the target organism. This phenomena occurs as a result of species outcompeting one another when present in high concentration causing difficulty in pathogen detections. Adapting the detection approach in season 2, led to no false positives and detections were found to be more time effective. However, DNA level detection should not be the only detection method as dead organism's DNA would still be present and therefore back tracing a positive molecular result to determine viability of the organism is crucial to confirm the presence of the hazard. Combining molecular and viable detection methods in a food safety assessment strategy provides a clear diagnostic framework for future studies.

5. CONCLUSION

The decreasing microbial load through the supply chain suggests that external environmental changes influences the survivability of bacteria on the pear carpoplane. The dominant residential bacteria on Packham's Triumph reflected both harmful and beneficial bacteria organisms. After CA storage the bacterial diversity was found to be lower. Foodborne pathogens (*Listeria monocytogenes*, *Salmonella* spp.) were detected at harvest but not thereafter. A food safety management system risk assessment tool confirmed an average to advanced-average food safety output (Chapter 5). The microbial data was therefore supportive of the effective food safety management system. A defining statement on the status of a healthy Packham's Triumph pear is reflected in the bacterial diversity and absence of foodborne pathogens. Future research should focus on other cultivars and the impact of processing technologies on the bacterial biome.

6. REFERENCES

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CHAPTER 5

ASSESSMENT OF FOOD SAFETY MANAGEMENT SYSTEMS ON PEAR PRODUCTION FARMS IN THE WESTERN CAPE PROVINCE

ABSTRACT

A total food safety management system (FSMS) consists of several principles, guidelines and standards within a specific producer's farm and postharvest environment. These systems aim to assure and maintain safe consumable fresh produce through the control of chemicals, microbiological and physical contaminants. The implementation of a complex FSMS does not always have the desired outcome or assurance as interpretation of standards differ between countries, industry sectors and even producers. A number of parameters contribute to FSMS that identify areas where human error may occur due to the complexity of the management systems. In order to assess the level of effective interpretation in the FSMS, a horticultural safety management system (HSMS) diagnostics system has since been developed. This tool covers all the critical control points in order to identify risky areas where FSMS are lacking in design. The contribution to the success of the implemented FSMS is obtained by accurate detection of the critical topics and specific identified needs of each individual farm. The objective of this study was to evaluate a diagnostic tool to assess the HSMS on five commercial pear production farming situations of the Western Cape Province, South Africa. The result identifies problems in the HSMS design where improvements can enhance the food safety output of a farm. The overall results of the riskiness and food safety output systems were identified to be at an average to advanced levels with variations for different farming practises. The HSMS diagnostic tool highlighted that farms focused more on chemical residue levels when compared to microbial contamination. The reason for this is the export oriented nature of the industry and the effective regulatory chemical residue monitoring in the country. The value of the HSMS as a self-assessment tool was questioned as objectivity can be influenced by personal experience or perceptions. To conclude, the HSMS is a good tool to assess the riskiness and food safety output of the industry and of individual companies. However, an emphasis on microbial analysis should be realised in order to prevent possible outbreaks caused by human pathogenic microorganisms and standard/guidelines that should be implemented through proper testing and monitoring.

1. INTRODUCTION

A typical food safety management system (FSMS) include several formal food safety guidelines and standards. Unique regulatory points are implemented to cover the overall safety of the food product traded. These guidelines and standards are required for market access. Transparency within the documentation system of exporting companies allows for effective regulation and lowers trade barriers as the importing company complies with relevant food safety standards and regulations. A number of different developed guidelines, private schemes and standards are available for producers to include in their FSMS such as GLOBAL-GAP, ISO 22000. Choice of the guidelines and standards vary depending on retailer specifications. Food safety management systems are designed to lower and/or limit the spread of infectious human pathogenic microorganisms and reduce the risk of hazardous chemicals (Jaxsens *et al.*, 2009a; Luning *et al.*, 2006 and Tsalo *et al.*, 2007). Fresh produce industries implement several voluntary food safety systems to ensure compliance with market access. Not all food safety management systems are compulsory, rather voluntary but *de facto* compulsory. Food safety principles that have been adopted universally and have been integrated in all FSMS are hazard analysis and critical control points (HACCP), which focus on the prevention of physical, chemical and biological hazards.

South African markets require several different food safety compliance requirements for trade of commodities on local as well as international markets. GLOBAL-GAP has been *de facto* compulsory to all farmers exporting to retailers in European markets. A greater demand for healthier food and an increase in production and trade has resulted in a better food safety regulatory system (Jacxsens *et al.*, 2009b). A FSMS diagnostic tool has thus been developed to support compliance and adoption systems and assess food safety riskiness of farmers (Luning *et al.*, 2011). The overall outcome of this FSMS diagnostic tool is to indicate areas that could improve and decrease the risk of pathogenic microbial contamination.

In the horticultural field, safety management systems have been implemented based on best agricultural practices. Often these programs require practices which include prerequisite programs such as hygiene and good agricultural practices (GAP). Therefore the horticultural safety management systems (HSMS) diagnostic tool addresses critical aspects of the production process in terms of microbiological and chemical standard operating procedures and the efficacy in the HSMS. The outcome of the HSMS analysis shows areas where the

farmers can improve, have adopted or lacking in control systems. Since there is no single blueprint for all scenarios on all farms, each situation should be assessed and evaluated on its own according to those specific production practices (Semos and Kontogeorgos, 2007).

The aim of this study was to evaluate the diagnostic tool to assess the HSMS on commercial pear production and export farms in the Western Cape Province, South Africa. The model HSMS self-diagnosis tool (Kirezieva *et al.*, 2013) was used to perform as an indicator of the pear production farm industry to determine compliance levels within the industry. The result of this case study will allow recommendations to be made to producers to more effectively implement food safety and to strengthen international and domestic trade.

2. METHODOLOGY

2.1. FARMER SURVEY AND DATA COLLECTION

Due to the reluctance of farmers the microbial assessment was only completed successfully by five pear producing farms in the Western Cape Province, South Africa. The assessments were performed as an interview with the farm or quality manager based on questionnaires (Lunning *et al.*, 2013) during a Food Safety Workshop held in Grabouw, Western Cape Province. All farmers that participated were voluntary and identities were kept confidential in agreement.

2.2. FOOD SAFETY MANAGEMENT SYSTEM

The details of the full assessment are outlined in Lunning *et al.* (2013), for the purpose of completeness, a summary of each section is included in this dissertation (Appendix 3). The assessment has six basic principles. A total of five parts are included in the self-assessment tool under which numerous questions covering essential topics have been included. The main principles were grouped into the three core parts; the context of the farms (Part I), the control (Part II) and assurance activities (Part III) implemented in the FSMS. Part 0 covers an introduction section reviewing the production farm characteristics and Part IV covers the performance of internal and external FSMS. Answers were recorded in a weight scale with the associated responses. Each of the questions has a low, medium or high risk factor that describes the best suited scenario to the farming practice at hand. A radar diagram was then constructed according to specific weights selected (Figure 5.1 to 5.4).

2.3. ASSESSMENT OF CONTEXT CHARACTERISTICS

Under Part I (assessment of context characteristics) lies four assessment sections grouped into two parts that cover the product and process (Part I a) and organisation characteristics and chain environment characteristics (Part I b). In the product characteristics, the questions cover the possibility that initial materials could become contaminated through microbial organisms, the potential that pesticide could be prepared with contaminated water (Coghan, 2000; Guan *et al.*, 2001) or the potential that the final product delivered could be contaminated. Similarly the process characteristics assess the potential that the production and environmental conditions could lead or encourage microbial or chemical contamination. The organisation and chain characteristics evaluate the competency of the workforce and possibility of human error.

In this section the selection of one of three situations that best suit their conditions is required. In Part I, Situation 1 is a low risk factor, demonstrating that incoming planting material used to cultivate products are not associated with contamination and are considered safe to produce, the environmental conditions are suited to limit growth or survival and the company workforce has been trained accordingly with a working FSMS. Situation 2 and 3 are of medium to high risk factor. In choosing these situations the company has room for improvement.

Results in the contextual evaluation are shown displayed on the radar diagram in red, a smaller red area displayed conclude a minor risk level of the company (Figure 5.1). The more risky the contextual situation the more easily a food safety problem may arise, which will put higher demands on the HSMS.

The mean value of the product and process characteristics and for the organisation and chain characteristics was calculated. The overall score was assigned by interpreting the mean score as 1 (low risk) if the mean score of characteristics is 1 to 1.2, score 1-2 (low-medium risk) if mean score of characteristics is 1.3 to 1.7, score 2 (medium risk) if mean score of characteristics is 1.8 to 2.2, score 2-3 (medium-high risk) if mean score of characteristics is 2.3 to 2.7 and score 3 (high risk) if mean score of characteristics is 2.8 to 3.0.

2.4. ASSESSMENT OF CORE SAFETY CONTROL ACTIVITIES

Core safety control activities are those activities that contribute to the realisation of a safe product by evaluation of the performance of both technological and human processes and taking corrective actions when necessary. Core safety control activities consist of four critical aspects covering the designs of 1) preventative measures, 2) intervention processes and 3) monitoring designs as well as 4) the operation of the designed preventative measures, intervention processes and monitoring systems. Part II (core safety control activities) analyse the FSMS applied in the limiting of cross contamination through direct contact of workers, equipment, and the appropriate FSMS instated. The actual control activities focus on the performance of the management systems on equipment, production practices and decision making.

In Part II one of four case scenarios are selected that best describe the core activity followed within their production plan. The preventative and processes focus on aspects regarding how the company have set requirements with regards to the condition of the equipment, storage facilities and the handling of initial material received. It also covers detailing how and when sanitation and farming processes are done. The manner in how and when processes are completed is also covered within this section. Close relationship with stakeholders are key to the success of the farm. Assessing of operation at preventive measures, intervention process and monitoring systems describes the confidence level between the producer and stakeholder. The transparency between these two partners is assessed through the monitoring systems and clear paper trail of the production practices.

A choice of four different situations is presented at each of the core safety activities questioned. Each of the four situations represents the functionality of the systems design and of the monitoring system. Situation 1, low level monitoring and design would typically describe of the company has a limited plan or facilities available for proper food safety management. Situation 2 asses a basic level of systems that is known with little to no in-depth background, normally reactive. Workers with no experience on the management system design or no knowhow of proper monitoring systems will fall in this situation. Situation 3 can be considered as the advanced level, where topics related to in-depth knowledge and which are managed according to proper food safety management

specifications. Typically the design of the systems would also be proposed from an expert point of view. Situation 4, highly advanced level is associated with accreditation of methodologies, certification and implementation of systems, management of workers as experts in the field with extended experience in problem solving and adaptation.

Results for Part II are displayed as a radar diagram, the performance scale represented by a larger green area indicating good core safety control activities. To rate the core safety control activities, overall levels were assigned by averaging the activity levels. The overall level that was assigned was interpreted by the mean score, level 1 (basic) if mean score of activities is 1 to 2.2, level 1-2 (basic-average) if mean of score of activities is 2.3 to 2.7, level 2 (average) if mean score of activities is 2.8 to 3.2, level 2-3 (average-advanced) if mean score of activities is 3.3 to 3.7 and level 3 (advanced) if mean score of activities is 3.8 to 4.0.

2.5. ASSESSMENT CORE ASSURANCE ACTIVITIES

The assessment validates and verifies the efficacy of systems designed and processes implemented. The company/farm should be flexible enough to change according to feedback from the validation and verification processes and accommodate the changes from unforeseen external inputs. Proper observations, recordkeeping and scientific backing will ensure a fluent flow within the company/farm.

Scoring in the questionnaire of Part III (core assurance activities) is similar to Part II with four scenarios to choose from that suit the company's profile. Situation 1 indicates that nothing has been done to the question or topic at hand. The company would typically have no set processes; no recordkeeping and low performance throughout. Situation 4 suggests that the company is highly advanced in evaluating the FSMS using specific criteria and that improvements are not necessary. The food safety risk at this level would be extremely low as constant changes and testing within the company is conducted, as a result leading to safer foods being sold. The weight scales in Part III are similarly represented as in Part II. Again, to rate the core assurance control activities, overall levels were assigned by averaging the activity levels. The overall level that was assigned was interpreted by the mean score as in Part II.

2.6. ASSESSMENT OF FOOD SAFETY PERFORMANCE

Inspection services, audits and accreditation of internal and external performances on food safety within the company are analysed in Part IV (food safety performance). The company would want to have a low risk factor as this covers the global status of the company. With regular and proper third party audits the company is objectively analysed. Once the produce and company has been analysed the product can be delivered to the market as a low food safety risk factor. The weight scales in Part IV are represented as in Part II and III.

Therefore results in Part I should not have high scoring with little to no colour in the radar diagram whereas Part II – IV one typically is looking for a colourful radar diagram indicating low food safety risk factors. To obtain an overall food safety output, overall levels were assigned by averaging the activity levels and the scores were the same as Part II.

3. RESULTS AND DISCUSSION

All farms in the study have between 10 and 49 permanent workers and between 50 and 249 temporary workers, producing, harvesting and packaging mainly deciduous fruit. The overview of five farms from the Western Cape Province on general farming questions concluded that all farms analysed were certified to the voluntarily standard of GLOBAL-GAP. One of the farms where in addition to GLOBAL-GAP also certified to HACCP (SANS 10330) and British Retail Consortium (BRC) standard and the Tesco private standards (Appendix 3).

3.1. ASSESSMENT OF CONTEXT CHARACTERISTICS (PART I)

Data collected from the self-assessment tool allowed the determination of the riskiness due to the contextual factors which include product, production, organisation and chain characteristics associated with the production of pears. The following questions were answered the same for all five farms and were subsequently excluded from the radar diagrams but were used to calculate the overall risk level. The pesticide risk of initial material were all placed into medium risk situation, mycotoxin risk of initial materials and microbial contamination of the production system were all placed into high risk situation. The microbial risk of initial materials were placed into a medium risk situation, except farm 4 which was placed into a low risk situation. The following questions were placed into situation 1 for all five farms 1) food safety information exchange, 2) inspection authorities,

3) specificity of external support and 4) specificity of the food safety legal framework. Questions where one out of the five farms answered variably were also not included in the radar diagrams. These included technical staff and information systems which were placed into situation 2 for four farms and for Farm 4 into situation 1 also the supplier relationship was placed into situation 3 for all four farms except for Farm 5 which was situation 1.

The combination of a higher risk product and production context with a lower risk organisation and chain context results in a medium to high contextual risk. The overall contextual risk result showed that Farm 1, 2, 4 and 5 were rated to be producing a medium risk product and Farm 3 was rated as producing a high risk product (Table 5.1).

Product and production factors that lead to all farms producing a medium to high risk product include the lack of testing for mycotoxins in the initial materials, climatic conditions with open field cultivation increasing the risk for microbial contamination in the production system (Figure 5.1). However risk factors like climatic conditions cannot be changed or altered therefore would need greater emphasis on preventative and intervention measures to ensure a reasonable food safety output score. In a recent study (Kirezieva *et al.*, 2015) using the self-diagnostic tool it was found that produce (including leafy greens, berries, fruit, and other fresh produce) produced by 118 different producers from countries around the world all resulted in medium to high risk products when the product and production characteristics are taken into account. Similar factors as found in this study contribute to high riskiness of the produce (Kirezieva *et al.*, 2015).

Organisation and chain contextual factors can be controlled and influenced by each farmer/company. The overall scoring of the organisation and chain contextual characteristics was at low-medium risk level for Farm 4 and 5 and at a medium risk level for Farm 1, 2 and 3 (Table 5.1). High risk organisational characteristics such as employee involvement where operators have no involvement in the FSMS, therefore often lack commitment and motivation to implement the FSMS and also formalisation of communication with regards to the FSMS resulted in a more risky organisational context. Proper food safety training of technical staff was also conducted on all farms, therefore lowering the riskiness. Farm 4 and Farm 5 (Figure 5.1) have shown to have the best overall organisation and chain conditions. In this section all farms indicated that there were low supplier relationships, therefore increasing the riskiness of the chain. In order to decrease the chain risk the importance of regulatory authorities, international quality assurance

requirements and legal frameworks needs to be realised for all farms (Figure 5.1). Organisation and chain characteristics in this study are similar to Kirezieva *et al.* (2015) from producers around the world. However, technical staff in this study have more specific knowledge on food safety with some producers implementing external food safety expertise to implement the FSMS as well as the food safety information exchange and most farms have dedicated individuals and teams within the management position that drive the FSMS.

3.2. ASSESSMENT OF CORE SAFETY CONTROL ACTIVITIES (PART II)

Information gathered from the self-assessment tool allowed the farms to be characterised into different levels of control. The basic assumption behind the FSMS self-assessment tool indicated that a very risky contextual situation requires a more advanced control activity. The following questions were answered the same for all five farms and were subsequently excluded from the radar diagrams but were used to calculate the control activity level. The sanitation program and personal hygiene requirements for all five farms were placed into the average level and advanced levels were evaluated for the methods used to assess pesticides. Questions where one out of the five farms answered variably were also not included in the radar diagrams. Maintenance and calibration program were at the advanced level for four of the farms and for Farm 4 were rated at the average level. Farm 4 used advanced level of control for incoming material and the other four farms used average levels. Supplier control and corrective action at average levels were implemented for four farms but a basic level was implemented for Farm 5. Control of the organic fertiliser program and the risk analysis at Farm 3 was at a basic level, where all other farms were at average levels.

The overall score of the mean control activities showed that Farm 4 and 5 have average-advanced level of activities, Farm 2 was rated as average and Farm 1 and 3 were rated as having basic control activities (Table 5.1). The control activity design of Farm 5 was rated as basic-average and due to the average-advanced nature of the operation of the control activity it results in an increased overall score (Table 5.1). Farm 4 was rated as having an average control activity design but due to the average-advanced control activity operation it contributed to an increased overall score (Table 5.1). In the opposite scenario for Farm 1 and Farm 3 the control activity design were rated as basic-average but due to basic rating of the operation of the control activities, the overall score decreased (Table 5.1). Farm 2 had average rating from the design, operation and overall mean of the control activities.

Table 5.1. The mean ratings of five farms on all assessments contributing to the mean food safety risk output

	Mean product and process	Mean organisation and chain	Mean contextual	Mean control activities design	Mean control activities operation	Mean control activities	Mean assurance activities	Mean food safety output
Farm 1	2.5	1.9	2.2 medium	2.5	1.8	2.1 basic	2.6 basic-average	3.1 average
Farm 2	2.4	1.8	2.1 medium	3.0	3.0	3.0 average	2.2 basic	3.4 average-advanced
Farm 3	2.5	2.1	2.3 high	2.4	1.8	2.1 basic	2.6 basic-average	3.1 average
Farm 4	2.1	1.4	1.8 medium	3.2	3.6	3.4 average-advanced	3.3 average-advanced	3.4 average-advanced
Farm 5	2.1	1.4	1.8 medium	3.1	3.4	3.3 average-advanced	3.6 average-advanced	3.9 advanced

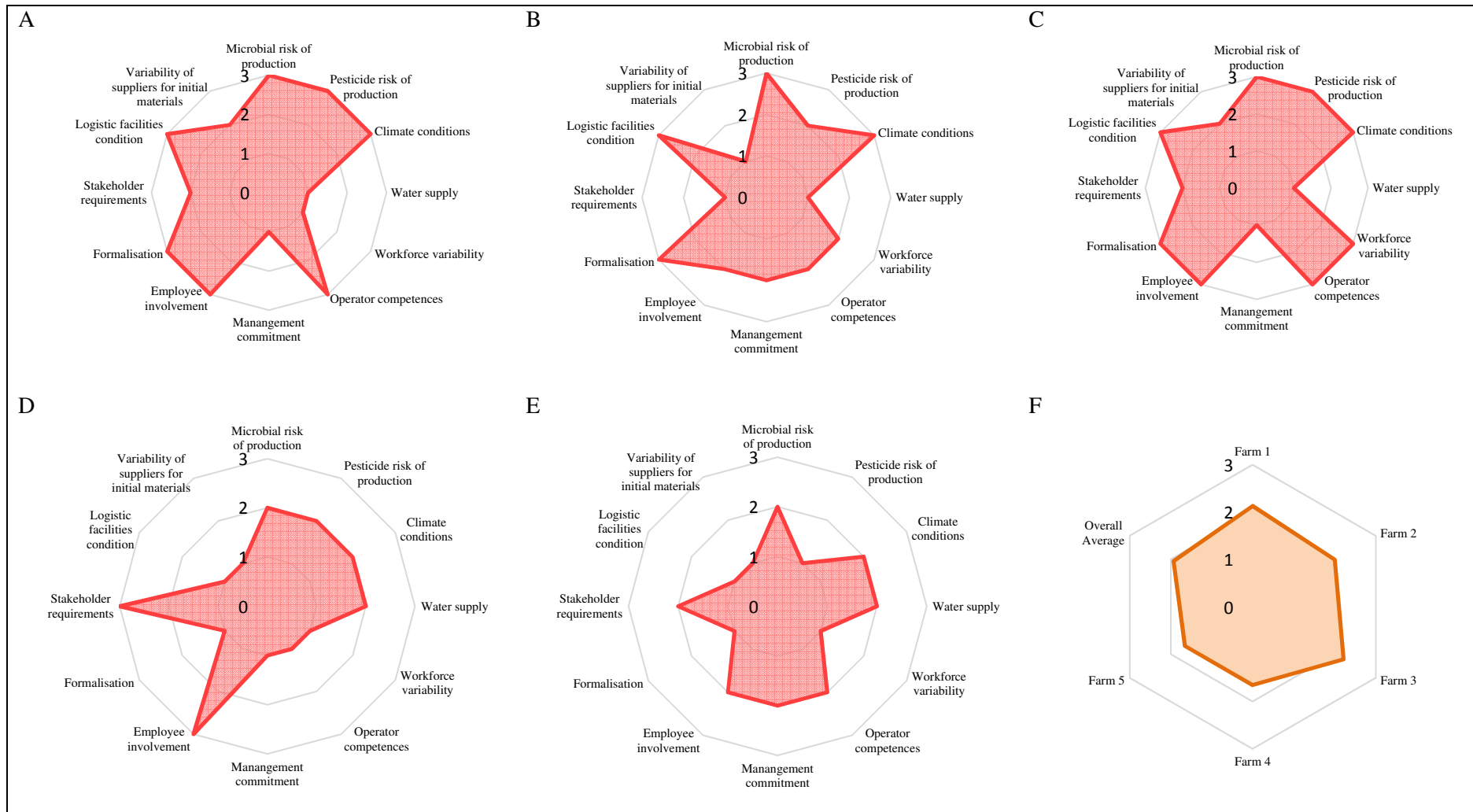


Figure 5.1. Assessment of product and production as well as organisation and chain characteristics from Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5 (E). The overall average assessment level of each farm and combined farms (F).

Low (1), medium (2) and high (3) risk situations for product and process characteristics correspond to low, potential and high chance of microbiological or chemical contamination as well as correspondence to supportive, constrained and lacking administrative chain conditions or low, restricted and high dependence on other chain actors (Kireziewa *et al.*, 2015)

On average the design of the preventative measures, intervention methods and system design resulted in average control activity design. Factors that decreased the scores for the control activity design include the lack of proper microbial sampling plan decreasing the information being contributed to improve food safety control, only two farms had a sampling plan based on in-house knowledge and utilise the system on a spot check bases (Figure 5.2). In the EU and other countries tested the majority of farms did not have microbial sampling plans in place (Kirezueva *et al.*, 2015). Microbial sampling and testing is not a requirement for fresh produce farms.

Methods used to assess the presence of foodborne pathogens on the majority of EU and non-EU farms were at low levels (Kirezueva *et al.*, 2015), we found that this factor was rated as basic to average levels on pear production farms. In this study hygienic design which only meet basic hygiene requirements, non-specific partial physical intervention and methods to assess equipment also contributed to a lower control activity design score.

All farms were assessed to be using highly advanced methods to assess pesticide compliances, this was also observed on EU farms however not on non-EU farms (Kirezueva *et al.*, 2015). South African pear producing farms that export to the EU comply with EU standards with regards to pesticide spraying and testing to ensure that maximum residue level on fruit are below threshold levels in order for the product to move into the export market, therefore this factor scores similarly to that of EU farms. Samples collected for testing of pesticides are done so by a third party external accredited laboratory. The pesticide program was thus considered on advanced levels on three of the five farms where the two farms utilized advanced spraying programs based on site specific scientific background. These two farms comply with standards focussed on application of pesticides, certified pesticide usage and exact application (timing and amount) of sprays.

Farm 4 had the highest scoring operation of control activities with an advanced level, followed by and Farm 5 with an average-advanced scoring operation of control activities, then followed by Farm 2. Farm 1 and 3 had basic control activities operating levels. All farms in this study conduct *ad hoc* hygiene performance of equipment and facilities. In contract the majority of producers studied in the EU and other countries as outlined by Kirezueva *et al.* (2015) had no information about on-farm hygienic performance.

Farms 1 and 3 showed that no stable system regarding storage and equipment performance measurements were present. Farm 5 does not have packaging facilities therefore could not attain high scores regarding package equipment as well as packaging capacity, therefore further decreasing the overall score. Packaging equipment not being in place is rated the same as not knowing the performance of the packaging equipment. In this study some farms use a communal or a consortium packhouse, therefore this result shows a lower level of control. Kirezieva *et al.* (2015) found that EU countries had stable performance of the on-farm storage or cooling facilities, where as non-EU countries had mostly storage or cooling facilities with little information on the performance. In this study it was evaluated that the storage or cooling facilities on the farm had automatic conditions to control for temperature and humidity with no analysis of deviations.

3.3. ASSESSMENT OF CORE ASSURANCE ACTIVITIES (PART III)

The following questions were answered the same for all five farms and were subsequently excluded from the radar diagrams but were used to calculate the risk level. Compliance to procedures, hygienic performance, documentation system and record keeping systems were considered at the average level for all five farms. An average level of availability of procedures was considered for all farms, except for Farm 5 where it was at the advanced level. Farm 2 recorded basic levels for feedback information to modify HSMS whereas all other farms were at the advanced level.

The overall rating of the core assurance activities followed the same trend as the core safety control activities. Farm 2 was rated as having basic assurance activities, Farm 1 and 3 were rated as having basic-average assurance activities and Farm 4 and 5 were rated as average-advanced assurance activities (Table 5.1).

Product safety is obtained through proper system requirement activities. Due to retail chains imposing quality and safety standard, all farms are pro-actively involved with stakeholder requirements by implementation of proper quality assurance systems (*i.e.* HACCP, BRC and ISO 22000) that should increase safety of marketable products. Typically these farms implement the systematic use of feedback information to allow the modification of HSMS systems as new legislation and international standards are adapted.

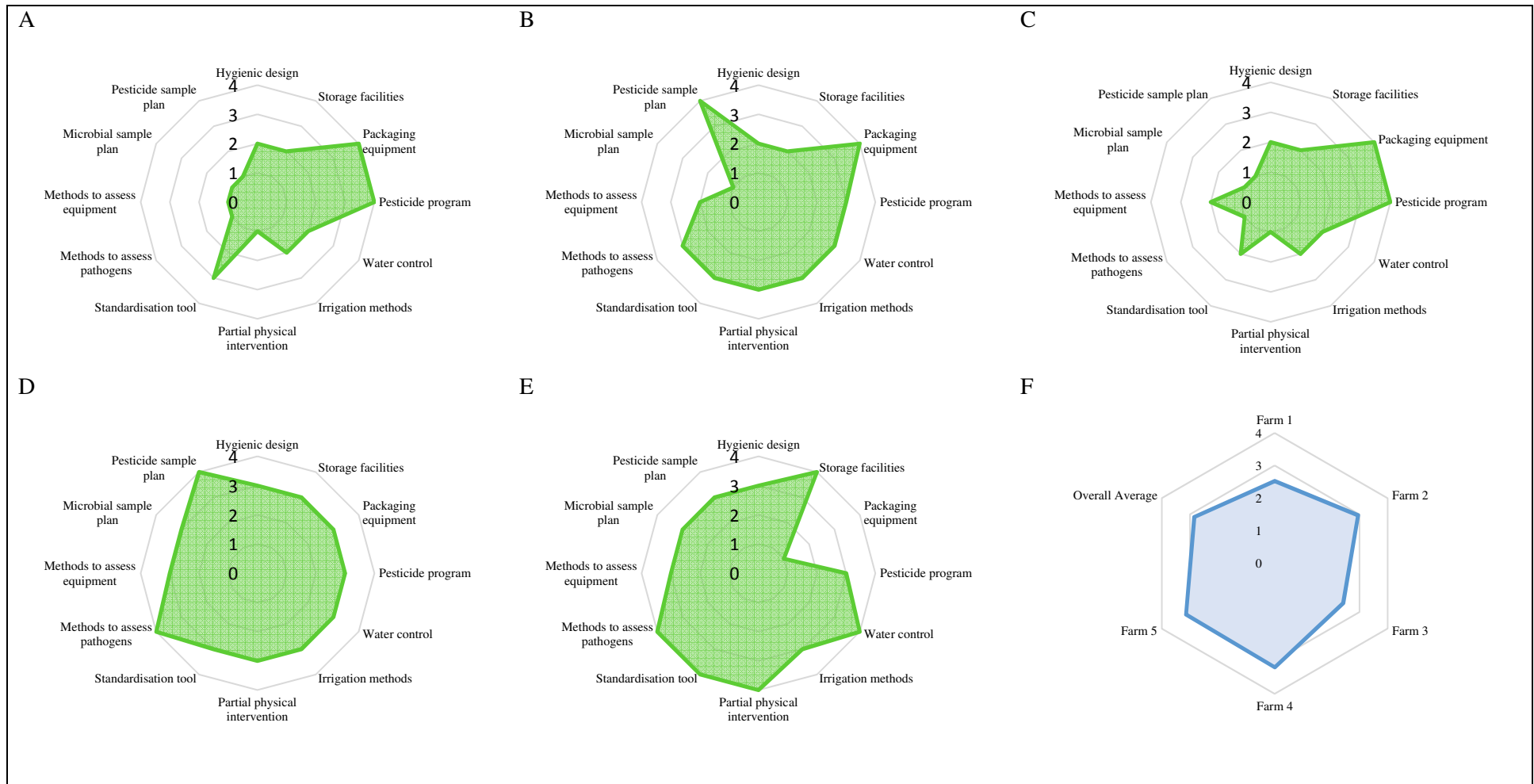


Figure 5.2. Assessment of control activities design from Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5 (E). The overall average assessment level of each farm and combined farms (F).

Situation levels represented by low level (1) - absent, not applicable, unknown; basic level (2) - lack of scientific evidence, use of company experience/history, variable, unknown, unpredictable, based on common materials/equipment; average level (3) - best practice knowledge/equipment, sometimes variable, not always predictable, based on generic information/guidelines for the product sector; advanced level (4) - scientifically underpinned (accurate, complete), stable, predictable, and tailored for the specific food production situation (Kirezieva *et al.*, 2015).

Farm 5 scored the highest with regard to the core assurance activities due to a number of factors, including the feedback information to the HSMS, prevention measures, intervention processes validation, monitoring process validation and people related performance validation were all scored at the advanced level, all other factors were scored as average-advanced (Figure 5.4).

All farms in this study, as well as farms in the study conducted by Kireziova *et al.* (2015) recorded well-structured record keeping and documentation systems, which positively add to the product safety. All core assurance activities in this study were rated similarly to EU farms assessed by Kireziova *et al.* (2015).

3.4. ASSESSMENT OF FOOD SAFETY PERFORMANCE (PART IV)

The following questions were answered the same for all five farms and were subsequently excluded from the radar diagrams but were used to calculate the food safety output level. Microbial complaints, product sampling to confirm use of pesticides, judgment criteria of pesticides and non-conformities were placed into the advanced level for all the farms and visual quality complaints were placed considered average. Farm 5 had advanced FSMS evaluation whereas all other farms had average FSMS evaluation.

The overall food safety output indicated that Farm 5 had advanced food safety indicators, Farm 2 and 4 had average-advanced food safety output and Farm 1 and 3 had average food safety output (Table 5.1).

All farms have medium risk initial materials (Figure 5.1) and Farm 1 and 3 have high pesticide production risk, Farm 2 and 4 have medium risk and Farm 5 has low pesticide production risk (Figure 5.1). All farms have average or advanced pesticide sampling plans that are well structured on company level and regular monitoring on a sector level. Methodology used to assess pesticides are all at advanced levels with results being judged on legal, external party and internal guidelines. Farm 1 and 3 have limited chemical complaints and Farm 2, 4 and 5 have no chemical complaints. All these factors lead to increased FSMS output. Kireziova *et al.* (2015) found that the majority of companies (93 of the 118) studied demonstrated average and advanced levels of pesticide management.

Farm 1 and 3 both have medium microbial risk of initial materials and the microbial risk of production is high (Figure 5.1 A and C), these two farms do not have a microbial sampling plan nor methods to assess microbial contamination (Figure 5.2 A and C) therefore have a low level performance with regards to sampling and judgment of microbiological analysis within the internal FSMS assessed (Figure 5.4 A and C). Farm 4 and Farm 5 have low and medium initial material microbial risk, respectively, and medium microbial production risks (Figure 5.1 D and E). Both farms have average and advanced control measures for the microbial sampling plan and pathogen detection methodology (Figure 5.2 D and E). Farm 5 have advanced microbial judgment levels with results that are obtained being compared to legal criteria, specifications of external parties as well as internal guidelines, therefore leading to a better FSMS output. Farm 4 only conducts *ad hoc* sampling on the final product and compares the results to legal criteria and specifications from external parties. Farm 2 has medium initial material microbial risk and high production risk (Figure 5.1 B) and does not have a sampling plan in place and all methods used to assess microbial pathogens were culture based in a non-certified laboratory (Figure 5.2 B). However in the analysis of the FSMS this farm rated that structured microbial sampling is conducted on the final product and only legal criteria are used to assess the microbial results obtained (Figure 5.4 B). For all farms no microbiological complaints were received (Figure 5.4).

The external FSMS are evaluated by third party auditors against quality assurance standards; this eliminates any bias results to that company (Figure 5.4). Chemical and visual complaints are restricted or have been received, resulting in good quality FSMS implementation. No non-conformities regarding microbiological indicators, pesticides or mycotoxins were registered, therefore a good food safety performance can be expected (Figure 5.4). Kirezieva *et al.* (2015) found that companies in the EU had restricted numbers of non-conformities and non-EU companies in the study had no formal system in place to register non-conformities. The findings reflected in this study have indicated that the self-assessment of the food safety management systems can lead to bias in the farms favour, especially with sensitive questions. This is illustrated by Farm 1, Farm 2 and Farm 3 who rated themselves as having a highly advanced performance level for non-conformities which indicates that no non-conformities regarding microbiological food safety, hygiene indicators and pesticides or mycotoxins were received but none of these farms test for mycotoxins and only some indicated that microbiological assessment was done. Further development of the

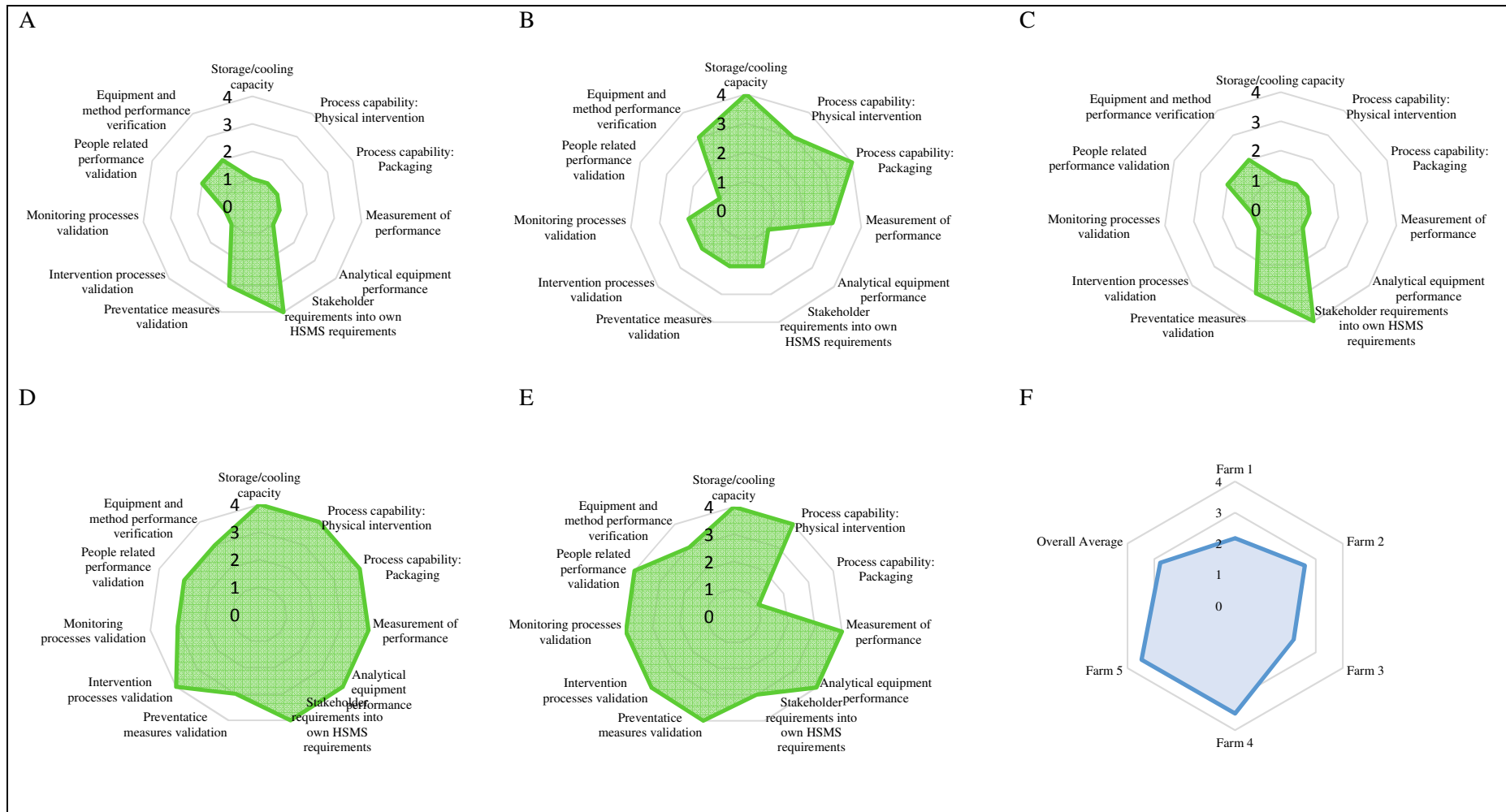


Figure 5.3. Assessment of control activities operation of Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5 (E). The overall average assessment level of each farm and combined farms (F).

Situation levels represented by low level (1) - absent, not applicable, unknown; basic level (2) - lack of scientific evidence, use of company experience/history, variable, unknown, unpredictable, based on common materials/equipment; average level (3) - best practice knowledge/equipment, sometimes variable, not always predictable, based on generic information/guidelines for the product sector; advanced level (4) - scientifically underpinned (accurate, complete), stable, predictable, and tailored for the specific food production situation (Kirezieva *et al.*, 2015).

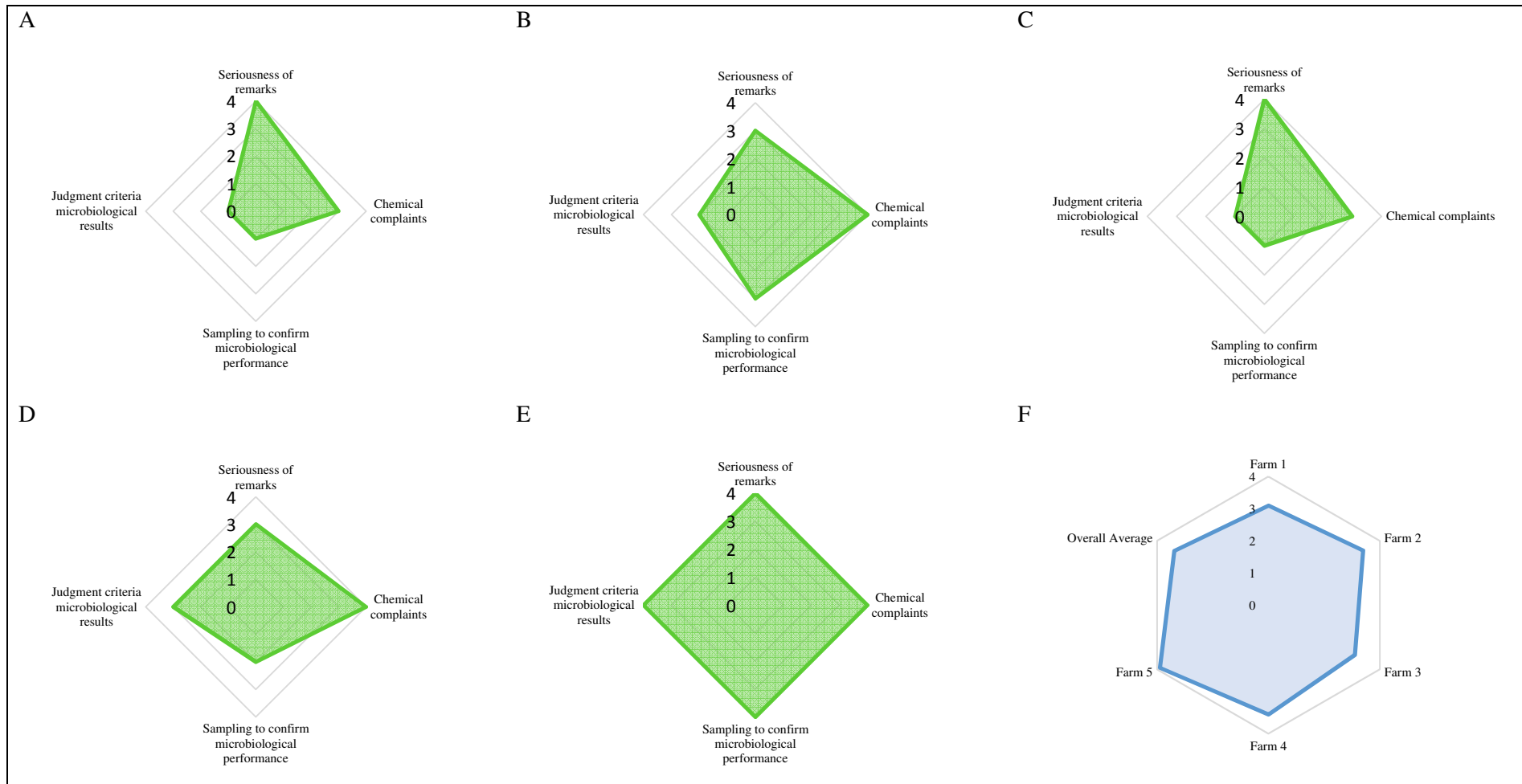


Figure 5.4. Assessment of food safety performance, external and internal food safety performance indicators of Farm 1 (A), Farm 2 (B), Farm 3 (C), Farm 4 (D) and Farm 5 (E). The overall average assessment level of each farm and combined farms (F).

Scenario levels represents (1) - absent, not applied, unknown; poor output (2) - ad-hoc sampling, minimal criteria used for evaluation, various food safety problems due to different problems in the activities; moderate output (3) - regular sampling, several criteria used for evaluation, restricted food safety problems mainly due to one (restricted) type of problem in the activities; good output (4) - systematic evaluation, using specific criteria, no safety problems (Kirezieva *et al.*, 2015).

self-assessment tool should include microbiological, mycotoxin and pesticide non-conformities as separate questions to improve the food safety output accuracy.

4. CONCLUSION

Application of the FSMS diagnostic tool reflect all major performance levels associated with the production farm. The results and conclusions regarding each farm have unique characteristics to the specific on-farm scenarios and are scored accordingly. Therefore a uniform direct application to all the different types of farms cannot reflect the true management system and performance level of a single given farm (Semos and Kontogeorgos, 2007). The CDC (2013) found that ten sites monitored for over a 13 year period had no positive or negative change in the resulted human microbial pathogenic infections after implementing a better FSMS. The current data on outbreaks suggests that the implemented FSMS are not enough to limit infections and spread of human pathogenic microorganisms. The need for a definite food safety assessment is needed to cover all aspects of the applied FSMS. In this study it has been found that in order to evaluate compliance levels of pear production systems, the self-assessment tool needs to be adapted to suit the commodity and its final use.

Three main issues that needed to be assessed according to the tool for fresh produce include pesticide residue levels, mycotoxin presence and microbial contamination (Kirezieva *et al.* 2013). In order for fruit to be exported into various global markets fruit are tested for pesticide residue levels in compliance with international standards. Mycotoxins are only considered a problem and a chemical hazard when fruit enter the processing arena. In this study pears were used for whole fruit sale and therefore mycotoxin testing is not required. However, some of this fruit will be desired for juicing and in that case the bulk shipment should be tested prior to processing. We therefore propose that in the assessment of fresh produce product safety from farms that contribute to whole fruit sale, mycotoxin testing should not be included. For the pear farms in the current study removing all mycotoxin related question did not have a significant effect on the final food safety output, however the potential does exist that this could change the control activity level and overall food safety output from a lower to a higher level. At primary production the FSMS is a result of the implementation of good agricultural and hygienic practices. During the implementation of good agricultural and hygienic practices there is no requirement for microbial testing of fresh produce. This is not a recommendation within the EU, nor for produce produced within the USA. Most companies do not have knowledge

and awareness about microbiological hazards within the fresh produce production arena. On the other hand, within the processing and trade arena the FSMS includes HACCP-based principles, good manufacturing and hygiene practices, where microbial and mycotoxin testing becomes important. Future work should include thorough microbial and pesticide testing in parallel to the risk assessment to be able to make direct statements on current implemented management systems.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

The South African pear industry exports 48% (181 928 tonnes) of total whole-pear fruit produced annually (Hortgro, 2013). The fruit surface microbial ecology, food safety and production risk assessment focused studies for pears has to the authors knowledge not been investigated. The carpo/plane microbial status interlinks public health, microbial ecology and food safety to consumers of unprocessed fruit. In this study we described overall bacterial diversity on pear fruit surfaces. Microbial organisms reside naturally on plant surfaces and can be introduced through external sources. Not only natural residing organisms but also foodborne pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium) could contaminate and have been found to attach and colonise different food sources (Centres for Disease Control and Prevention, 2014). The ability of microorganisms to survive in a pear production chain depends highly on an organisms' ability to overcome host defence mechanisms and to adapt to fluctuations in environmental conditions as well as the ability to rapidly attach and colonise the surface (Collignon & Korsten, 2010; Sapers *et al.*, 2005). A proper scientific evaluation of fruit surfaces is needed in order to determine the microbial flora of a healthy pear. The microbial load and the presence of identified microorganisms present on fruit surfaces, contributes to a better understanding of pre- and post-harvest factors affecting microbial ecology on a healthy pear surface. A diagnostic tool assessing the food safety management systems (FSMS) within a typical pear production supply chain further identified the shortcomings of farming practices and contributed to determining the potential link between food safety hazards and current management systems for more effective risk management studies.

The influence of temperature typically controls the growth rate of microbial organisms, along with other factors. The optimum growth temperature range residential microbes were found to be similar to that of pear surface and atmospheric temperatures measured. *Curtobacterium flaccumfaciens* (Funke *et al.*, 2005), *Pantoea agglomerans* (Jung *et al.*, 2002), *Pseudomonas azotoformans* (Iizuka & Komagata, 1963), *P. syringae* (Young *et al.*, 1977), *Serratia marcescens* (Hejazi & Falkiner, 1997) were some of the dominant microbial organisms identified in this study to have these optimal growth temperature ranges. The clustering of the associated bacterial species resulted in three dominant phyla, these include Firmicutes,

Actinobacteria and Proteobacteria (divided into the Beta- and Gamaproteobacteria classes). Dominant Microbacteriaceae account to 46% of dominant species identified in this study. Leff and Fierer (2013) reported that Microbacteriaceae were dominantly present on peach surfaces (16.4 %) though unculturable techniques were applied.

The importance of survival and the attachment mechanisms of foodborne pathogens is described in a study by Solomon and Matthews (2006). Findings showed that even though the pathogens were able to adhere to the fruit surfaces (Chapter 2), several factors such as, chemical drenching, environmental and storage conditions as well as extended fruit storage periods may reduce the survivability of foodborne pathogens through the pear production chain. The non-corrugated, smooth surfaced with wax scales and without trichomes pear fruit surface presents a suitable niche to be populated by the residing microbes (Chapter 2, Roszak & Colwell, 1987). Inoculated foodborne pathogens are capable of colonising and surviving the fruit surfaces. The capability to cause a foodborne illness does not only depend on the survivability of the foodborne pathogen but is also influenced by the infectious dose ingested at the point of consumption. In this study it was found that *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium but not *Staphylococcus aureus* were able to survive at a high enough infectious doses to potentially cause an illness. Therefore it is important to determine how the foodborne pathogens interact and survive on fruit and vegetable surfaces as well as where contamination occurs along the supply chain in order to implement the appropriate control steps required within the production and transport environments.

The high number of recorded foodborne pathogen outbreaks have been traced back to the production stages concluding that the probability of foodborne pathogens able to survive up to the market stages are high, resulting in foodborne illness if contaminated produce are consumed (Todd *et al.*, 2009). Not only in this study have *E. coli* O157:H7 and *Salmonella* Typhimurium attachment structures been observed within a couple of minutes of pear surface exposure but also by Collignon and Korsten (2010) on peaches and plums. Residential epiphytic bacteria were speculated to influence *E. coli* resulting in low attachment in a study done on cherry tomatoes and strawberries (Ziuzina *et al.*, 2014). Residential microorganisms identified on pear fruit included both bacteria that are potentially harmful to humans and plants, as well as bacteria containing beneficial properties. Bacterial species isolated in this study has been identified to have plant and non-plant specific pathogenic properties (Braun-Kiewnick *et al.*, 2000; Janisiewicz & Buyer, 2010; Patten & Glick, 2002). Knowledge of the supply chain could

contribute to implementation of practices to reduce post-harvest losses and contamination of foodborne pathogens. Also early detection of foodborne pathogens reduces the potential health risk to consumers of raw unprocessed fruit. The most common microbial contamination sources are water, wind-blown dust, physical handling from pickers and contact surfaces within the packhouse.

Proper food safety production management systems implemented could drastically lower the risk of a possible foodborne disease outbreak. A diagnostic tool developed (Lunning *et al.*, 2011) and adapted (Kirezieva *et al.*, 2015) has highlighted some factors within the production system that increase and decrease the risk within a typical pear production farming system in the Western Cape Province. The overall risk findings of four pear producing farms were found to be on average to advanced management levels with minor variations between different farming company practices. Findings shown that the adapted Horticulture Safety Management System is a good tool for self-assessment to obtain an overall view of the level of riskiness of a company and the effectiveness of its management system. The emphasis should therefore still be focused on foodborne pathogen detection and prevention as outbreaks have led to mortalities to consumers of contaminated products.

The South African Department of Health does not have a standard for allowable or acceptable aerobic bacterial counts on raw consumed fruit and vegetables surfaces. Data on fungal, yeast, coliforms and foodborne pathogens (including *E. coli*, *Salmonella* spp. and *L. monocytogenes*) are expressed as CFU/g, which is not a true reflection on the total surface microbial numbers present (Department of Health, 2006). This study has been the first to present a true representation of the microbial status of a natural healthy pear. Concluding that the microbial load of an unprocessed pear is defined as an aerobic bacterial concentration of less than 5 log CFU/cm². In addition, no foodborne pathogens should be present in order to be classified as a healthy pear. Pears sampled after drenching (before CA) as well as after CA storage were classified as natural healthy pears. Future studies should investigate the impact of variable environmental factors, lower inoculation loads as well as host response to the interaction of foodborne pathogens, natural occurring biocontrol microorganisms, and host specific plant pathogenic and residential epiphytic microorganisms on the fruit surfaces. The combination of molecular and viable detection methods in a food safety assessment strategy provides a clear diagnostic framework. Other cultivars and the impact of processing technologies on the bacterioflora should also be considered in future research.

Microbial ecology studies are and will be revolutionised with culture-independent studies of the microbiome of plant surfaces. Future research should focus on the metagenomics approach to determine the complete microbiome of pears as well as to determine the effect that different farming practices have on the microbiome of pear fruit. In addition to investigating the culture independent microbiome of pear fruit, one can gauge the range of foodborne pathogens and bacterial plant pathogens found on the pear carpoplane.

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APPENDIX 1

Table 1: Controlled atmosphere storage conditions and maturity index values of five pear cultivars (Findlay & Combrink, 2013)

Cultivar	Release Criteria			Over Mature Standard		Atmospheric Conditions				Storage Period (Months)
	Firmness (kg)	% TSS	Acid %	Firmness (kg)	Acid %		O ₂ (%)	CO ₂ (%)	Temp (°C)	
Packham's Triumph	8	11.5	0.3	5	0.2	Opt.:	1,5	2,5	-0,5	9
						Max	2,0	3,0	0,0	
						Min	1,0	1,0	-0,5	
Forelle	6.8	13.5	0.3	4.5	0.2	Opt.	1,5	0,0 - 1,5	-0,5	7
						Max	2,0	1,5	0,0	
						Min	1,0	0,0	-0,5	
William Bon Chretien	10.5	11	0.35	7.2	0.3	Opt.	1,0	0,0	-0,5	4
						Max	1,5	0,0	0,0	
						Min	1,0	0,0	-0,5	
Beurre Bosc	8.1	12.5	0.3	4.5	0.2	Opt.	1,5	1,5	-0,5	4
						Max	2,0	2,0	0,0	
						Min	1,0	1,0	-0,5	
Doyenne du Comice	6.8	12	0.35	3.6	0.25	Opt.	1,0	1,0	-0,5	6
						Max	1,5	1,5	0,0	
						Min	1,0	1,0	-0,5	

*TSS – Total Soluble Solids

APPENDIX 2

Table 1. Pear surface temperatures of two consecutive seasons at harvest stages

Pears Harvested		Farm 1 9:00 am	Farm 2 11:37 am	Farm 3 11:00 am	Farm 4 13:20 pm
Season 1 (2013)	Replicate 1	13.2 ± 0.3	18.2 ± 2.4	20.0 ± 0.9	26.9 ± 1.3
	Replicate 2	11.8 ± 0.2	18.2 ± 4.1	18.9 ± 0.9	27.0 ± 0.8
	Replicate 3	11.2 ± 0.5	20.1 ± 4.4	20.9 ± 2.3	22.9 ± 0.4
	Replicate 4	14.0 ± 1.5	15.4 ± 1.5	17.0 ± 1.1	24.4 ± 1.0
Pears Harvested		Farm 1 10:05 am	Farm 2 11:40 am	Farm 3 13:27 pm	Farm 4 14:51 pm
Season 2 (2014)	Replicate 1	29.5 ± 4.6	20.3 ± 0.9	18.9 ± 0.5	20.7 ± 2.0
	Replicate 2	23.1 ± 5.2	19.7 ± 0.7	17.3 ± 0.9	21.4 ± 2.7
	Replicate 3	20.7 ± 1.7	19.7 ± 0.7	18.6 ± 0.2	20.6 ± 1.6
	Replicate 4	20.6 ± 0.6	19.5 ± 0.7	17.6 ± 4.0	21.7 ± 2.1

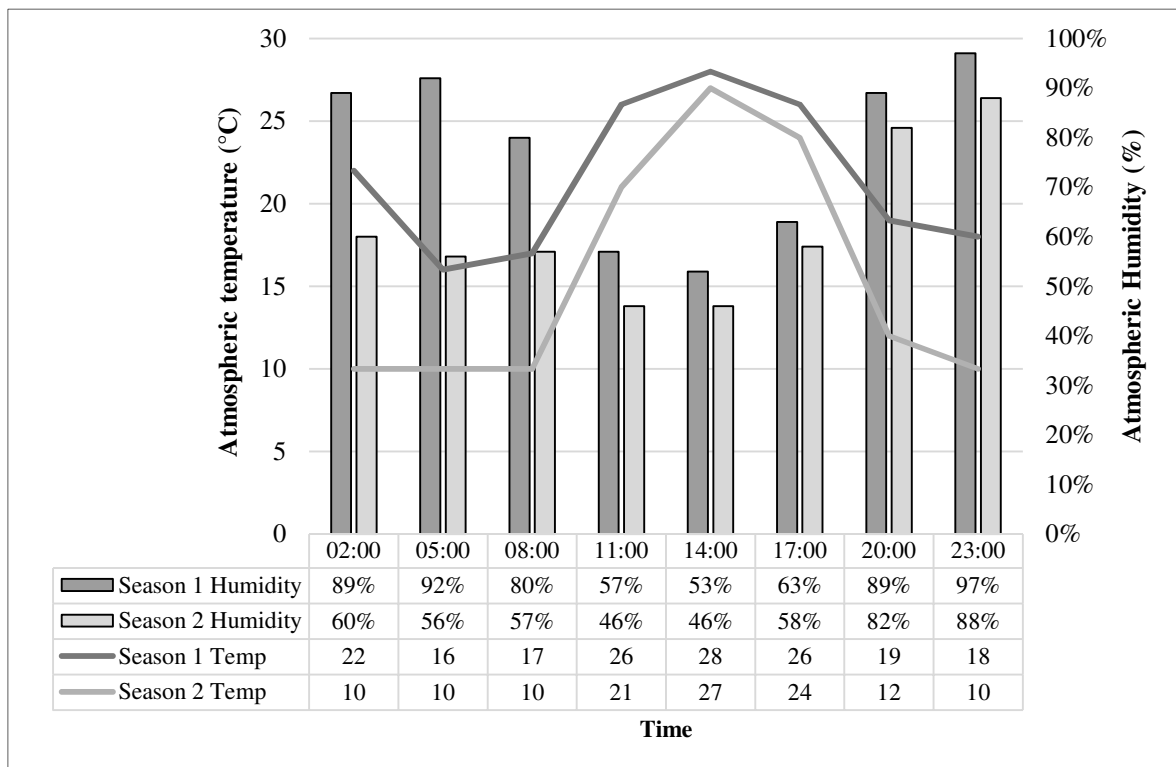


Figure 1. Weather data of two consecutive seasons on harvest day (18/2/2013 and 19/5/2014). (<http://www.worldweatheronline.com/>).

APPENDIX 3

	FARM 1	FARM 2	FARM 3	FARM 4	FARM 5
Part 0: Introduction and selection of Representative Production Unit (RPU)					
What is the ownership of your farm (e.g. independent producer, in a cooperative, out-grower/contract farmer)?*	Independent Producer	Cooperative	Independent Producer	Independent Producer	Independent Producer
Location of your farm	Elgin, Western Cape, South Africa	Elgin, Western Cape, South Africa	Elgin, Western Cape, South Africa	Elgin, Western Cape, South Africa	Grabouw, Western Cape
Total number of permanent employees in the company.	10-49	10-49	10-49	10-49	10-49
Total number of temporary employees in the company.	50-249	50-249	10-49	50-249	50-249
Products cultivated on the farm.	Apples, Pears, Peaches and Plums	Apples and Pears	Apples and Pears	Apples and Pears	Apples, Pears, Plums, Wine Grapes
What type of cultivation system do you have (e.g. greenhouse, open field)*?	Open Field	Open Field	Open Field	Open Field	Open Field
Are your products sold as organic production?	No	No	No	No	No
Do you have a combined production of cereals or animal by-products?	Yes	No	No	No	No
Which Quality Assurance (QA) standards/guidelines have been implemented*?	GLOBAL-GAP	GLOBAL-GAP	GLOBAL-GAP	GLOBAL-GAP, HACCP, BRC, Tesco Standards, Tesco Nurture	GLOBAL-GAP
For which QA standards is your company certified*?	GLOBAL-GAP	GLOBAL-GAP	GLOBAL-GAP	GLOBAL-GAP, BRC, Tesco Standards, Tesco Nurture	GLOBAL-GAP
Did the owner/manager of the farm undergo training on food safety/quality management*?	No	Yes	Yes	Yes	Yes
Which specific product is made in this production unit? (e.g. lettuce, spinach, apple, berries)	Pears	Pears	Pears	Pears	Pears
Who are the major customers of this specific product group? (e.g. auctions, open market, wholesalers, retailers, food processing companies, etc.)	Informal Market, Fresh Market, International Market, Wholesalers, Retailers, Food Processing Companies	International Market, Wholesalers, Retailers, Food Processing Companies	Informal Market, Fresh Market, International Market, Wholesalers, Retailers, Food Processing Companies	Wholesalers, Retailers	International Market
What are the initial materials that you use for these products? (e.g. seeds, seedlings, small trees for cultivation)	Certified Small Trees	Certified Small Trees	Certified Small Trees	Certified Small Trees	Certified Small Trees

	FARM 1	FARM 2	FARM 3	FARM 4	FARM 5
What is packaging/storage concept used for this product group? (e.g. no packaging, crates, bags, bulk containers, CA, etc.)	Packaged Boxes, Bags	Not Packed, Bulk, Crates, Juiced	Packed in Boxes and Not Packed	Crates, Bags, Bulk containers, CA.	Packed in Boxes and Not Packed
What are major activities to cultivate this product (e.g. planting, fertilization, irrigation, harvesting, packaging)	All	All	All	All	All
What are major units used for this product group (e.g. cultivation area/field, packaging area, storage facility, water storage, compost storage area, etc.)	Cultivation Area, Storage Facility, Holding Dam/Borehole, Compost Storage Area, Pesticides and Fertiliser Facilities	N/A	Cultivation and Storage, Holding Dam, Borehole, Pesticides and Fertiliser Facilities	Cultivation Area/Field, Packaging Area, Drenching (Post Harvest Chemical Treatment Plant), Storage and Cold Storage Facilities, Water Storage	Cultivation Area
What are major equipment/machines used of this product group (e.g. harvesting machine, packaging machine, weeding machine, pesticide sprayer, etc.)?	Pesticide Sprayers, Transport Tractors, Trucks, Mowers	Weeding Machine, Pesticide Sprayer, Transport Tractors, Trucks	Pesticide Sprayers, Transport Tractors, Trucks	Tractors, Picking Bags, Picking Bins, Contaminant Ladders, Forklifts, Trucks, Packshed Facility Machine, Pesticide Sprayer, Grass Cutting Machine, Weed Sprayer, Snail Pellet Spreader.	Weeding Machine, Pesticide Sprayer, Transport Tractors, Trucks

	FARM 1	FARM 2	FARM 3	FARM 4	FARM 5
Part I: Assessment contextual factors					
A. Assessment of product characteristics					
In which situation would you place the initial materials in respect to microbiological contamination?	Situation 2	Situation 2	Situation 2	Situation 1	Situation 2
In which situation would you place the initial materials in respect to pesticide contamination?	Situation 2	Situation 2	Situation 2	Situation 2	Situation 2
In which situation would you place the initial materials in respect to mycotoxin contamination?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
In which situation would you place the final product in respect to microbiological contamination?	Situation 3	Situation 3	Situation 3	Situation 2	Situation 2
In which situation would you place the final product in respect to pesticide contamination?	Situation 3	Situation 2	Situation 3	Situation 2	Situation 1
B. Assessment of process characteristics					
In which situation would you place susceptibility for microbial contamination of the production/cultivation system ?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
In which situation would you position the climate conditions in which your RPU operates, in respect to microbiological and chemical contamination?	Situation 3	Situation 3	Situation 3	Situation 2	Situation 2
In which situation would you position the water supply in respect to microbiological and chemical contamination?	Situation 1	Situation 1	Situation 1	Situation 2	Situation 2
C. Assessment of organization characteristics					
In which situation would you place your company with regards technological staff ?	Situation 2	Situation 2	Situation 2	Situation 1	Situation 2
In which situation would you place the variability of workforce composition ?	Situation 1	Situation 2	Situation 3	Situation 1	Situation 1
In which situation would you place requirements on operator competences ?	Situation 3	Situation 2	Situation 3	Situation 1	Situation 2
In which situation would you place management commitment of your company?	Situation 1	Situation 2	Situation 1	Situation 1	Situation 2
In which situation would you place employee involvement ?	Situation 3	Situation 2	Situation 3	Situation 3	Situation 2
In which situation would you place formalization to support decision-making in your company?	Situation 3	Situation 3	Situation 3	Situation 1	Situation 1
In which situation would you place information systems to support food safety (management system) decisions in your company?	Situation 2	Situation 2	Situation 2	Situation 1	Situation 2
D. Assessment of chain environment characteristics					
In which situation would you place requirements of stakeholders ?	Situation 2	Situation 1	Situation 2	Situation 3	Situation 2
In which situation would you place supplier relationships with respect to the major suppliers of critical materials for your RPU?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 1
In which situation would you place your food safety information exchange with the major suppliers of critical materials for your RPU?	Situation 1	Situation 1	Situation 1	Situation 1	Situation 1
In which situation would you place the conditions of the logistic facilities used till the product reach the next chain actor?	Situation 3	Situation 3	Situation 3	Situation 1	Situation 1
In which situation would you place inspections of food safety authorities in your country in respect to your RPU?	Situation 1	Situation 1	Situation 1	Situation 1	Situation 1
In which situation would you place the variability of suppliers for initial materials for cultivation in your farm/company?	Situation 2	Situation 1	Situation 2	Situation 1	Situation 1
In which situation would you place the specificity of external support in respect to your RPU?	Situation 1	Situation 1	Situation 1	Situation 1	Situation 1

	FARM 1	FARM 2	FARM 3	FARM 4	FARM 5
In which situation would you place the specificity food safety legal framework in your country in respect to your RPU?	Situation 1	Situation 1	Situation 1	Situation 1	Situation 1
PART II: Assessment core safety control activities					
E. Assessment of preventive measures design					
At which situation would you place the hygienic design of equipment and facilities relevant for your RPU?	Situation 2	Situation 2	Situation 2	Situation 3	Situation 3
At which situation would you place your maintenance and calibration program relevant for your RPU?	Situation 4	Situation 4	Situation 4	Situation 3	Situation 4
At which situation would you place the storage facilities relevant for your RPU?	Situation 2	Situation 2	Situation 2	Situation 3	Situation 4
At which situation would you place the sanitation program(s) relevant for your RPU?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
At which situation would you place the personal hygiene requirements relevant for your RPU?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
At which situation would you place the incoming material control relevant for your RPU?	Situation 3	Situation 3	Situation 3	Situation 4	Situation 3
At which situation would you place your packaging equipment relevant for your RPU?	Situation 4	Situation 4	Situation 4	Situation 3	Situation 1
At which situation would you place the supplier control at your RPU?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 2
At which situation would you place the organic fertilizer program relevant for your RPU?	Situation 3	Situation 3	Situation 2	Situation 3	Situation 3
At which situation would you place the pesticide program relevant for your RPU?	Situation 4	Situation 3	Situation 4	Situation 3	Situation 3
At which situation would you place the water control relevant for your RPU?	Situation 2	Situation 3	Situation 2	Situation 3	Situation 4
At which situation would you place the irrigation method relevant for your RPU?	Situation 2	Situation 3	Situation 2	Situation 3	Situation 3
F. Assessment of intervention processes design					
At which situation would you place your partial physical intervention , relevant for your RPU?	Situation 1	Situation 3	Situation 1	Situation 3	Situation 4
G. Assessment monitoring system design					
At which situation would you place the analysis of CCP/CPs with respect to your RPU?	Situation 3	Situation 3	Situation 2	Situation 3	Situation 3
At which situation would you place your standards and tolerances design with respect to your RPU?	Situation 3	Situation 3	Situation 2	Situation 3	Situation 4
At which situation would you place analytical methods to assess pathogens?	Situation 1	Situation 3	Situation 1	Situation 4	Situation 4
At which situation would you place analytical methods to assess pesticides?	Situation 4	Situation 4	Situation 4	Situation 4	Situation 4
At which situation would you place measuring equipment to monitor process/ product status in your company/RPU?	Situation 1	Situation 2	Situation 2	Situation 3	Situation 3
H. Assessment of operation of preventive measures, intervention process and monitoring systems					
At which situation would you place sampling plan for microbial assessment?	Situation 1	Situation 1	Situation 1	Situation 3	Situation 3
At which situation would you place sampling plan for pesticide assessment?	Situation 1	Situation 4	Situation 1	Situation 4	Situation 3
At which situation would you place corrective actions?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 2
At which situation would you place actual availability of procedures* in your RPU?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 4
At which situation would you place the actual of compliance to procedures by agricultural workers in your RPU?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
At which situation would you place actual hygienic performance of equipment and facilities?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
At which situation would you place the actual storage/cooling capacity?	Situation 1	Situation 4	Situation 1	Situation 4	Situation 4

	FARM 1	FARM 2	FARM 3	FARM 4	FARM 5
PART III: Assessment core assurance activities					
I. Assessment of setting system requirements activities					
At which situation would you place the actual process capability of partial physical intervention ?	Situation 1	Situation 3	Situation 1	Situation 4	Situation 4
At which situation would you place the actual process capability of packaging processes?	Situation 1	Situation 4	Situation 1	Situation 4	Situation 1
J. Assessment validation activities					
At which situation would you place the actual performance of measuring equipment with respect to your RPU?	Situation 1	Situation 3	Situation 1	Situation 4	Situation 4
At which situation would you place the actual performance of analytical equipment (both microbiological and chemical) relevant for your RPU?	Situation 1	Situation 1	Situation 1	Situation 4	Situation 4
K. Assessment of verification activities					
At which situation would you place the translation of stakeholder requirements into own HSMS requirements related to your RPU?	Situation 4	Situation 2	Situation 4	Situation 4	Situation 3
At which situation would you place the systematic use of feedback information to modify HSMS related to your RPU?	Situation 4	Situation 2	Situation 4	Situation 4	Situation 4
L. Assessment of documentation and record-keeping to support food assurance					
At which situation would you place validation of preventive measures ?	Situation 3	Situation 2	Situation 3	Situation 3	Situation 4
At which situation would you place validation of intervention processes (partial physical intervention)?	Situation 1	Situation 2	Situation 1	Situation 4	Situation 4
PART IV: assessment of food safety performance					
M. EXTERNAL Food Safety Performance indicators					
At which situation would you place validation of monitoring systems with respect to your RPU?	Situation 1	Situation 2	Situation 1	Situation 3	Situation 4
At which situation would you place verification of people related performance ?	Situation 2	Situation 1	Situation 2	Situation 3	Situation 4
At which situation would you place verification of equipment and methods related performance ?	Situation 2	Situation 3	Situation 2	Situation 3	Situation 3
At which situation would you place documentation with respect to your company?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
At which situation would you place your record keeping system with respect to your company?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
N. INTERNAL Food Safety Performance indicators					
How would you typify your Food Safety Management System evaluation ?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 4
How would you indicate seriousness of remarks of the HSMS evaluation?	Situation 4	Situation 3	Situation 4	Situation 3	Situation 4
How would you typify the hygiene related and microbiological food safety complaints of customers?	Situation 4	Situation 4	Situation 4	Situation 4	Situation 4
How would you typify the chemical safety complaints of customers?	Situation 3	Situation 4	Situation 3	Situation 4	Situation 4
How would you typify the (visual) quality complaints by your customers?	Situation 3	Situation 3	Situation 3	Situation 3	Situation 3
How would you typify your product sampling to confirm microbiological performance?	Situation 1	Situation 3	Situation 3	Situation 2	Situation 4
Which judgment criteria are used to interpret microbiological results?	Situation 1	Situation 2	Situation 1	Situation 3	Situation 4
How would you typify your product sampling to confirm use of pesticides ?	Situation 4	Situation 4	Situation 4	Situation 4	Situation 4
Which judgment criteria are used to interpret pesticide testing results?	Situation 4	Situation 4	Situation 4	Situation 4	Situation 4
How would you typify your non-conformities ?	Situation 4	Situation 4	Situation 4	Situation 4	Situation 4

