Control of *Listeria monocytogenes* in an avocado processing facility

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Declaration

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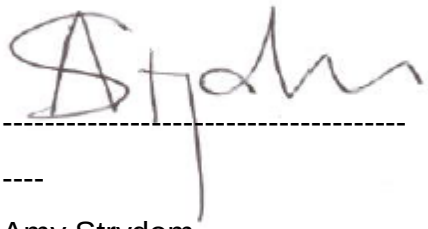

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To my parents

Alwyn and Elna Strydom

who made this possible

Abstract

Listeria monocytogenes contamination of food is a growing concern for the food industry since it is the causative agent of human listeriosis. Despite increased awareness and strict microbiological standards for this pathogen, countries such as France, Austria and Germany have reported increases in listeriosis outbreaks. The research in this thesis shows how *Listeria* contamination in a South African avocado processing was almost eradicated. The first aim of this project was to isolate and genetically type the *L. monocytogenes* strains isolated from the facility. Pulsed field gel electrophoresis (PFGE) was used to group a subset of strains (n=80) according to the digestion of their genomes with the restriction enzyme *Alu*I. These results were compared to polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the *inl*A gene (n=140). The results of the two methods compared well with each other but both indicated a lower genetic diversity among the isolates than expected. These strains were isolated over a period of four years and only two major groups were identified with the PFGE and the PCR-RFLP resulted in only three banding patterns. Also, the origin of the bacterial contamination could not be identified, but results indicated that cross contamination played a role in the persistence of these bacteria in this food processing facility.

Secondly, the commercial product Listex™ P100 was assessed for possible use as a biocontrol agent in the facility. The host range of the phage product was determined based on 239 *L. monocytogenes* strains isolated from this facility, but only 26.7% of these strains were susceptible to the bacteriophage. The strains were also analysed for serotype and no correlation was observed between typing methods or isolation source and date of isolation of the strains, as well as the susceptibility to the phage product. This could indicate that cross contamination played a role in the transfer of bacterial cells since these strains were distributed randomly in the facility. Inoculation of the phage product with *L. monocytogenes* T162 in brain heart infusion (BHI) broth resulted in significant reductions in the bacterial concentration. However, activity of the phage in avocado pulp and guacamole was very low and no significant reduction in the *L. monocytogenes* concentration was measured.

Lastly, the population of the *Listeria* strains in the facility were continuously monitored over five years. The final products and processing environment, including floors, equipment, work areas and personnel were tested on-site for *Listeria* with the ISO 11290-1 method. Based on the prevalence of *Listeria*, the facility introduced new strategies in processing to counter cross contamination. Results from the 2014 guacamole production season showed almost complete eradication of *Listeria* spp. in final products (0.17%, n=1170) and the processing facility (0.79%, n=1520). These results indicate that successful management of *Listeria* spp. in an avocado processing facility can be accomplished with in-house monitoring of the bacterial population and subsequent adjustments to the processing system.

The results from this project indicated that the cause of contamination by *L. monocytogenes* in the facility was due to cross contamination, although a strict quality control system was followed. Despite low genetic variability between the *L. monocytogenes* strains, the commercial phage product was only effective against 26.7% of strains tested. This is surprising since literature reported very high percentages of susceptible strains to this specific product. Although bacteriophage biocontrol with Listex^{TM} P100 was not effective in this facility, it cannot be concluded that this will be the case for other facilities. Also, bacteriophage product with a broader host range such as a cocktail of different phages, may work well in the processing environment to minimise transfer of bacterial cells to the final product. Control of *L*. *monocytogenes* will, however, only be effective if the processing conditions counter cross contamination.

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Chapter 1

Introduction

Listeria monocytogenes is a food-borne pathogen that can cause listeriosis, a severe human infection (Farber & Peterkin, 1991; Jeffers et al., 2001). Almost all food products can act as a vector for *L. monocytogenes* transmission, but recently particular reference has been made to ready-to-eat food products (Jamali, Chai, & Thong, 2013; Sant'Ana, Franco, & Schaffner, 2014; Strydom & Witthuhn, 2015). Due to several outbreaks in the United States (Graves et al., 2005; Orsi et al., 2008), Austria and Germany (Fretz et al., 2010), as well as Canada (Farber, Kozak, & Duquette, 2011), microbiological regulatory criteria for food products relating to *L. monocytogenes* have become extremely strict. The U.S. Department of Agriculture (USDA) for example, requires no viable *L. monocytogenes* cells in 25 g ready-to-eat (RTE) food products (Jami, Ghanbari, Zunabovic, Domig, & Kneifel, 2014). This has consequently resulted in large amounts of product recalls due to *L. monocytogenes* contamination, which has significant economic impact.

Despite the growing awareness and strict regulation, control of these bacteria in a food processing facility seems impossible. The ability of *L. monocytogenes* to grow at refrigeration temperatures and to form biofilms, are reasons for the persistence of these bacteria in processing environments. As a result, *L. monocytogenes* presents an interesting challenge and the pathogenicity and characteristics of these bacteria have been researched extensively (Cossart & Toledo-Arana, 2008; Cossart, 2007; Strydom & Witthuhn, 2015). Recently, the presence of prophages in some *L. monocytogenes* strains have been suggested to influence the persistence of these bacteria in food processing environments, although this has not been confirmed (Verghese et al., 2011).

Bacteriophage typing of *Listeria* in the 1990's opened a door to bacteriophage biocontrol of these pathogens in the food industry. The first phage product approved by the United States Food and Drug Administration (FDA) was ListShield™ (formerly known as LMP-102), targeting *L. monocytogenes* in food related environments. Only a month later another *Listeria*-targeted phage product, Listex™, was awarded

Generally Regarded As Safe (GRAS) status by the FDA. Preliminary research indicates promising results for using bacteriophages to control *L. monocytogenes* (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005; Guenther & Loessner, 2011; Hong, Choi, Lee, & Conway, 2015; Leverentz et al., 2003; Rossi et al., 2011). However, reviewers warn that practical implementation of a bacteriophage treatment in a processing facility will be more complex (Bouvier & Maurice, 2011; Denes & Wiedmann, 2014; Goodridge & Abedon, 2003). Additionally, data regarding the control of an entire *L. monocytogenes* population in one specific facility is needed, since most studies only focus on one or two strains which are known to be susceptible to a specific bacteriophage (Strydom & Witthuhn, 2015).

A different approach to controlling *L. monocytogenes* in food processing facilities was proposed by Parisi et al. (2013). This included a facility-based approach consisting of regular monitoring of the *L. monocytogenes* population. It has been proposed that the main reason for *L. monocytogenes* persistence in processing facilities is the inability of food product manufacturers to remove these bacteria from the environment (Carpentier & Cerf, 2011). It is clear that quality control programs are not sufficient to control *Listeria* spp. inside food processing facilities and consistent monitoring of the processing environment, as well as the final product by the manufacturers might aid in the management of these bacteria.

This thesis investigates the persistence of *Listeria* spp. in a South African avocado processing facility. The *Listeria* population was monitored over five years based on prevalence during production of guacamole, which is more or less from May to September each year. The aims of this project was to firstly analyse the *L. monocytogenes* strains isolated from the facility using genetic typing. Pulsed field gel electrophoresis (PFGE) was used to group a subset of strains according to digestion of their genomes with *Alu*I and this was compared to typing based on the *inl*A gene with polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Secondly, the commercial product Listex^{TM} P100 was assessed for possible use as a biocontrol agent in the facility. The host range of the phage product was determined based on 240 *L. monocytogenes* strains and the activity of the phages in avocado pulp and guacamole was analysed. Lastly, an In-house Monitoring System was developed during which the population of the *Listeria* strains in the facility were continuously monitored on-site. The data obtained were used to modify the processing conditions to specifically counter cross contamination and the transfer of

bacterial cells from low risk areas to high risk areas, where the food product is exposed.

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Chapter 2

Literature Review

Listeria monocytogenes: A Target for Bacteriophage Biocontrol

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Comprehensive REVIEWS in Food Science and Food Safety

Listeria monocytogenes: A Target for Bacteriophage Biocontrol

Amy Strydom and Corli R. Witthuhn

Abstract: *Listeria monocytogenes* is a growing concern in the food industry as it is the causative agent of human listeriosis. There are many research articles concerning the growth, survival, and diversity of *L. monocytogenes* strains isolated from food-related sources, elucidating the difficulty in controlling these bacteria in a food-processing facility. Bacteriophage biocontrol of *L. monocytogenes* strains was introduced in 2006, through the first commercial bacteriophage product targeting *L. monocytogenes* ListShield™. This review focuses on the use of bacteriophage biocontrol to target *L. monocytogenes* in the food industry, specifically direct application of the bacteriophages to food products. In addition, we discuss characteristics of these bacteria that will have a significant influence on the effective treatment of bacteriophages such as genetic diversity between strains prevalent in one facility. There are many positive results of phage treatments targeting *L. monocytogenes* in food; however, success of *in vitro* studies might not be reproducible in practice. Future studies should focus on creating experimental design that will imitate the conditions found in the food industry, such as a stressed state of the targeted bacteria. *In situ* evaluation of bacteriophage treatment of *L. monocytogenes* will also be necessary because the presence of these bacteria in a processing facility can vary greatly regarding genetic diversity. The potential use of phages in the food-processing facility as a biosanitizer for *L. monocytogenes*, as well as the use of lysins to target these bacteria should also be explored. Despite the exciting research avenues that have to be explored, current research shows that biocontrol of *L. monocytogenes* is feasible and has potential to positively impact the food industry.

Keywords: bacteriophages, biocontrol, *Listeria monocytogenes*

Introduction

Listeria monocytogenes can cause listeriosis when transmitted to humans, usually via contaminated food products (Farber and Peterkin 1991). These pathogens are ubiquitous in nature and can contaminate the food processing line at any point. Food products that are traditionally prone to *L. monocytogenes* contamination include raw or processed dairy products, fish, meat, and vegetables (Farber and Peterkin 1991). Recently, ready-to-eat (RTE) food products, as well as fresh fruit have been implicated in *L. monocytogenes* contamination because the biggest outbreak of listeriosis in the United States was due to contaminated cantaloupes (FDA 2011). Food products that cause the biggest concern are those products that do not undergo a heat treatment or rely on refrigeration for control of *L. monocytogenes* because these bacteria can grow at refrigeration temperatures and even survive at −0.4 °C (Farber and Peterkin 1991).

In most countries, strict regulations for microbial standards are established to prevent retail of contaminated food products. However, control of unwanted bacteria in the food industry is extremely difficult because the food products provide a nutrient-rich niche for growth (Hagens and Loessner 2010). In addition, consumers pressure the industry for "natural" food products, free of chemicals and preservatives (Xi and others 2011). Undoubtedly, the pressure for natural products and the high microbiological standards for food products have increased difficulty in controlling contamination by *L. monocytogenes* (Ivanek and others 2005).

Bacteriophages, the viruses of bacteria, can act as natural antimicrobials against food pathogens in the food industry (Goodridge and Abedon 2003). These phages infect specific bacteria and use the genomic material of the bacteria to produce new phages, ultimately destroying the bacterial cell. The first 2 commercial phage products approved by the U.S. Food and Drug Administration (FDA) target *L. monocytogenes* in food products (Sulakvelidze 2013). These bacteria are one of the most studied foodborne pathogens, and therefore useful to evaluate the antimicrobial potential of bacteriophages (Cossart 2007). This review aims to evaluate the presence of *L. monocytogenes* in the food industry and the feasibility of using bacteriophages for biocontrol.

L. monocytogenes regulation

The rate of *L. monocytogenes* infection is not as high as that by other foodborne pathogens, but the mortality rate of listeriosis has been reported to be the third highest in the United States, making it a very serious public health threat (Scallan and others 2011). In 1991, it was suggested that listeriosis will be the leading fatal foodborne infection in the United States (Gellin and others 1991).

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Governmental bodies responded to this threat by implementing strict regulation for food products concerning *L. monocytogenes* contamination. The U.S. Dept. of Agriculture (USDA) has a strict zero tolerance policy (no viable cells detected in 25 g) for *L. monocytogenes* in RTE food products. The current regulation states that contamination with *L. monocytogenes* is adulteration of food products, thus giving the USDA grounds for legal action against food manufacturers (Kraiss 2008) According to the commission regulation of the European Union (EU; European Commission 2005), the concentration of *L. monocytogenes* should be kept below 100 cfu/g in food products and be absent in 25-g samples of RTE food products that can support the growth of the bacteria or is intended for infants or special medical purposes. Regulations in Canada, New Zealand, and Australia also differentiate between RTE food products that can support the growth of *L. monocytogenes* (absent in 25-g sample) and other food products (<100 cfu/g; Jami and others 2014).

Regardless of these strict regulations, many countries have reported increases in cases and outbreaks of listeriosis. The Advisory Committee of Microbiological Safety of Food (ACMSF) reported an increase of listeriosis in individuals older than 60 y in the U.K. since 2000 (ACMSF 2009). Surveillance of listeriosis in France showed the highest incidence in 2007 since reporting listeriosis became mandatory in 1998 (Goulet and others 2008). Allerberger and Wagner (2009) reported an increase of invasive listeriosis in Austria, as well as other European countries such as the Republic of Ireland and Germany. In an attempt to assess the global burden of listeriosis, de Noordhout and others (2014) used a meta-analysis to assess epidemiological data of listeriosis incidences from 1990 to 2012. They estimated that, in 2010, listeriosis resulted in 23150 illnesses and 5463 deaths worldwide. In addition, *L. monocytogenes* is responsible for an estimated US$2.6 billion cost of illness in the U.S., as well as a loss of 9400 quality-adjusted life-years per annum (Hoffmann and others 2012).

The regulations regarding *L. monocytogenes* also have a major impact on the food industry and in 2005 it was estimated that the cost of product recalls due to *L. monocytogenes* contamination in the United States was between US$ 1.2 and 2.4 billion (Ivanek and others 2005). In addition, many food manufacturers treat a positive result for *Listeria* spp. as a positive result for *L. monocytogenes*, which greatly increases the loss of food products and the economic burden. Clearly, additional control measures are needed to lessen the economic and health burdens of this pathogen.

L. monocytogenes in the food industry

The genus *Listeria* contains 10 species, namely *L. monocytogenes*, *Listeria marthii, Listeria innocua, Listeria seeligeri, Listeria welshimeri, Listeria ivanovii, Listeria grayi, Listeria rocourtiae* (Graves and others 2010; Leclercq and others 2010), *Listeria fleischmannii* (Bertsch and others 2013), and *Listeria weihenstephanensis* (Halter and others 2013). Three *Listeria* strains isolated from cheese in Switzerland were designated as a novel species, *L. fleischmanni*. Interestingly, cell wall binding domains from *Listeria* phage endolysins could bind to these strains, indicating the relatedness to the *Listeria* genus (Bertsch and others 2013). At the same time, 2 strains isolated from a fresh-water plant were designated as novel species, *L. weihenstephanensis* (Halter and others 2013). Strains from these novel species did not display hemolysis and can be classified as avirulent (Leclercq and others 2010; Bertsch and others 2013; Halter and others 2013). Thus far, virulence factors have only been identified in *L. monocytogenes*, *L. ivanovii*, and *L.* seeligeri (Gouin and others 1994). However, only *L. monocytogenes*, which is a very diverse species with 4 lineages and 13 serotypes, is associated with human illness (Orsi and others 2011). The differences between strains of *L. monocytogenes* is of importance because not all are virulent, and they have different abilities to adapt to processing environments. Isolation and characterization of *L. monocytogenes* is mainly performed to determine the prevalence of this pathogen in the food industry, as well as to identify different serotypes, genetic diversity, presence of virulence genes, and the antimicrobial resistance of these strains. This information is very useful in risk assessment studies, especially because it sheds some light on the differences between *L. monocytogenes* strains. We will now discuss studies that characterized *L. monocytogenes* strains isolated from food-related environments since 2010, referring to prevalence, genetic variability, and influence of bacteriophages (Table 1).

Prevalence of *L. monocytogenes* in the food industry

Prevalence of *L. monocytogenes* in food products is mostly low as 14 studies (Table 1: 2, 6, 8, 10, 11, 12, 13, 15, 18, 21, 22, 23, 25, and 27) indicated positive samples below 10.0% with the lowest incidence at 1.42% ($n = 6270$; Table 1: 15) positive samples. Only 5 studies (Table 1: 16, 17, 19, 21, and 26) indicated *L. monocytogenes* contamination of samples between 10% and 20% and 1 study reported a 55% ($n = 100$; Table 1: 24) incidence. Interestingly, the highest incidence of *L. monocytogenes* was found in blue-veined cheese rind ($n = 100$) (Table 1: 24), whereas the lowest incidence was reported from 6270 samples taken from various sources in cheese-producing facilities over at least 4 y (Table 1: 15). The size of prevalence studies and the variation in sources such as food product, swabs from personnel and food processing facility (food contact areas and nonfood contact areas), as well as the nearby environment have an influence on the results and make it difficult to compare independent studies. However, the highest level of contamination for specifically cheese product samples in study 15 was 13.6%, which is still pointedly lower than the 55% reported in study 24 (Table 1). This may indicate that the cheese rind tested in study 24 might be very susceptible to *L. monocytogenes* contamination because the cheese pulp from the same samples was not contaminated.

The samples tested in these studies included various food products, including raw meat, seafood, dairy products, fresh produce, and RTE products. Recently, a review regarding *L. monocytogenes* contamination of seafood products was published, describing a diverse prevalence of these bacteria (Jami and others 2014). In the group of studies discussed in this paper, the frequency of *L. monocytogenes* also varied significantly in the same type of food product. In some cases (Table 1: 7 and 25), seafood products had the highest contamination levels when compared to other products tested, and then in other cases (Table 1: 2 and 18), the contamination levels of seafood was lower. Similar inconsistencies were reported for meat and poultry products (Table 1: 1, 13, 14, 20, 23, 25, 27, and 29). Fresh produce is very difficult to compare because there are so many variations in these food products, but overall there seems to be a high prevalence of *L. monocytogenes* among vegetables. Leafy greens (2), salads (12 and 17), and vegetable dishes (6) had some of the highest incidences of *L. monocytogenes* contamination when compared to other food products tested (Table 1). Interestingly, 1 study (Table 1: 11) reported that salad mixes and lettuce had a lower percentage of *L. monocytogenes* than mixed vegetables. It is likely that increased manipulation during preparation of the food

Table 1–Studies describing the prevalence of *L. monocytogenes.*

Number	Isolation source	Positive *L. monocytogenes* samples (%)	Reference
1	Raw meat	97	Shen and others (2013)
2	Various food products	20 (3.9)	Cetinkaya and others (2014)
3	Avocado processing facility and guacamole	140	Strydom and others (2013)
4	Various environmental and food sources	103	Lomonaco and others (2011)
5	Various food products	46	Chen and others (2011)
6	RTE food samples	10 (6.3)	Chen and others (2013)
7	RTE food samples, processing plants, human listeriosis cases	166 (4.6)	Lambertz and others (2013)
8	Dairy processing facilities	25 (4.6)	Parisi and others (2013)
9	Cheese	47	Acciari and others (2011)
10	Raw milk	18 (4.1)	Jamali and others (2013b)
11	RTE vegetable products	16 (3.1)	Sant'Ana and others (2012)
12	Various food products	554 (2.6)	Kramarenko and others (2013)
13	Duck meat and environment	15 (2.8)	Adzitey and others (2013)
14	Turkey meat	37 (20.5)	Erol and Ayaz (2011)
15	Cheese processing facility and environment	34 (1.4)	Almeida and others (2013)
16	Various food products	65 (12.4)	Wang and others (2013)
17	Raw and RTE food products	45 (11.4)	Jamali and others (2013a)
18	Various food products	59 (6.2)	Yu and Jiang (2014)
19	Raw and RTE food products	23 (16.4)	Marian and others (2012)
20	Chicken	202	Alonso-Hernando and others (2012)
21	Raw and RTE food products, environment, personnel swabs	4.83% (raw), 14.5% (RTE)	Fallah and others (2013)
22	RTE seafood	12 (4.8)	González and others (2013)
23	Raw and RTE meat products	66 (5.3)	Modzelewska-kapitu and Maj-sobotka (2014)
24	Blue-veined cheese rinds	100 (55)	Bernini and others (2013)
25	RTE meat and fish products	2 (5)	Kovačević and others (2012)
26	Raw and RTE chicken	51 (18.2)	Osaili and others (2011)
27	RTE poultry products	9 (3)	Meyer and others (2012)
28	Various samples	222	Fox and others (2012)
29	Beef samples	191 (17)	Khen and others (2014)
30	Water, clinical and milk	20 (2.8)	Soni and others (2013)

products can lead to a higher probability of contamination and, therefore, higher levels of the bacteria. Raw and processed dairy products mostly have a lower *L. monocytogenes* incidence when compared to other products (Table 1: 2, 6, and 7), although the highest prevalence reported in this paper was from cheese rind (Table 1: 24). The variation in prevalence in similar food products indicates that the food matrix is not the only factor in the persistence of these pathogens. The implementation and management of a quality control system and integrity of the cold chain also plays important roles in controlling *L. monocytogenes* contamination of processing facilities and food products.

Food processing plants and environmental areas were also included in some of the studies (Table 1: 3, 4, 6, 7, 8, and 21). It is suggested that contamination of food products occurs mainly in the processing plant (Miettinen and Wirtanen 2006; Lomonaco and others 2009). In a few cases, cross-contamination from resident strains, which survive in harborage sites, was suggested to be the source of contamination, rather than raw ingredients (Table 1: 3, 7, and 21). In a recent review, harborage sites was found to be the main reason why *L. monocytogenes* strains persist in food processing facilities (Carpentier and Cerf 2011). The processing facility is usually more contaminated than the final products (Pak and others 2002). For instance, a higher level of *L. monocytogenes* contamination was found on nonfood contact areas (18.75%; $n = 32$) when compared to food products (2.4%; $n = 249$) and food contact areas (4.88%; $n = 266$; Table 1: 8). Floor drains, equipment, and personnel have all been implicated in cross-contamination of *L. monocytogenes* (Carpentier and Cerf 2011; Jami and others 2014; Table 1: 3, 4, 7, 8, and 21). This emphasizes the necessity to monitor food-processing facilities very closely for the presence of *L. monocytogenes*. However, other sources of contamination or at least initial contamination of a facility should not be ignored. Ruminants shed *L. monocytogenes* in their feces and can spread this pathogen further along the food chain (Hutchison and others 2004; Lyautey and others 2007). Filiousis and others (2009) found identical clones of *L. monocytogenes* in unrelated food products that were isolated from open-air markets in Greece. This may be due to contamination at the markets or the prevalence of dominant clones in the region. Either way, it remains important to avoid circumstances where cross-contamination can take place, whether it is during processing or retail.

Genetic variability in *L. monocytogenes* strains isolated from the food industry

General opinion is that serotyping of *L. monocytogenes* strains is not of particular use in subtyping studies because strains associated with human infection or isolated from food sources are mostly in lineage I and II, which harbors serotypes 1/2a, 1/2b, and 4b (McLauchlin and others 2004; Parisi and others 2013). However, all the major outbreaks of listeriosis involve strains from serotype 4b, which is not as frequently isolated compared to serotype 1/2a (Doumith and others 2004). It might, therefore, be of use to screen strains implicated in a listeriosis outbreak with serotyping before using pulsed-field gel electrophoresis (PFGE) in epidemiological investigations (Swaminathan and others 2001). In the studies described here (Table 1), the preferred method for serotyping is by multiplex PCR that separates only the major serotypes, namely: 1/2a (3a), 1/2b (3b, 7), 1/2c (3c), and 4b (4d, 4e; Doumith and others 2004). As only serotypes 1/2a, 1/2b, 1/2c, and 4b are commonly found in food-related or clinical sources many researchers

assume that these are the identities of the specific strains that were classified in the serotype groups described by Doumith and others (2004). Another method used is the conventional serotyping based on the commercially prepared antisera against somatic (O) and flagellar (H) antigens (Farber and Peterkin 1991).

Results form 12 independent studies (Table 1: 1, 2, 7, 8, 9, 10, 12, 16, 18, 21, 27, and 29) indicated that serotype 1/2a was most frequently isolated, regardless of isolation source or typing method. In all cases, serotype 4b was also isolated except from studies 9 and 27 (Table 1). Three studies indicated that the majority of the strains belonged to serotype 4b (Table 1: 6, 11, and 14). These strains were isolated from RTE foods in China (6), RTE vegetable products in Brazil (11), and fresh turkey meat in Turkey (14). This is of serious concern, especially because a ribotype was also identified in the Brazilian *L. monocytogenes* strains that have been implicated in listeriosis cases all over the world.

Although prevalence studies do not focus on serotyping of *L. monocytogenes* strains, some studies determine the serotypes of the bacterial strains when describing sensitivity or resistance to bacteriophages. Host ranges of 114 phages against strains, representing all the serotypes of *L. monocytogenes* ($n = 13$), indicated that strains belonging to serotype 4 and 1/2 were more susceptible to phages compared to strains in other serotypes (Vongkamjan and others 2012). These strains were all isolated from dairy silage and it is reasonable to assume that bacterial strains that are prevalent in food-related sources such as 1/2 and 4 will correlate with the bacteriophages in these environments. Another study also reported serotype 4 strains particularly sensitive to phages (Kim and others 2008). Interestingly, 9 of 12 phages were able to infect multiple *L. monocytogenes* serotypes, as well as other *Listeria* spp., including *L. invanovii, L. wellshimeri, L. seeligeri,* and *L. innocua*. However, strains belonging to *L. monocytogenes* serotype 1/2 often displayed resistance to these broad host range phages. At this point, there are not enough data to perceive a relationship between susceptibility of *L. monocytogene*s strains to bacteriophages and specific serotypes, but susceptibility of serotype 4 strains is, however, encouraging as these strains are most associated with listeriosis.

A more specific differentiation between *L. monocytogenes* strains than serotyping is needed to understand the ecology of these bacteria in the food industry. The ability to understand the contamination sources and routes inside a processing facility is necessary to implement control strategies, not to mention the identification of strains (and the sources of those strains) implicated in listeriosis outbreaks. Molecular subtyping methods such as PFGE have been critical in identifying the sources of listeriosis outbreaks (Graves and others 2005) and have also been used to evaluate the genetic diversity of the strains isolated from the food industry. In fact, 10 of 13 studies (Table 1: 1, 3, 4, 5, 7, 9, 13, 16, 18, and 27) described in this paper used PFGE to differentiate between strains. In addition, almost all of the studies used the Pulsenet-prescribed method, which includes both *ApaI* and *AscI* digestion (Swaminathan and others 2001). This is, however, time-consuming and expensive and other methods have been compared to PFGE results. Amplified fragment length polymorphism (AFLP; Table 1: 4, 5, 8, and 24), ribotyping (Table 1: 11) and restriction fragment length polymorphism (Table 1: 3) have all been reported to deliver good results compared to PFGE-typing of *L. monocytogenes* strain.

The genetic diversity of the *L. monocytogenes* strains reported by these studies seems contradicting, as some have reported very high diversities (Table 1: 1, 4, 5, 16, and 24) and others reported very low diversities (Table 1: 7, 9, 13, 18, and 27). The strains in these studies were isolated from different product types (Table 1: 1, 5, 7, 16, 18, and 27) and locations (Table 1: 1, 4, 5, 7, 9, 13, 16, 18, and 27) and included environmental (Table 1: 4, 7, and 13) and clinical (Table 1: 5) isolates. Furthermore, most of the strains were isolated over a period of at least 12 mo. Interestingly, the only study (Table 1: 24) that described the genetic variation in isolates from similar product types (cheese rinds produced from different cheese types produced in one facility) had a very high diversity according to AFLP results. This is also the study that presented the highest prevalence of *L. monocytogenes* and, therefore, it is probable that this specific facility had a high level of contamination with these bacteria. Regardless, it appears that the genetic diversity of *L. monocytogenes* also varies, like the dispersion of the pathogen in the food industry. This may present a problem for bacteriophage biocontrol because bacteriophages are host specific and it is advisable that the genetic diversity of the *L. monocytogenes* strains in a specific location be determined as a preliminary step to biocontrol.

Apart from prevalence and genetic variability within the *L. monocytogenes* species, there also exist differences between the pathogenicity of these strains. *L. monocytogenes* lineage I (particularly serotype 4b) is overrepresented in clinical strains, even when compared to lineage II, whereas lineages III and IV are rarely isolated and mostly from animal origin (Orsi and others 2011). It has been reported that lineage I strains have a 100-fold increased risk of causing listeriosis over lineage II and that strains from lineage II carry stop codons in the gene, *inlA*, which lowers the risk of infection (Chen and others 2006). The variability in pathogenicity has led to many studies that have identified virulence factors for each stage during infection, describing the entire infection process of *L. monocytogenes* (Cossart and Toledo-Arana 2008).

Some of the studies in Table 1 also reported strains with virulence factors (1, 6, 10, and 13). The presence of *inlA*, *inlC*, and *inlJ* was determined in studies 1 and 10 by a multiplex PCR method (Liu and others 2007). All but 1 strain in study 1 ($n = 97$) and all the strains in study 10 ($n = 18$) tested positive for these virulence markers. The first gene sequenced for *L. monocytogenes hlyA* with known virulence association (Cossart 2007) was reported in 100% of the strains tested in study 13 (Table 1). Furthermore, listeriolysin S-positive strains were identified in study 1 (Table 1) by targeting *llsX* with a PCR method (Clayton and others 2011). Twelve of 97 strains were positive for *llsX* and 5 (5/12) belonged in epidemic clone I. Strains belonging in epidemic clone I were associated with major listeriosis outbreaks in different countries and described as a "cosmopolitan clonal group" associated with virulence (Chen and Knabel 2007). Although these results do not explicitly confirm virulence, they do indicate the potential of these strains to cause infection. The differentiation between virulent and avirulent *L. monocytogenes* strains is critical and more studies should include descriptions of the virulence potential of strains isolated from the food industry. In fact, a risk assessment by the combined efforts of the FDA and USDA's Food Safety and Inspection Service (FSIS) included the varied virulence found among *L. monocytogenes* strains and concluded that the risk might significantly be lower due to the presence of avirulent strains (Whiting 2003).

Despite the genetic diversity seen between strains of *L. monocytogenes*, the pan genome (comparison of 16 strains representing all serotypes) of the species have been described as very stable, containing a high percentage conserved genes. Diversity in the pan-genome of the 16 *L. monocytogenes* strains were found to be influenced by prophage-related genes (Kuenne and others 2013). Although lysogeny in *Listeria* strains is very common (Loessner and Rees 2005), prophage genes are unevenly distributed among serotypes. Some strains are completely free or do not have

complete prophages such as WSCL 1001 and WSCL 1042 (Klumpp and Loessner, 2013; Klumpp and others 2014), as well as F2365 (Nelson and others 2004), L312 (Chatterjee and others 2006), and SLCC 2376 (Haase and others 2011) as found by Kuenne and others (2013). These particular strains belong to serotypes 1/2a, 4b, and 4c.

An interesting study recently showed the influence of a prophage in a critical step in the infection cycle of *L. monocytogenes*, where excision of the prophage from the *comK* gene activates the gene, expressing the ComK protein. In turn, this protein activates the Com system that is necessary for escape of the bacterial cell from the vacuole formed during phagocytosis (Rabinovich and others 2012). This is not the only function attributed to the prophage interrupting the *comK* gene. In some *L. monocytogenes* strains, a particular prophage in the *comK* gene might also have a role in persistence of these strains in processing facilities (Verghese and others 2011). This is somewhat contradictory to the review of *L. monocytogenes* persistence in processing facilities by Carpentier and Cerf (2011), who concluded that this species does not have unique abilities which facilitates persistence. The influence of phages on the genetic diversity and regulation of genes in *L. monocytogenes* is an interesting field of research with very little information and many questions.

L. monocytogenes bacteriophage biocontrol

Despite strict regulatory policies, *L. monocytogenes* still has a major detrimental influence in the food industry, causing food product recalls and disease outbreaks. In a recent review, Carpentier and Cerf (2011) discussed reasons why *Listeria* cells persist in processing facilities, maintaining that these pathogens possess no unique abilities to adapt to these environments, apart from their ability to grow at very low temperatures. They concluded that difficulty in controlling these bacteria are due to inability to identify and destroy harborage sites. It is clear that control measures in quality control systems and the implementation of these practices are not sufficient to manage these bacteria inside processing facilities. Preservation methods (chemical or physical) used for food products are also not sufficient to control growth after initial contamination. Additional methods are, therefore, needed to control these bacteria. The use of bacteriophages to aid in these problems is now often discussed and numerous reviews regarding biocontrol of bacteria in the food industry have been published. Here we will discuss the properties of bacteriophages and possible applications with the results of studies describing the effects of bacteriophage treatments on *L. monocytogenes* in food products.

Bacteriophages are natural bactericidal agents

The complete destruction of bacterial cells infected by lytic phages is one of the advantages over antibiotics, which are sometimes only bacteriostatic, such as tetracycline (Loc-Carrillo and Abedon 2011). Bacteriophage treatment of pathogenic or spoilage bacteria in the food industry have high potential, because some sanitizers are also bacteriostatic. Bacteriophages are a better control option compared to chemical food preservatives as they are natural enemies of bacteria. In addition, their specificity regarding targeted bacterial strains adds to the potential use in food products.

Since 2003, 11 studies have described the effect of bacteriophages on *L. monocytogenes* in food products (Table 2). All of these studies reported some success where the bacteriophage treatment reduced the *L. monocytogenes* counts under various circumstances. It is not certain if complete eradication of the bacteria in the food matrix has been accomplished, because enrichment culturing is not used to determine the *Listeria* counts after phage treatment. Carlton and others (2005) did report no regrowth of *L. monocytogenes* on cheese after 21 d after bacteriophage treatment but this is the only case where the results were confirmed by selective enrichment and subsequent plating of the product samples. In a few biocontrol studies, the bacteria counts dropped below levels of detection with direct plating, but rose again after prolonged incubation (10 to 22 d) (Guenther and others 2009; Bigot and others 2011; Guenther and Loessner 2011; Rossi and others 2011). Determination of *L. monocytogenes* concentration in biofilms with direct epifluorescence microscopy showed higher survival rates after bacteriophage treatment compared to when the concentrations were determined with conventional methods (Montañez-Izquierdo and others 2012). Nonetheless, even without complete eradication and different testing methods, bacteriophage treatment has a significant influence on *L. monocytogenes* in food products.

Host–phage interaction

Bacteriophages have to physically attach to their target bacteria before infection can take place. Bacteria, however, adapt very readily to their environments, which can result in structural changes of the cell membranes. These changes can influence the attachment of phages and, therefore, also the effectiveness of the phage treatment. A recent review reported on the phage-host interactions regarding responses of bacterial hosts to their environment (Denes and Wiedmann 2014). The bacterial condition in a food matrix or food-processing facility is discussed with specific reference to productivity of infection in host cells that are in a stationary or lag phase. The production of new virons would be much less in these cells compared to hosts cells in the exponential phase, because the amount of nucleic acid available in the host will have a direct influence on the amount of virons produced (Bouvier and Maurice 2011).

The food matrix will have an influence on the phage–host interaction, which in turn will impact the success of a bacteriophage treatment. Food products that have been tested with *L. monocytogenes* biocontrol include fresh fruit and fruit juice (Leverentz and others 2003; 2004; Oliveira and others 2014; Hong and others 2015), poultry products (Guenther and others 2009; Bigot and others 2011; Chibeu and others 2013), dairy products (Guenther and others 2009; Guenther and Loessner 2011; Soni and others 2012), fresh sausage (Rossi and others 2011), as well as RTE seafood, vegetables, and hot dogs (Guenther and others 2009). Greater reduction of *L. monocytogenes* in chocolate milk and mozzarella cheese brine was achieved compared to solid food products (Guenther and others 2009). This is probably due to unrestricted passive diffusion of phages in a liquid matrix, which is not possible on the surface of solid food products. Bacteria on the surface of food products such as chicken breast or cheese are more difficult to reach, and Guenther and others (2009) concluded that it was the limited diffusion and, therefore, lack of contact between phages and bacteria that reduced efficacy on solid food products.

The acidity of the environment also has an effect on the success of phage treatment. In apple fruit juice, no significant differences between control and phage-treated samples were recorded, whereas pear and melon juice samples had a reduction in *L. monocytogenes* counts. Similar results were seen when fresh-cut fruit slices inoculated with *L. monocytogenes* were treated with bacteriophages (Leverentz and others 2003; Oliveira and others 2014). No effect was observed on apple slices, whereas phage treatment on other fruit such as melons and pears reduced *L. monocytogenes* counts. In all cases, the pH of the apple juice or slices (pH of 4.4

Table 2–Bacteriophage biocontrol studies of *L. monocytogenes*.

Bacteriophages	Matrix	Reference
Phage LM-103[a], phage LMP-102[a], nisin	Fresh-cut red delicious apples fresh-cut honeydew melons	Leverentz and others (2003)
LMP-102[a]	Honeydew melon tissue	Leverentz and others (2004)
12 Phages isolated from 4 plants	Isolated from turkey processing plants and host ranges tested on BHI plates (spot tested)	Kim and others (2008)
A511, P100	RTE food products	Guenther and others (2009)
FWLLm1	BHI broth, RTE chicken breast	Bigot and others (2011)
A511	Soft-ripened cheese	Guenther and Loessner (2011)
P100[b]	Brazilian fresh sausage	Rossi and others (2011)
P100[b]	Biofilms on stainless steel surfaces	Montañez-Izquierdo and others (2012)
P100[b], LAE, PL-SD	Queso fresco cheese	Soni and others (2012)
P100[b], PL, SD	Cooked turkey, roast beef	Chibeu and others (2013)
P100[b]	Fresh-cut fruit, fruit juices	Oliveira and others (2014)
LMP-102	Fresh-cut melon	Hong and others (2015)

[a] Intralytix, Inc. (Baltimore, Md., U.S.A.).
[b] LISTEX™ (USA).
LAE, lauric arginate; PL, potassium lactate; SD, sodium diacetate.

or less) were lower than the other fruits. Phage titers have also been shown to decline rapidly in the acidic environment of apple juice (pH 3.7) when compared to melon and pear juice (pH 4.6 to 5.9; Oliveira and others 2014).

Bacteriophage treatment

One advantage in bacteriophage therapy is a once-off application or treatment, the theory being that the phages will multiply sufficiently to eradicate all target bacteria. This is, however, not possible in an environment such as food-processing facilities and food products, where there are low contamination levels, especially if the strains have a wide genetic make-up as seen in *L. monocytogenes*.

Studies with food products have shown that the first treatment of phages with the highest titer had the biggest influence on bacterial cells and that repeated doses did not have continuous bactericidal effects. Also, with or without repeated doses, the bacteria are reported to increase in numbers, although not as high as in control samples (Bigot and others 2011; Guenther and Loessner 2011; Soni and others 2012; Chibeu and others 2013). This is not always the case as no regrowth (after 8 d) of *L. monocytogenes* in melon juice or on cheese was reported after bacteriophage treatment (Carlton and others 2005; Oliveira and others 2014).

In all cases, the titer of the bacteriophages are a deciding factor in the success of the treatment. Some of the highest reductions in *L. monocytogenes* counts were reported when at least 10^8 pfu/unit phages were applied to the food products (Leverentz and others 2004; Guenther and others 2009; Guenther and Loessner 2011). *L. monocytogenes* counts on fresh melon tissue had a 6.7 log unit decrease when a phage treatment (10^8 pfu/mL) was added 1 h before bacterial inoculation (Leverentz and others 2004). In the liquid products (chocolate milk and mozzarella cheese brine) tested by Guenther and others (2009), bacterial counts dropped below levels of detection after a 10^8 pfu/g bacteriophage treatment, whereas the control samples reached between 10^4 and 10^5 cfu/mL. In the same study, the bacterial counts on solid food products (hot dogs, sliced turkey meat, smoked salmon, seafood, sliced cabbage, and lettuce leaves) were reduced up to 5 log units. In addition, when different concentrations of phage treatments were tested, the higher concentration always had a greater effect (Guenther and others 2009; Bigot and others 2011; Guenther and Loessner 2011). Even when bacteriophages were used to target *L. monocytogenes* biofilms on stainless steel coupons, the higher concentrations had a greater reduction in bacterial cells (Montañez-Izquierdo and others 2012).

Combination treatments

A few studies have reported the effect of bacteriophage treatment in combination with chemicals such as nisin, a bacteriocin that has generally regarded as safe (GRAS) status (FDA 2001). Leverentz and others (2003) recorded a reduction of 5.7 log units of *L. monocytogenes* on melon slices with a phage treatment in combination with nisin. Nisin creates nonselective pores in the bacterial plasma membrane which can possibly aid the bacteriophage since bacterial cells with weakened membranes are more susceptible to infection and lysis. As nisin is more active at a lower pH, it is potentially a good addition to bacteriophage treatments of acidic food products such as fruit or fruit juices. Especially, as bacteriophage treatment in fruit juices with higher acidities have not been found to be as successful when compared to fruit juice with a higher pH (Leverentz and others 2003; Oliveira and others 2014).

Addition of lauric arginate (LAE) resulted in a greater decrease in *L. monocytogenes* levels in cheese compared to a bacteriophage treatment, although a regrowth of the bacteria was detected at 4 °C. The addition of potassium lactate-sodium diacetate (PL-SD) to samples with either LAE or a bacteriophage treatment prevented the regrowth of *L. monocytogenes*, although PL-SD alone had no effect on the bacteria concentration (Soni and others 2012). Chibeu and others (2013) tested the effect of bacteriophages on *L. monocytogenes* on cooked turkey and roast beef samples, treated during processing with PL and PL-SD, respectively. Only roast beef samples with bacteriophage and PL-SD treatments did not have bacterial cell regrowth after 28 d at 4 °C. Addition of PL-SD has been found to be listeriostatic rather than listeriocidal (Vogel and others 2006), but it increased the effect of the bacteriophage treatment on cheese and beef samples, indicating the potential in using a combination of bacteriophages and other antimicrobials.

L. monocytogenes biocontrol in food processing facilities

The presence and persistence of *Listeria* in food-processing facilities is a major problem as it is one of the main sources of contamination of food products. The potential use of bacteriophages as biosanitation agents, as part of hurdle technology, has been suggested but not investigated specifically for *L. monocytogenes* (Mahony and others 2011). These pathogens readily form

biofilms on stainless steel and other surfaces, and this is believed to be one of the reasons why these bacteria can be so persistent in processing facilities, as the biofilms can protect the cells from sanitizers in harborage sites (Orgaz and others 2013). Targeting mature biofilms is essential in removing resident strains from processing facilities, as this is a source of contamination of *L. monocytogenes*. The close proximity of the cells in a biofilm should provide bacteriophages easy access to cells for secondary infection; however, biofilms are surrounded by extracellular polymeric substance (EPS) consisting of bacterial polysaccharides, which protects the cells from antimicrobial agents (Chan and Abedon 2015). Some bacteriophages have polysacharases or polysaccharide lyases that can degrade the EPS and provide entrance to the cells in the biofilm. Montañez-Izquierdo and others (2012) found that the rate of biofilm degradation is dependent on the physiological state of the cells, rather than the EPS barrier. Either way, bacteriophage P100 (Listex™) have been used to destroy listerial biofilms on stainless steel coupons by significantly reducing the cell counts up to 5.4 log/cm^2 (Soni and Nannapaneni 2010).

L. monocytogenes resistance to bacteriophages

One of the main reasons for the popularity in bacteriophage research is the resistance that bacteria have developed against available antibiotics. In the food industry resistance to sanitizers is also frequently discussed and bacteriophages are thought to induce resistance at a slower rate, as these viruses are the natural predators of bacteria. There are, however, ways in which bacteria can avoid lyses by phages including adsorption reduction, postinfection blocks of the virus, and abortive infections where both the bacterial cell and the bacteriophage die (Hyman and Abedon 2010).

Restriction modification (RM) systems that play a role in phage resistance have been identified in specific *L. monocytogenes* strains. First, a specific RM system has only been identified in strains from epidemic clone II and contains a restriction endonuclease that is expressed at 30 °C and down-regulated at higher temperatures. This system enforces phage resistance in *L. monocytogenes* strains at 30 °C or less, but not at 37 °C (Kim and Kathariou 2009; Kim and others 2012). Two RM systems, which are more widespread, have been identified in *L. monocytogenes* strains from lineage I, II, and III. Evidence that these RM systems can defend the bacterial cell against phage infection is given by Lee and others (2012). In addition, the genomic content of lytic, as well as lysogenic phages were checked for the presence of recognition sites for these RM systems, and it seems that lytic phages might have reduced susceptibility to these RM systems.

Some biocontrol studies have tested the development of resistant *L. monocytogenes* strains to bacteriophages used to treat the bacteria in food products (Carlton and others 2005; Guenther and others 2009; Guenther and Loessner 2011; Chibeu and others 2013). Strains recovered from bacteriophage-treated samples were infected again with the same bacteriophages and checked for resistance to phages. Only clones (3/10) of one *L. monocytogenes* strain were resistant to secondary bacteriophage infection (Guenther and others 2009). In all other cases no resistance was detected to bacteriophages and the bacteria were always destroyed. However, a recent study in Austria found resistance of *L. monocytogenes* strains to Listex™ P100 (2.7%; n = 486) (Fister and others 2015). The strains were isolated over a period of 15 y from 59 dairy processing facilities, some of which used Listex™ P100 as a control agent for *L. monocytogenes*. Resistance of *Listeria* strains to the bacteriophage were associated with the use of Listex™ P100 in the facilities, indicating adaptation of the strains to this specific phage.

Detection of phage-resistant strains after bacteriophage treatment in facilities (Fister and others 2015) is troubling and should serve as an early warning sign about the consequences for using phages. However, strategic application of bacteriophages can aid in preventing the development of bacterial resistance, and availability of current technology enables the monitoring of developing resistance in bacteria. Bacteriophage treatments that include multiple virus strains have been a suggestion to prevent or slow the development of resistant strains and this will also aid in targeting a more genetically variable population as one could find in a processing facility (Sulakvelidze 2013). Using bacteriophages in combination with other antimicrobials as an additional hurdle might also slow resistance. Another option is to use bacteriophage treatments as emergency measures and not routinely (Fister and others 2015). Meaden and Koskella (2013) give a review on the risk of using bacteriophages in natural environments, with a focus on the development of bacterial resistance to phages. Given the possibility of inducing resistance with the use of bacteriophages, future applications should be carefully approached to prevent mistakes similar to when bacteriophages were first discovered.

Choosing a phage

To date, many listeria phages have been identified, although most were isolated during typing studies and, therefore, not characterized for biocontrol application. Klumpp and Loessner (2013) give an updated review of the different listeria phages and their unique characteristics. Most phages are temperate belonging to the family siphoviridae, although there are exceptions. This certainly has implications for biocontrol intentions as temperate phages can integrate into the host DNA and change the genomic make-up. Also, many listeria phages have been found to be capable of generalized transduction during which small pieces of the bacterial host DNA is packaged into the phage head during assembly (Hodgson 2000). Phages intended for biocontrol should, therefore, be obligate lytic with no option for exchange of genetic material.

All evidence indicates that bacteriophages are harmless to humans (Hagens and Loessner 2010) and bacteriophage studies in humans and rats have revealed no adverse effects in test subjects (Bruttin and others 2005; Carlton and others 2005). Although legislation regarding the use of bacteriophages is still unclear, the European Commission stated that bacteriophages should be considered as food additives or as substances used for reducing surface contamination when used on food products (Andreoletti and others 2009). There are, in fact, a few ways in which phage-based products can be regulated; these are described by Sulakvelidze (2013). Hagens and Loessner (2010) listed specific attributes, which phages for food applications should possess, including being issued GRAS status by the U.S. FDA. The first phage product that was approved by the FDA was ListShield™ (formerly known as LMP-102), which was used in several biocontrol studies (Leverentz and others 2003, 2004; Hong and others 2015). Only a month later, another *Listeria*-targeted phage product, Listex™, was awarded GRAS status by the U.S. FDA.

Apart from the genetic requirements and regulatory approvals for phage-based biocontrol products, there are also the challenges of manufacturing a high-titer product and application of this product in a food-processing environment or food product which will have a significant reduction, if not a complete eradication of the targeted bacteria. Although almost all biocontrol studies have not tested for complete eradication of *L. monocytogenes* after bacteriophage treatment, they do report significant reduction in

contamination levels. A risk assessment from the FDA and USDA's FSIS found that even a ten-fold reduction in *L. monocytogenes* levels will have a significant effect on the mortality rate of listeriosis (Whiting 2003). If the same results can be accomplished in the processing facilities, bacteriophage treatments will certainly have a massive effect on the safety of food products. However, even in the controlled experiments there are many factors influencing the success of bacteriophage treatment, such as bacterial contamination load and diversity of targeted strains, or rather lack of diversity. In the *L. monocytogenes* biocontrol studies, only one *L. monocytogenes* strain was tested (Leverentz and others 2003, 2004; Bigot and others 2011) or in some cases 2 strains (Guenther and others 2009; Guenther and Loessner 2011). This does not represent a diverse bacterial population and certainly not cells that are under stress, which would be the case in the processing environment.

Use of bacteriophage endolysins to control *L. monocytogenes*

Bacteriophages use lysins or endolysins to degrade the peptidoglycan layer of the bacterial cell wall to facilitate DNA injection, as well as release of newly formed virons. These lysins comprise a diverse group of enzymes that can hydrolyze the peptidoglycan bonds and include amidases, endopeptidases, glycosidases, and carboxypeptidases. The lysins produced by bacteriophages that target Gram-positive bacteria such as *L. monocytogenes* also have cell wall binding domains that strengthen the affinity of the substrate to the enzyme (Callewaert and others 2011). Phage lysins can lyse peptidoglycan of Gram-positive bacteria from the outside, and can therefore be used as antimicrobials. However, research regarding biocontrol of *L. monocytogenes* with lysins in food products is not as frequently published as direct bacteriophage treatments. One study did test a lysin, LysZ5, against *L. monocytogenes* in soy milk. The bacteria were reduced by 4 log units after only 3 h at 4 °C. In addition, the lysins were active against *L. innocua* and *L. welshimeri*, but not *Staphylococcus aureus* and *Enterococcus feacalis* (Zhang and others 2012). Other endolysins from *Listeria* phages have been characterized but their lytic ability has not been shown in food-related matrixes (Zimmer and others 2003; Klumpp and others 2008; Dorscht and others 2009).

Conclusion

L. monocytogenes continues to be a problem for the food industry despite numerous regulations. Persistence of these pathogens in the processing environment, as well as retail shops, is a major concern and regular sanitizers are not always able to kill the bacteria, as seen by the prevalence studies discussed in this review. Additional methods for controlling *L. monocytogenes,* such as treatment with bacteriophages, are needed to aid food manufacturers in meeting the strict regulations for food products. Reductions of *L. monocytogenes* in food products through bacteriophage treatments have been reported. There are, however, many factors that influence the success of these treatments, such as the food matrix itself and the bacteriophages used in the treatment. Liquid food products are treated more successfully, probably due to dispersal of the phages and treatment with higher phage titers (10^8 pfu/mL) always have better results. Only a few studies reported complete eradication of the bacteria, although only one used enrichment culturing to determine bacterial counts after bacteriophage treatment. The reductions are, however, significant and will have an effect on the risk that is presented by *L. monocytogenes*.

Factors that will have a collective influence on the success of a bacteriophage include genetic diversity and distribution throughout a food processing facility or in a food product. In addition, these cells will probably not be in exponential phase or protected by biofilms or the food matrix. No reports of *in situ* bacteriophage biocontrol of *L. monocytogenes* have so far been published, but it can be expected to be less successful than *in vitro* experiments, since *L. monocytogenes* can vary genetically in the environment. The specificity of bacteriophages can be a disadvantage in this case and a phage with a broad host range or a cocktail of phages such as the product ListShield^{TM} is needed to target environmental *L. monocytogenes* strains. Biocontrol with endolysins might also be more successful in treating a more genetically variable population. Evaluation of the *Listeria* population in a specific food processing facility might be necessary before a bacteriophage treatment is applied.

Based on results from the surveillance and biocontrol studies discussed in this review, we have concluded that phage treatments have the potential to positively impact the food industry. However, strategic implementation of bacteriophages or bacteriophage-derived products is necessary for the successful and sustainable control of *L.* monocytogenes, with the monitoring of resistant strains. To be successful, bacteriophage treatments should also be implemented as part of an existing quality control system in hurdle technology. Finally, the use of bacteriophages in the food industry may also aid in development of therapeutic phage products.

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Chapter 3

Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE

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Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE

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ABSTRACT

The aim of this study was to subtype *Listeria monocytogenes* strains present in an avocado processing facility using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLPs) and pulsed-field gel electrophoresis (PFGE). *The inlA* gene of the *L. monocytogenes* isolates was PCR amplified and cut with the restriction endonuclease *Alu*I or *Tsp*509I. Initial investigation of 64 *L. monocytogenes* isolates indicated that *Alu*I was more discriminating and therefore an additional 76 isolates were subtyped with PCR-RFLPs using *Alu*I. The PCR-RFLP with *Alu*I resulted in three banding patterns. Subtyping of the isolates using PFGE was carried out by macrorestriction of the genomic DNA with *Apa*I and *Asc*I. The PFGE fingerprints generated by *Apa*I contained 5 clusters and resulted in better differentiation between the strains than digestion with *Asc*I. The majority of strains were grouped in cluster I ($n = 22$) and in cluster II ($n = 50$). All of the clusters contained product, as well as environmental isolates and thus no correlations with specific sources of contamination could be established. The results of this study indicated that both molecular subtyping methods, PCR-RFLP and PFGE, were sensitive and specific enough to assess the diversity among the majority of the *L. monocytogenes* isolates. This is the first study in which *L. monocytogenes* isolates from a South African avocado processing facility were characterised with PCR-RFLP and PFGE. Results clearly point to the need for increased hygienic measures to prevent the dissemination of this pathogen during avocado processing.

1. Introduction

Listeria monocytogenes are Gram-positive bacteria that can cause listeriosis, meningitis, septicaemia, encephalitis and abortion (Jeffers et al., 2001). These pathogens are predominantly transmitted to humans via contaminated food products (Farber & Peterkin, 1991). Fresh cantaloupe was identified as the cause of the recent (September to November 2011) listeriosis outbreak in the United States which resulted in 146 illnesses and 30 deaths (FDA, 2011). Avocado halves and pulp of approximately 78,000 pounds was also recalled during September 2011 due to contamination with *L. monocytogenes* (FDA, 2012).

In recent years there has been an increase in the documented incidences of *L. monocytogenes* contaminated fresh produce and ready-to-eat foods, making it critical to maintain high quality production processes and products. The South African avocado industry is a multi-million Rand industry, exporting 49,714 ton and selling 22,654 ton avocados locally from July 2010 to June 2011 (Anonymous, 2012). Additionally, the production of guacamole from avocados in South Africa has increased to 4500 tons produced in 2011 (Anonymous, 2011).

L. monocytogenes have previously been isolated from avocados and guacamole (Arvizu-Medrano, Iturriaga, & Escart, 2007; Estelal & Sofos, 1993). The survival and growth characteristics of these bacteria in avocado pulp and processed guacamole were determined in 2002 (Iturriaga, Arvizu-Medrano, & Escartín, 2002). However, the molecular characterisation of *L. monocytogenes* strains prevalent in avocado processing environments has not been attempted. This would provide valuable insight into the prevalence of these pathogens and can be important for the effective control and the prevention of product contamination (Liu, Ainsworth, Austin, & Lawrence, 2003; Roche et al., 2001). Molecular characterisation can be accomplished by subtyping *L. monocytogenes* strains with methods such as amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and ribotyping (Hyytiä-Trees, Cooper, Ribot, & Gerner-Smidt, 2007). The aim of this study was to determine

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the *L. monocytogenes* subtypes present in a South African avocado processing facility with PFGE and PCR-RFLP. This is the first report on the characterisation of *L. monocytogenes* strains isolated from an avocado processing facility. Given the South African avocado export market, this research will provide valuable data to maintain high quality products and meet export quality expectations.

2. Materials and methods

2.1. Microbial strains, media and growth conditions

A collection of 140 *L. monocytogenes* isolates received from an avocado processor were analysed. These isolates were obtained from final product and environmental samples over a period of four seasons. The environmental samples were taken from areas inside the processing facility such as the floors, drains, equipment, conveyer belts and storage rooms as well as food processor's boots, hands and cleaning facilities. The recommended *L. monocytogenes* NCTC 7973 was used as the type strain (Jones & Seeliger, 1983). *L. monocytogenes* isolates were retrieved from glycerol stocks (70%) kept at −80 °C. A volume of 100 μl was inoculated into 10 ml brain heart infusion (BHI) broth (Merck, Cape Town, South Africa) and incubated at 37 °C for 48 h. The isolates were then streaked on BHI agar and incubated at 37 °C for 48 h.

2.2. DNA isolation

A modified method by Wang and Levin (2006) was used to isolate DNA. One colony from each strain obtained after growth on BHI agar as described above was transferred to 250 μl double strength (2X) TZ lysis solution (TZ consists of 2.0% Triton X-100 (Merck) and 2.5 mg ml^{-1} sodium azide (Merck) in 0.1 M Tris-HCl pH 8.0) in boil-proof microcentrifuge tubes. The samples were then boiled for 10 min, cooled and centrifuged for 5 min at 10 000 × *g*. A volume of 200 μl of the supernatant was transferred to a sterile microcentrifuge tube and used as DNA template in the PCR amplification reactions.

2.3. PCR-RFLP

For PCR, the primer pair seq01 (5′-AAT CTA GCA CCA CTG TCG GG-3′) and seq02 (5′-TGT GAC CTT CTT TTA CGG GC-3′) was used to amplify a 733-base pair (bp) fragment of the *inlA* gene (Rousseaux, Olier, Lemaître, Piveteau, & Guzzo, 2004). Each PCR reaction was performed in a total reaction volume of 50 μL containing 1X reaction buffer with 1.5 mM $MgCl_2$ (Super-Therm, Southern Cross Biotechnologies, Cape Town, South Africa), 0.8 mM dNTPs (Southern Cross Biotechnologies), 0.5 μM of each primer, 1.25 U *Taq* DNA polymerase (Super-Therm) and 2 μL of DNA template. The PCR was performed in an Eppendorf Mastercycler Personal (Merck, Hamburg, Germany) and the cycling conditions were as follows: initial denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and elongation at 72 °C for 2.5 min; and a final elongation at 72 °C for 7 min.

Each restriction digest was performed in a total reaction volume of 30 μL containing either 1 U FastDigest *Alu*I or FastDigest *Tsp*509I (Fermentas, Inqaba Biotechnical Industries, Pretoria, South Africa), 10 μL amplicon, 2 μL FastDigest Green buffer and 17 μL distilled water. The *Alu*I samples were incubated at 37 °C for 10 min and the *Tsp*509I samples at 65 °C for 10 min. Restriction products were then separated on a 5% agarose gel containing ethidium bromide (1.6 μL 100 mL^{-1}) for 1 h at 100 V. A 100 bp ladder (Fermentas) was used as a size indicator and 1X Tris-borate EDTA (TBE; Sambrook, Fritsch, & Maniatis, 1989) was used as the buffer. The resulting gel was viewed using an ultraviolet transilluminator (Vilber Lourmat, Marne-La-Vallée, France).

2.4. PFGE

2.4.1. Preparation of agarose plugs

Sterile swabs moistened with sterile TE buffer (Sambrook et al., 1989) were used to transfer colonies from the BHI agar plates to sterile McCartney bottles containing 4 mL TE buffer. The optical density (OD) at 610 nm was measured using a Beckman Coulter DU 530 Life science UV/Vis spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) and adjusted until a reading of 1.00 was obtained for each sample by adding TE buffer or cells. This was done to ensure approximately equal concentrations of cells in the plugs. A 200 μL volume of the cell suspensions (OD = 1.00) were transferred to microcentrifuge tubes and 10 μL lysozyme (20 mg mL^{-1}) (Merck) was added to each tube, the tube was gently mixed and incubated at 55 °C for 15 min in a water bath. A 10 μL volume of a 20 mg mL^{-1} proteinase K (Merck) solution was added to each tube and gently mixed. A 200 μL volume of melted 1% SeaKem Gold agarose (Whitehead Scientific, Cape Town, South Africa) was added to the 220 μL cell suspension, mixed and immediately dispensed into plug moulds (Bio-Rad Laboratories, Johannesburg, South Africa). The plugs were allowed to set at room temperature for 15 min. The plugs were then removed from the moulds and placed into 5 mL proteinase K/cell lysis buffer containing 5 mL cell lysis buffer (50 mM Tris and 50 mM EDTA, pH 8.0 containing 1% sarcosyl) and 25 μL proteinase K stock solution (20 mg mL^{-1}) and incubated in a 54 °C shaker water bath for 2 h with constant agitation. The proteinase K/cell lysis buffer was replaced with 15 mL sterile TE buffer and incubated in a 50 °C shaker water bath for 30 min. This washing procedure was repeated three times. Plugs were then transferred to 5 mL sterile TE buffer and stored at 4 °C until use.

2.4.2. Restriction digestion

A sterile scalpel was used to cut a 2.0–3.0 mm piece of the *L. monocytogenes* embedded agarose plug. This plug was placed in a microcentrifuge tube containing 200 μL 1X Buffer B or 1X Buffer Tango (Fermentas Life Sciences) for digestion with *Apa*I or *Asc*I (*Sgs*I) (Fermentas Life Sciences), respectively and left to equilibrate at room temperature for 15 min. The buffer was removed and 200 μL restriction enzyme solution (20 μL 10X Buffer B, 2 μL BSA (Fermentas Life Sciences), 50 U *Apa*I and 173 μL ddH_2O for *Apa*I digestion, or 20 μL 10X Buffer Tango, 2 μL BSA, 40 U *Asc*I and 174 μL ddH_2O for *Asc*I digestion) was added to cover the plugs. Plugs were incubated at 37 °C for 3 h in a water bath. After restriction enzyme digestion, the restriction enzyme solution was removed and the plugs were immediately loaded onto the agarose gel.

2.4.3. Pulsed-field gel electrophoresis

The plugs were loaded into a 1% Megabase agarose gel (Bio-Rad Laboratories). The wells were filled with melted 1% SeaKem Gold agarose (Whitehead Scientific) and left to set at room temperature for 15 min. A CHEF DRII system (Bio-Rad Laboratories) was used for the electrophoretic separation. Three litres of 0.5X TBE buffer was poured into the chamber and allowed to cool to 14 °C. The current-switching parameters were as follows: 4 s initial switch time, 40 s final switch time, 200 V (6 V cm^{-2}), buffer temperature at 14 °C and a total run time of 18 h. After electrophoresis, the gel was stained in 500 mL dH_2O containing 40 μL ethidium bromide (10 mg mL^{-1}) for 30 min, followed by destaining in 500 mL dH_2O for 2 h. The separated PFGE fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat) and the captured images were used for fingerprint analysis.

2.4.4. Analysis of PFGE gel images

Captured gel images were analysed using BioNumerics version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium). Groupings of the PFGE fingerprints were performed by means of the Dice similarity coefficient (s_D) and the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) (Sneath & Sokal, 1973). The s_D value is the similarity value and an indication of the relative correlation between the strains of one cluster. A *L. monocytogenes* isolate used as reference standard was included in multiple lanes of every gel for normalisation purposes.

3. Results

3.1. PCR-RFLP

The primers seq01 and seq02 were selected to amplify a 733 bp fragment of the *inlA* gene of *L. monocytogenes* isolates (Rousseaux et al., 2004). The *inlA* gene of all *L. monocytogenes* isolates evaluated in this study, as well as the type strain NCTC 7973, was successfully amplified. Two restriction endonucleases, *Alu*I and *Tsp*5091, were evaluated in a preliminary restriction digestion of 64 isolates (isolated in seasons 2007–2009) for PCR-RFLP grouping of *L. monocytogenes* strains. *Alu*I yielded three different profiles containing 7–8 bands that ranged in size from 44 to 241 bp for all the *L. monocytogenes* isolates and the type strain. Restriction digestion with *Tsp*509I yielded two different profiles with four bands each, ranging in size from 43 to 281 bp for the 64 *L. monocytogenes* isolates and the type strain (Fig. 1).

Based on these results, an additional 76 isolates (obtained in 2010) were evaluated with only the *Alu*I restriction endonuclease. After the final PCR-RFLP digestions, a total of 140 RFLP profiles were obtained based on the *inlA* gene of *L. monocytogenes* strains isolated from the processing facility. The majority of these isolates ($n = 74$) and the NCTC 7973 strain, grouped in the *Alu*I profile I. Fifty-five isolates showed the banding pattern typical of *Alu*I profile II, and only 11 isolates presented the *Alu*I profile III banding pattern. All three profile types contained isolates from both product and environmental origins.

3.2. PFGE

The suitability of the restriction endonucleases *Apa*I and *Asc*I for PFGE analysis of *L. monocytogenes* was evaluated using 64 isolates (obtained from seasons 2007–2009) from an avocado processing facility, as well as the type strain NCTC 7973. The initial clustering analyses (*Apa*I, *Asc*I and a combination of *Apa*I and *Asc*I) showed that the 65 isolates grouped into four clusters (Table 1). The most fragments were observed in the fingerprints obtained after digestion with *Apa*I. The combined fingerprint clustering analysis of the *Apa*I and *Asc*I PFGE profiles also revealed four clusters. The Dice coefficient values of this combined analysis were lower than those values obtained for the isolates of the analysis with only *Apa*I (Table 1).

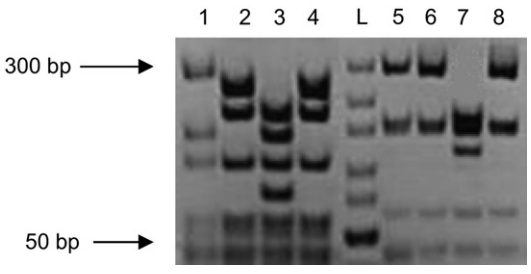

Fig. 1. Restriction digestion products for the PCR-RFLP of *L. monocytogenes* isolates separated on a 5% (m/v) agarose gel. Lanes 1–4: *Alu*I digests of isolates 43 (*Alu*I profile III), 61 (*Alu*I profile I), 62 (*Alu*I profile II), and 64 (*Alu*I profile I); Lane L: 50 bp DNA ladder; lanes 5–8: *Tsp*509I digests of isolates 43 (*Tsp*509I profile I), 61 (*Tsp*509I profile I), 62 (*Tsp*509I profile II), and 64 (*Tsp*509I profile I).

Table 1
Initial PFGE analysis of 64 *L. monocytogenes* isolates.

Clusters	*Apa*I		*Asc*I		*Apa*I and *Asc*I	
	n[a]	s_D[b]	n[a]	s_D[b]	n[a]	s_D[b]
I	48	82.8	50	61.7	48	67.3
II	15	70.2	12	67.75	15	59.1
III	1	–	1	–	1	–
IV	1	–	2	52.63	1	–

[a] n = number of isolates.
[b] s_D = Dice similarity coefficient.

Based on these results an additional 16 isolates (obtained in the 2010 season) were performed with the *Apa*I restriction endonuclease, after which a dendrogram containing the PFGE profiles of the 80 isolates (seasons 2007–2010) and the type strain NCTC 7973 was constructed (Fig. 2). Inclusion of the additional 16 isolates resulted in 5 clusters. Cluster I contained 22 strains that grouped together with a s_D of 68.4%. Cluster II contained 50 isolates and grouped at a s_D of 72.07% and both clusters III ($n = 2$) and IV ($n = 3$) had high s_D values of 88.89% and 93.29%, respectively. Finally, cluster V containing the type strain and two other isolates had a very low s_D value of 50.07%.

4. Discussion

4.1. PCR-RFLP

Preliminary restriction digestion based on a 733 bp fragment of the *inlA* gene of 64 *L. monocytogenes* isolates indicated that *Alu*I allowed a more detailed discrimination of strains (or comparison of strains) strains when compared to restriction enzyme *Tsp*5091 (Fig. 1). An additional 76 isolates were therefore evaluated with the *Alu*I restriction endonuclease. Although a total of 140 isolates (obtained over a period of 4 years from the production environment as well as the final product) were evaluated, no relationship between the PCR-RFLP profiles and the origin of the isolates was found.

This was also the case in two separate studies where *L. monocytogenes* strains isolated from human, food and environmental sources were analysed with the same primer pair and restriction endonucleases (Rousseaux et al., 2004; Tamburro, Ripabelli, Fanelli, Grasso, & Sammarco, 2010). Rousseaux et al. (2004) performed PCR-RFLP analysis on 37 *L. monocytogenes* strains and Tamburro et al. (2010) analysed 105 *L. monocytogenes* isolates from humans, food and environmental origin in Italy. In all cases, digestion with *Alu*I resulted in more subtype profiles than digestion with *Tsp*5091, but no correlation between the origin of the isolate and PCR-RFLP composite profiles existed in either of these studies. It seems, therefore, that the PCR-RFLP described in these studies is not suitable for epidemiological studies.

4.2. PFGE

The initial clustering analyses of 64 *L. monocytogenes* isolates indicated that *Apa*I digestion was more differentiating than digestion with *Asc*I and digestion with a combination of the restriction endonucleases. Therefore, an additional 16 isolates (obtained in the 2010 season) were analysed only with the *Apa*I restriction endonuclease. The dendrogram based on the total 80 PFGE *Apa*I profiles

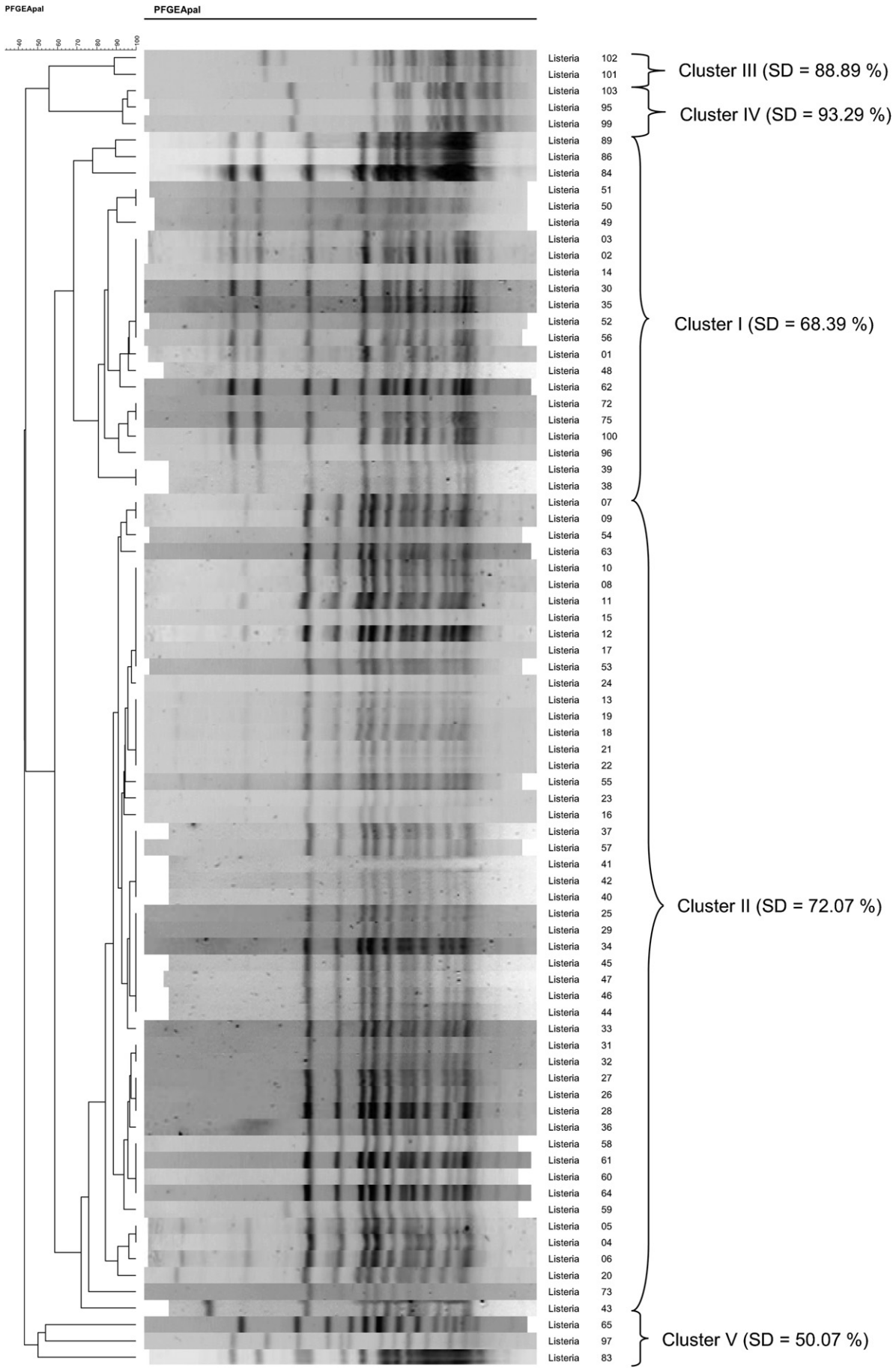

Fig. 2. Dendrogram obtained by UPGMA analysis of the Dice similarity coefficient (s_D) of the PFGE fingerprints with the restriction enzyme *Apa*I of 65 *L. monocytogenes* isolates.

(seasons 2007–2010) and the type strain NCTC 7973 showed five clusters (Fig. 2).

Cluster I contained more environmental isolates ($n = 14$) than product isolates ($n = 8$) and these isolates were isolated during all four production years. Cluster II contained isolates obtained from products ($n = 35$), as well as the production environment ($n = 15$). Three isolates obtained from avocado fruits grown in the same geographical region (Limpopo, South Africa) were also included in this group. Only two isolates grouped in cluster III and were obtained from the production environment (isolated in 2010) and as these clustered at a high s_D value of 88.89% they were regarded to be clonally related. The three isolates in cluster IV (isolated in 2010) also grouped at a very high s_D value of 93.29% and this also indicated a clonal relationship. The similarity of the strains in cluster IV thus points to possible cross-contamination, as they were obtained from samples taken from the electric box of the tub filler, the tub filler conveyer belt and a clerk's hands in the processing facility. The isolates which grouped in cluster V were obtained from a factory worker operating the blast freezers, a hand wash basin and also included the type strain. The s_D value (50.07%) of this cluster was, however, too low to indicate any clonal relationship between the isolates and the band patterns were quite dissimilar indicating a high genetic diversity of these isolates amongst each other.

The results of this study indicate that there is cross-contamination of *L. monocytogenes* strains between the production environment (including staff) and the final product. There seems to be a large reservoir of strains present in the processing facility (presented in cluster II), but there also appears to be an influx of new isolates which are being introduced into the processing plant by the processing of fruit each season. It was, however, not possible to track the exact contamination routes, as strains from clusters I and II were isolated from both product and environmental isolates. Other studies have reported the contamination sources and routes of *L. monocytogenes* with PFGE. Borucki et al. (2005) identified the silage and faecal samples as the main sources of *L. monocytogenes* contamination on a dairy farm with PFGE. The contamination routes of *L monocytogenes* in a fish processing facility were also identified with RAPD by Vogel, Huss, Ojeniyi, Hrens, and Gram (2001). The subtypes identified by the RAPD were confirmed with PFGE. It may be possible to track the *L. monocytogenes* strains in the avocado processing facility with PFGE profiles of more strains isolated from the outside environment as well as the supplying avocado farms.

Furthermore, it appears to be impossible to control the cross-contamination between the environment, workers and the product, as the product is not submitted to a heat processing step. This has important implications on the hygienic practices and hygiene regiment in the processing facility, which should be re-evaluated and re-designed to prevent the contamination of the final product. Separation between high risk (final product) and low risk (raw avocados and movement between inside and outside the facility) areas in a processing facility will for example prevent the spread of this pathogen in the avocado products.

Similarities were observed when the *Alu*I PCR-RFLP results obtained in this study were compared to the clustering analysis results obtained by PFGE. All of the isolates grouped in identical groups except for four isolates. Isolate 65 and the *L. monocytogenes* type strain NCTC 7973 grouped in cluster V, but had an *Alu*I profile I which corresponded with the cluster II grouping obtained with PFGE. Furthermore, strains 83 and 97 clustered in cluster V with the type strain, but exhibited a PCR-RFLP profile II which corresponded to the PFGE cluster I. Isolate 96 also had a PCR-RFLP profile I, but clustered in PFGE cluster IV, as opposed to PFGE cluster II. This might be an indication that although there is a 95% correlation between the PCR-RFLP and PFGE results, some differences between *L. monocytogenes* strains are not located at the *inlA* gene, which was the target gene for the PCR-RFLP, but elsewhere in the genome. As PFGE is a method that targets the complete genome of the micro-organism, it has a higher probability of identifying clonal differences between the strains and track the origin and movements of strains (Gerner-Smidt et al., 2006; Graves et al., 2005).

5. Conclusion

Although all of the isolates in this study were from the same processing facility, diversity among the isolates, as previously suggested documented in other studies, were far less than expected. Five clusters were identified, of which four had high similarities and showed possible clonal relationships between the strains of each cluster. All of the clusters also contained product, as well as environmental isolates and thus no correlations with specific sources of contamination could be established.

Both molecular subtyping methods, PCR-RFLP and PFGE, were sensitive and specific enough to assess the diversity among the majority of the *L. monocytogenes* isolates. PCR-RFLP could, therefore, be used as a screening step to identify certain *L. monocytogenes* subtypes before PFGE analysis is performed. The method should not take the place of PFGE in epidemiological studies. This is the first study which characterised *L. monocytogenes* isolates from a South African avocado processing facility with PCR-RFLP and PFGE. The results clearly show a potential risk associated with South African produced Avocado and thus a need for increased hygienic measures exists to prevent the dissemination of this pathogen during processing.

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Chapter 4

Assessment of Listex™ P100 for biocontrol of *Listeria monocytogenes* strains isolated from a South African avocado processing facility

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Assessment of Listex™ P100 for biocontrol of *Listeria monocytogenes* strains isolated from a South African avocado processing facility

Abstract

Listeria monocytogenes persistence in the food industry is a major concern as these bacteria can cause listeriosis. Additional management methods to quality control programs are needed to aid food manufacturers to meet regulations regarding these pathogens. Bacteriophage biocontrol is one such method that has received attention in the last decade. This study aimed to determine the feasibility of using a commercial phage product, Listex™ P100, to control persistent *L. monocytogenes* strains in an avocado processing facility. Bacterial strains were isolated over a period of five years and tested for susceptibility to the P100 phage product. Only 26.7% (n=240) of the strains were lysed by the phage. Activity of the bacteriophage resulted in a 4 log unit reduction ($P < 0.05$) in bacterial cells in brain heart infusion broth. However, there was no significant reduction in *L. monocytogenes* concentrations when inoculated with the phage product in avocado pulp, as well as the final guacamole product. These results indicate resistance of most *L. monocytogenes* strains isolated from this specific facility to the Listex™ P100 product and emphasise the complexity of bacteriophage biocontrol of bacterial strains in a processing facility.

Keywords

Listeria monocytogenes
Bacteriophage
Listex™ P100
Guacamole
Biocontrol

1. Introduction

Listeria monocytogenes is a well-known food-borne pathogen that has received attention due to its persistence in food processing facilities (Carpentier & Cerf, 2011) and economic losses due to product recalls (Ivanek, Gröhn, Tauer, & Wiedmann, 2005). These bacteria

can cause an invasive infection, listeriosis, which has been reported to have the third highest mortality rate of all food-borne diseases in the USA (Scallan et al., 2011). The largest listeriosis outbreak occurred in 2011 due to contaminated cantaloupes. During this outbreak 147 cases, including 33 deaths were reported across 28 states in the USA (CDC, 2011). Consequently, product recalls due to *L. monocytogenes* contamination in the USA drastically increased. From 1 January to 30 April 2015 the US Food and Drug Administration (FDA) reported 29 product recalls due to *L. monocytogenes* contamination. These products included a wide variety of raw and ready-to-eat foods such as ice cream, frozen vegetables, fresh pasta salad, soybean sprouts, organic chopped spinach, Granny Smith apples, caramel apples and smoked salmon (FDA, 2015).

Listeria phage P100 was isolated from sewage in a German dairy processing plant in 1997 (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005). This bacteriophage is commercially available from MICREOS Food Safety (previously EBI Food Safety) as a food processing aid (Listex^{TM} P100) for the control of *L*. *monocytogenes*. The product has been granted Generally Regarded As Safe (GRAS) by the FDA and has been approved by United State Department of Agriculture, Canada Health, and Food Standard of Australia and New Zealand (Anon, 2015). Phage P100 has been used in numerous studies which proved the effectiveness of control of *L. monocytogenes* in food products such as chocolate milk, chicken, fresh cut fruit and cheese, as well as in disruption of biofilms (Carlton et al., 2005; Chibeu et al., 2013; Guenther, Huwyler, Richard, & Loessner, 2009; Oliveira et al., 2014; Rossi et al., 2011; Soni, Desai, Oladunjoye, Skrobot, & Nannapaneni, 2012; Soni & Nannapaneni, 2010).

Listeria phage P100 belongs in the morphological group *Myoviridae* with an isometric capsid and contractile tail. It is classified in the subfamily *Spounavirinae* with another virulent *Listeria* phage A511. Both these bacteriophages are virulent with broad host ranges (Habann et al., 2014; Klumpp et al., 2008). The host ranges of bacteriophages intended for biocontrol is of importance since many phages can infect only a limited number of bacterial strains. To target an entire species in a food processing environment, either a phage with a sufficiently broad host range should be used, or a cocktail of phages with different host ranges which would increase the range of bacterial

strains susceptible to infection (Goodridge & Abedon, 2003). The prevalence and level of contamination of *L. monocytogenes* strains in a facility can vary greatly (Strydom & Witthuhn, 2015) and the possibility exists that even a bacteriophage with a broad host range will not be able to lyse all the strains that occur in one facility. There is little information on how effective bacteriophages are against an entire population of *L. monocytogenes* strains isolated from one environment. The aim of this study was to determine how efficient a virulent, broad host range phage such as phage P100 would be in controlling *L. monocytogenes* in an avocado processing facility with a six year history of contamination. *Listeria monocytogenes* strains were firstly isolated from the facility and 152 of these strains were characterised according to serotype. Phage P100 host range was determined against 239 of these isolated *L. monocytogenes* strains. Infectivity and activity of P100 were determined using three *L. monocytogenes* strains (T162, T182 and LS-20) which showed susceptibility to the phage. Finally, it was also attempted to isolate new bacteriophages from the facility.

2. Materials and methods

2.1 Isolation and characterisation of *Listeria monocytogenes*

Listeria monocytogenes strains were isolated from the final product, as well as the avocado processing environment between 2008 and 2013. Samples were enriched in half Fraser broth and then Fraser broth. Positive samples were streaked out onto *Listeria* Selective Agar (Oxford formulation, LSO) (Oxoid, Basingstoke, Hampshire, UK), Brilliance *Listeria* Agar (Oxoid) and RAPID'L.mono Agar (Bio-Rad). All isolates identified as *L. monocytogenes* were stored in 30% glycerol stocks at -80 °C. DNA was isolated from the strains with a DNeasy Blood and Tissue kit (Qiagen) after a pre-treatment. Firstly, strains were grown in Brain Heart Infusion (BHI) broth (Merck) for 24 h (37 °C) after which 2 mL of the cultures were centrifuged at 8000 *g* for 10 min. The supernatants were removed and replaced by 100 µl Triton X (Sigma) and 900 µl lysis buffer (100 mM Tris.Cl; 50 mM EDTA, 1 % SDS). These mixtures were vortexed for 1 min. The kit's instructions were followed and isolated DNA was stored at -20 °C. One hundred and fifty two *L. monocytogenes* strains were classified according to serotype based on the multiplex PCR described by Doumith, Buchrieser, Glaser, Jacquet, & Martin (2004).

2.2 Infection cycle of *Listeria monocytogenes* by P100

The infection cycle and subsequent lysis of *L. monocytogenes* LS20 by Listex™ P100 was recorded by first preparing solutions containing 2 mL overnight host culture (BHI, 37 °C). These were infected with 2 mL phage preparation (Listex™ P100) and 1 mL sterile BHI was added. The solutions were then fixed post infection after 10 min, 3 h and 6 h intervals with 5 mL of a 0.1 M sodium phosphate-buffered 3% gluteraldehyde (ALS) solution (pH 7) for 3 h. This was followed by a secondary fixative containing 1% osmium tetroxide (ALS) for 1 h in a similarly buffered solution. The cells were rinsed twice with the same buffer (0.1 M sodium phosphate) and then dehydrated in a series of acetone (ALS) dilutions of 50%, 70%, 95% for 20 min each, followed by two final dehydrating steps in 100% acetone (1 h). The dehydrated cells were then embedded in epoxy resin (Spurr, 1969) and polymerised at 70 °C for 8 h in transmission electron microscope embedding moulds. A Leica ultramicrotome EM UC7 (Vienna, Austria) was used to prepare roughly 60 nm thick sections of the epoxy resin with a glass knife and double staining was performed using uranyl acetate, dissolved in distilled water to obtain a saturated concentration (ca. 6%) and lead citrate (Reynolds, 1963) for 3 min each, after which the sections were rinsed three times with distilled water. These sections were viewed with a Philips (FEI, The Netherlands) CM100 Transmission Electron Microscope (TEM) at 60 V.

2.3 Listex™ P100 host range analyses

Listeria monocytogenes strains (n=239) isolated from the avocado processing facility between 2008 and 2013, as well as the *L. monocytogenes* type strain NCTC 7973 (Jones & Seeliger, 1983) were used to determine the host range of phage P100. Strains were grown at 37 °C in BHI for three hours after which lawns were prepared on BHI agar plates (3% agar). Five µL Listex™ P100 preparation (10^9 pfu/mL) was spotted on these lawns and plates were left at room temperature for 24 h and afterwards analysed for lysis zones or plaque formation. Additionally, the P100 phage was grown on *L. monocytogenes* T162 for 24 h in BHI broth. The bacteria were killed with chloroform (v/v) and removed from the suspension with centrifugation at 8 000 *g*, for 15 min. The resulting P100 phage suspension had a concentration of 10^6 pfu/mL and was also spotted (5 µL) on the bacterial

lawns. Each strain was tested at least three times for susceptibility to the phage preparation and a positive lysis result was recorded when single plagues or a lysis zone was observed.

2.4 Isolation of novel *Listeria monocytogenes* bacteriophages

In an attempt to isolate bacteriophages specific to the *L. monocytogenes* strains isolated from the avocado processing facility, waste water (n=10) from the facility's reservoir was collected and screened for phage activity according to Loessner & Busse (1990). However, sterilisation of the samples with a 0.22 µm filter, as well as a 45 µm filter, continuously lowered phage concentrations until finally, no plaques were observed when phage solutions were plated on BHI agar with *L. monocytogenes* T182. Additional waste water samples from the avocado processing facility (n=6), as well as waste water from a local milk pasteurising plant (n=7), a butchery (n=3) and a sewage plant (n=12) were screened for phage activity with a modified method (van Twest & Kropinsky, 2009). Twenty mL of a water sample (not filtrated or sterilised) was added to 15 mL BHI in a sterile 50 mL Falcon tube (Lasec, SA). One mL host culture grown overnight (*L. monocytogenes* strains LS 20, T151 or T182) was added to the Falcon tube and incubated at room temperature overnight. The following day, 1 mL chloroform (Sigma, SA) was added to each sample and after quick mixing, the Falcon tubes were left for 30 min at room temperature. Chloroform and debris from the water were then removed by centrifugation at 8 000 *g* at 4 °C for 15 min. Supernatants were transferred to sterile Falcon tubes and stored at 4 °C. To increase presumptive phage concentrations, 800 µL BHI was added to a microcentrifuge tube together with 100 µL host culture (the same strain used in first step) grown for 3 h incubation at 37 °C, and 100 µL of sterilised phage suspension. This was left for 15 min at room temperature and then 200 µL was plated on BHI agar plates (3 g agar in 800 mL broth). The plates were incubated over night at room temperature and checked for plaque formation or lysis zones. To purify phages, single plaques were picked up by pressing the opening of a pipet tip into the agar surrounding a plaque and transferring the agar plug to a sterile tube with 800 µL BHI. This was mixed lightly and left for 30 min to elute phages. These suspensions were then sterilised with chloroform (100 µL) for 10 min and centrifuged for 15 min at 8 000 *g*. The

supernatant was transferred to a new tube and phages were purified by repeating this until three single plaques were sterilised and plated with the corresponding host culture.

2.5 Listex^{TM} P100 activity against *Listeria monocytogenes*

The activity of P100 was tested against *L. monocytogenes* T162 in BHI broth, as well as against *L. monocytogenes* T162 in the avocado pulp and the guacamole. Firstly, two test tubes with 9 mL BHI broth were inoculated with 1 mL exponential phase culture of *L. monocytogenes* T162 to a final concentration of 10^6 cfu/mL. To the control test tube 1 mL dH_2O was added and to the other, 1 mL Listex^{TM} P100 phage preparation was added to a final concentration of 10^7 pfu/mL. These test tubes were incubated at 37 °C. The concentrations of the *L. monocytogenes* were measured at incubation, 2, 4, 6, 15 and 24 h, by taking a 1 mL sample from each test tube and plating a dilution series of that sample on LSO. Plates were incubated at 37 °C for 48 h after which bacterial counts were noted. Guacamole samples (100 g in sterile food grade bags) were inoculated with *L. monocytogenes* T162 to a final concentration of 10^6 cfu/g. Distilled water was added to the control samples and Listex^{TM} P100 was added to the tested samples to a final concentration of 10^7 pfu/g. Each bag was placed in a stomacher for 1 min to disperse inocula. These samples were then incubated at room temperature and the bacterial concentration was measured after 21, 24 and 27 h. Ten gram samples were weighed, diluted in 90 mL physiological solution (70% NaCl) and mixed in a stomacher for 1 min. A dilution series of these samples were then plated on LSO and bacterial counts were taken after 48 h incubation at 37 °C. Growth of *L. monocytogenes* T162 in avocado pulp was similarly tested and the bacterial concentrations were measured after 21, 24 and 27 h incubation at room temperature.

2.6 Statistical analyses

Significant differences between *L. monocytogenes* counts were determined with a Student's T-Test at a 95% level of significance.

3. Results and Discussion

3.1 Typing of *Listeria monocytogenes* strains

Assessment of the *L. monocytogenes* strains based on the multiplex PCR identified 85 strains in the serotype group 1/2a (3a), 47 strains as 1/2c (3c) and 1 strain as 4b (4d, 4e) according to the description in Doumith et al. (2004) (Table 1, Supp. Table 1). This is a similar distribution of *L. monocytogenes* strains isolated from the food industry reported in other studies, apart from the fact that no strains were identified as serotype 1/2b (Cetinkaya et al., 2014; Shen et al., 2013; Strydom & Witthuhn, 2015). For 11 strains, only the *prs* gene was amplified with the multiplex PCR, therefore only indicating *Listeria* spp. and 8 strains had indistinguishable profiles (Table 1, Supp. Table 1).

3.2 Infection of *Listeria monocytogenes* with phage P100

It was possible to visualise the infection cycle of Listex^{TM} P100 in *L. monocytogenes* LS20 (Fig. 1). Attachment of viral particles to bacterial cells and injection of viral DNA into the bacterial cell were noted after only 10 min. It was also possible to view DNA being injected into the bacterial cell after 3 h. New viral particles and lysis of cells were seen after 6 h. It is difficult to say how many new virons are formed in each cell, since only segments of specific cells can be seen. The production of new virons that can further infect and kill surrounding bacteria is one of the advantages discussed in bacteriophage biocontrol. However, host-phage interaction is different in circumstances where the bacterial cells are stressed. For example, exponentially growing cells have more nucleic acid available for production of new viral particles compared to a cell in a stressed state. Membrane activity and energy levels in bacterial cells can also influence phage infectivity (Bouvier & Maurice, 2011).

3.3 Host range analysis of Listex^{TM} P100

Only 26.7 % (n = 239 and NCTC 7973) of *L. monocytogenes* strains were susceptible to the P100 phage (Table 1), whereas 12 strains showed spontaneous lysogeny. This is interesting since other studies describing *L. monocytogenes* susceptibility to this particular bacteriophage reported much higher percentages such as 95 % (n=250) of food borne *Listeria* isolates (Carlton et al., 2005; Fister et al., 2015). Also, it has been reported that the spot test method can overestimate the host range, since other factors such as bacteriophage lysins in a preparation or resistance due to prophages can influence

Table 1 Listex™ P100 susceptible *L. monocytogenes* strains

Number	Strain	Source	Date	P100	Serotype	PFGE[a]
1	#5 IMB	Drain	2009	x	1/2a	II
2	#8 IMB	Unknown	2009	x	4b	*
3	Ap 12074 160/09 (19)	Product	2009	x	1/2c	*
4	B51	Waste water	2012	x	*	*
5	E1	Waste water	2012	x	*	*
6	F3	Waste water	2012	x	*	*
7	L04	Product	2008	x	*	I
8	L17	Ribbon Blender	2008	x	1/2a	II
9	L37	Product	2008	x	1/2a	II
10	LS-20	Processing environment	2013	x	*	*
11	LS-27	Processing environment	2013	x	*	*
12	M01	Unknown	2009	x	1/2a	*
13	M02	Unknown	2009	x	1/2a	*
14	M03	Unknown	2009	x	1/2a	*
15	M04	Unknown	2009	x	*	*
16	M06	Unknown	2009	x	1/2a	*
17	M07	Unknown	2009	x	*Listeria*	*
18	M10	Unknown	2009	x	1/2c	*
19	M15	Unknown	2009	x	1/2c	*
20	M18	Unknown	2009	x	*	*
21	M20	Unknown	2009	x	1/2c	*
22	M28	Floor store room	2009	x	*	II
23	M43	Unknown	2009	x	1/2c	*
24	M45	Unknown	2009	x	1/2c	*

Table 1 Listex™ P100 susceptible *L. monocytogenes* strains

Number	Strain	Source	Date	P100	Serotype	PFGE[a]
25	M48	Unknown	2009	x	1/2c	*
26	M60	Unknown	2009	x	1/2c	*
27	M63	Unknown	2009	x	*	*
28	M64	Table	2009	x	1/2a	II
29	M65(1)	Unknown	2009	x	1/2a	*
30	M65	Product	2009	x	1/2a	II
31	M67	Unknown	2009	x	1/2c	*
32	M73	Unknown	2009	x	1/2a	*
33	M75	Unknown	2009	x	1/2a	*
34	M77	Unknown	2009	x	*Listeria*	*
35	M84	Unknown	2009	x	1/2c	*
36	T005/10	Table leg	2010	x	1/2c	*
37	T006/10	Boot captive floor low risk area	2010	x	1/2c	*
38	T008/10	Drain boot captive	2010	x	1/2c	*
39	T010/10	Drain	2010	x	1/2a	*
40	T011/10	Drain boot captive high risk area	2010	x	1/2c	*
41	T012/10	Tubfiller machine roller	2010	x	1/2a	*
42	T013	Floor below tables	2010	x	1/2c	*
43	T014	Hose pipe	2010	x	1/2c	*
44	T015	Green hose pipe	2010	x	*	*
45	T040	Floor 4A	2010	x	*	*
46	T041	Floor 2A	2010	x	*	*
47	T049	Hand spray bottles	2010	x	*	*
48	T053	Washed clean bucket	2010	x	1/2c	*

Table 1 Listex™ P100 susceptible *L. monocytogenes* strains

Number	Strain	Source	Date	P100	Serotype	PFGE[a]
49	T058	Hand wash basins	2010	x	1/2c	V
50	T065	Floor 2A	2010	x	*Listeria*	*
51	T079	Blender controls	2010	x	*	*
52	T098	Bucket stand (mixing)	2010	x	1/2c	*
53	T125	Bucket standing (mixing)	2010	x	1/2c	*
54	T126	Scale	2010	x	1/2c	*
55	T130	Unknown	2010	x	1/2a	*
56	T135	Scale	2010	x	IP	*
57	T142	Waste bins below tables	2010	x	*	*
58	T151	Tubfiller controls	2010	x	1/2a	*
59	T161	Apron	2010	x	*	*
60	T162	Buckets on tables (with avo)	2010	x	IP	*
61	T182	Waste bins below table	2010	x	1/2a	*
62	T188	Paper towel container (boot captive)	2010	x	1/2a	*
63	T196	Floor bucket washing	2010	x	1/2a	*
64	Zesty	Product	2010	x	*	*

[a]Results from Strydom, Bester, Cameron, Franz, & Witthuhn, 2013
* = not tested
1/2a = 1/2a (3a)
1/2c = 1/2c (3c)
4b = 4b (4d, 4e)
Listeria = only *prs* gene was amplified during multiplex PCR (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004)
IP = indistinguishable profile

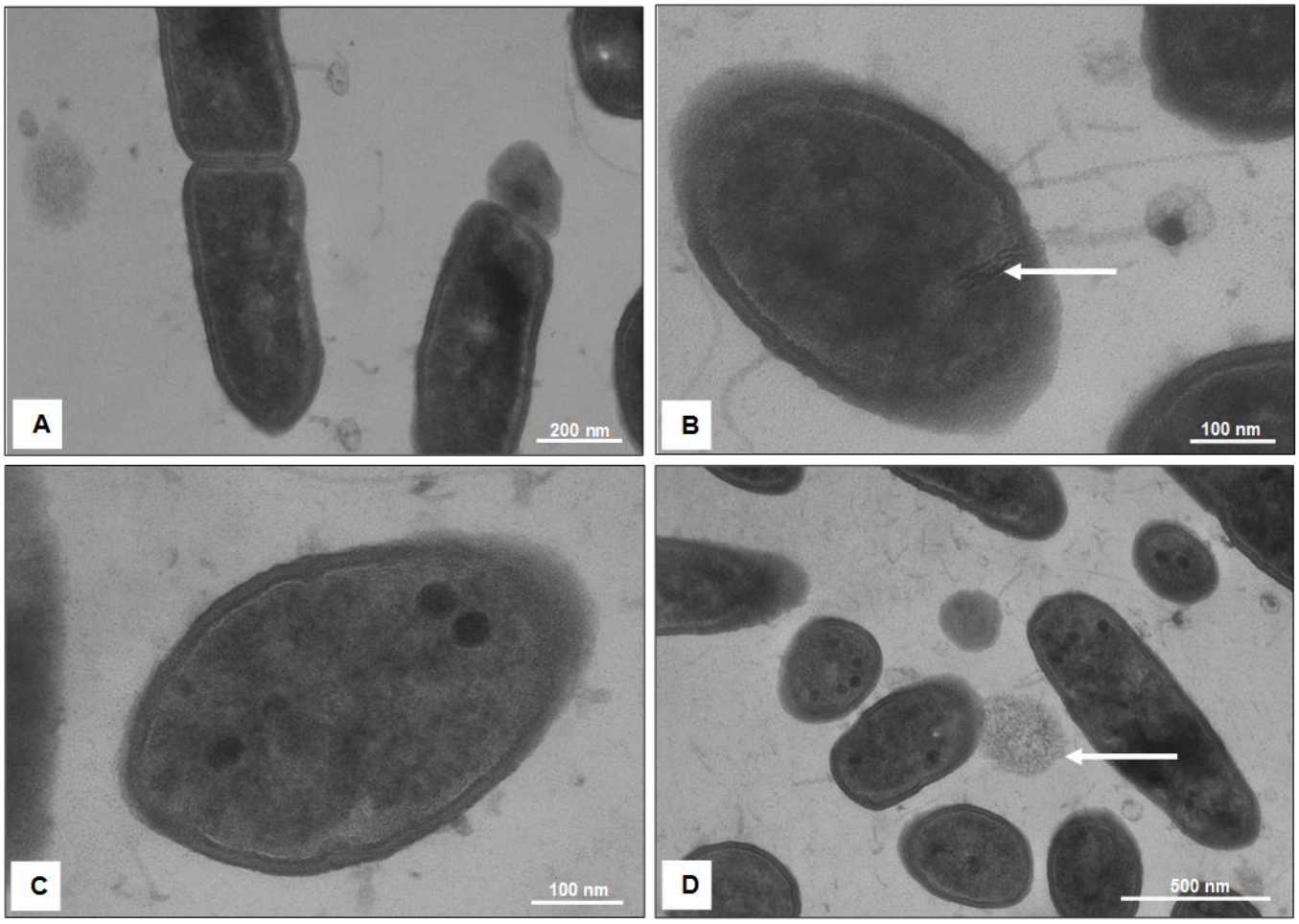

Figure 1. Infection cycle of Listex^{TM} P100 visualised in an epoxy resin with TEM. Bacterial cells (cultures grown overnight at 37°C) were infected with phage (Listex^{TM} P100) and the solutions were fixated at different times with gluteraldehyde. **A**: Bacteriophages with empty capsids attached to listerial cells were observed after 10 min. **B**: Injection of DNA into bacterial cell (indicated with white arrow) (3 h). **C**: Formation of new virons in the bacterial cell (6 h). **D**: New virons and cell lysis (indicated with white arrow) (6 h).

susceptibility of strains (Khan Mirzaei & Nilsson, 2015). Resistance of the strains used in this study may also be attributed to presence of prophages since there were 12 bacterial strains that showed lysogeny without prior treatment with ultra violet light. Additionally, some of the strains such as *L. monocytogenes* T162, which were lysed by the phage product did have small bacterial colonies in the lysis zones, indicating resistant cells in those strains.

A closer look at these specific strains is, therefore, necessary to understand the resistance to Listex™ P100. In a previous study, 56 of these strains were typed with pulsed field gel electrophoreses (PFGE), using the restriction enzyme *Alu*I (Strydom, Bester, Cameron, Franz, & Witthuhn, 2013). The PFGE results indicated only two major genetic groups in the processing facility, which was less than expected from strains isolated over five years. Interestingly, bacterial strains that were resistant to Listex™ P100 were grouped in both PFGE groups, as with the susceptible strains (Table 1). The results of PFGE analysis of these strains could, therefore, not be used as indicators for susceptibility to this particular bacteriophage. Serotyping of 152 strains isolated from this facility identified serotypes 1/2a and 1/2c which are generally isolated from food borne sources. As expected, Listex™ P100 was able to lyse strains from serotypes 1/2a (19 of 46 strains), 1/2c (21 of 46 strains), as well as the one strain identified as serotype 4 (Carlton et al., 2005). However, of the 106 resistant *L. monocytogenes* strains which were also subjected to serotyping, 66 belonged to serotype 1/2a and 26 strains belonged to 1/2c. There was no relationship between date of isolation or isolation source of the strains, and susceptibility to Listex™ P100. There was not one specific site or one specific date during which strains belonging to one serotype were isolated, and there was no case where strains isolated from one specific area from the facility were all susceptible to the bacteriophage in question. This may indicate that cross contamination played a role in transfer of the *L. monocytogenes* strains in this specific facility since it seems that the bacterial strains were randomly dispersed in the facility.

Resistance of *L. monocytogenes* strains to their bacteriophages are interesting as only a few strains contain an intact CRISPR (clustered regularly interspaced short palindromic repeat) system. The impression is that only some strains of the genus *Listeria* can use this system for phage resistance (Kuenne et al., 2013). Restriction modification

(RM) systems may play a role in resistance of *L. monocytogenes* and a few novel RM systems have recently been identified (Lee, Ward, Siletzky, & Kathariou, 2012). Specifically, in *L. monocytogenes* epidemic clone II strains, a RM system containing a restriction endonuclease which is down regulated at 37 °C, was identified. These *L. monocytogenes* strains are resistant to the specific bacteriophages at lower temperatures, but they become susceptible when grown at 37 °C (Kim & Kathariou, 2009; Kim et al., 2012). In the food industry, where the targeted *L. monocytogenes* strains contain this RM system or one with a similar temperature dependence, phage resistance will certainly be influenced by the processing and storage temperatures of the food product.

3.4 Isolation of novel bacteriophages

Many plaques and lysis zones were observed throughout the study and in total 26 samples indicated lysis. However, most of these plaques were cloudy indicating incomplete lysogeny or had small bacterial colonies inside the lysis zone, indicating resistant cells. It was possible to purify three phages, all isolated from the sewage water, but it was not possible to attain concentrations high enough for TEM analyses. Additionally, after storage at 4 °C for four weeks, these phages could not be activated. Although it was not possible to identify the type of bacteriophage or isolate DNA for genome analysis, it is probable that these phages were lysogenic. Most *Listeria* phages are lysogenic and belong to the genus *Siphoviridae* (Klumpp & Loessner, 2013).

3.5 Listex^{TM} P100 activity against *Listeria monocytogenes*

The activity of the P100 phage in BHI broth against *L. monocytogenes* T162 led to a reduction of almost log 3 cfu/mL after 6 h, whereas the bacterial control reached log 9.5 cfu/mL at that time (Fig. 2). The difference between the phage treated sample and the bacterial control sample after 6 h incubation was, therefore, log 5.5 cfu/mL ($P < 0.05$). With longer incubation, the phage treated sample showed increased concentration in bacterial cells of almost 2 log units, with a final concentration of 10^6 cfu/mL. This regrowth may be due to resistant cells in this specific bacterial strain which was also observed in the host culture analysis.

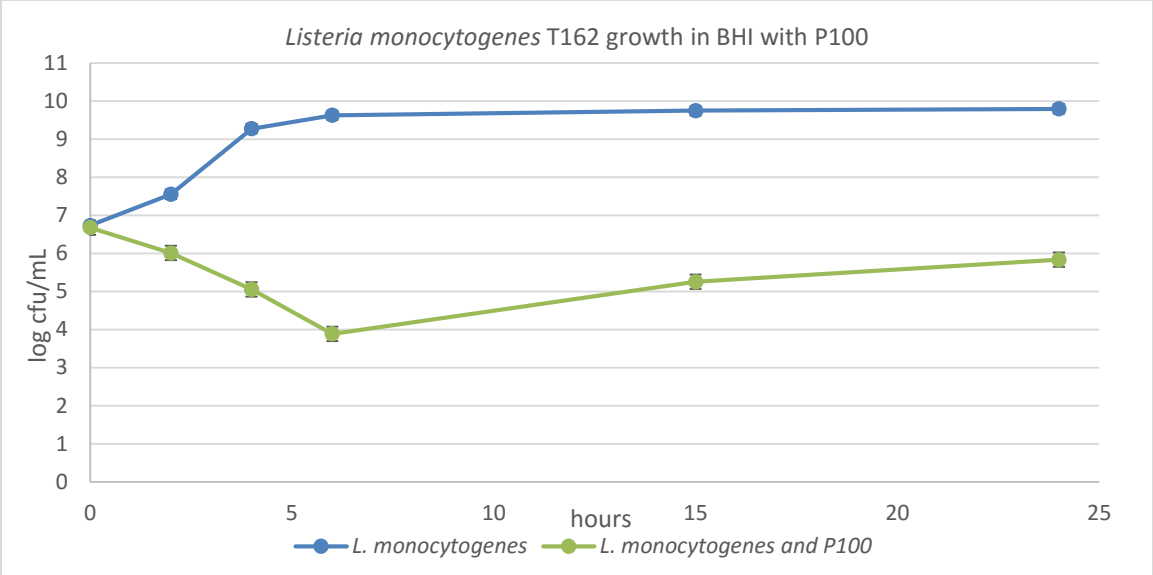

Figure 2. Growth of *L. monocytogenes* in BHI in the absence (blue) and presence (green) of Listex™ P100. Error bars indicate standard deviation. Significant difference ($P < 0.05$).

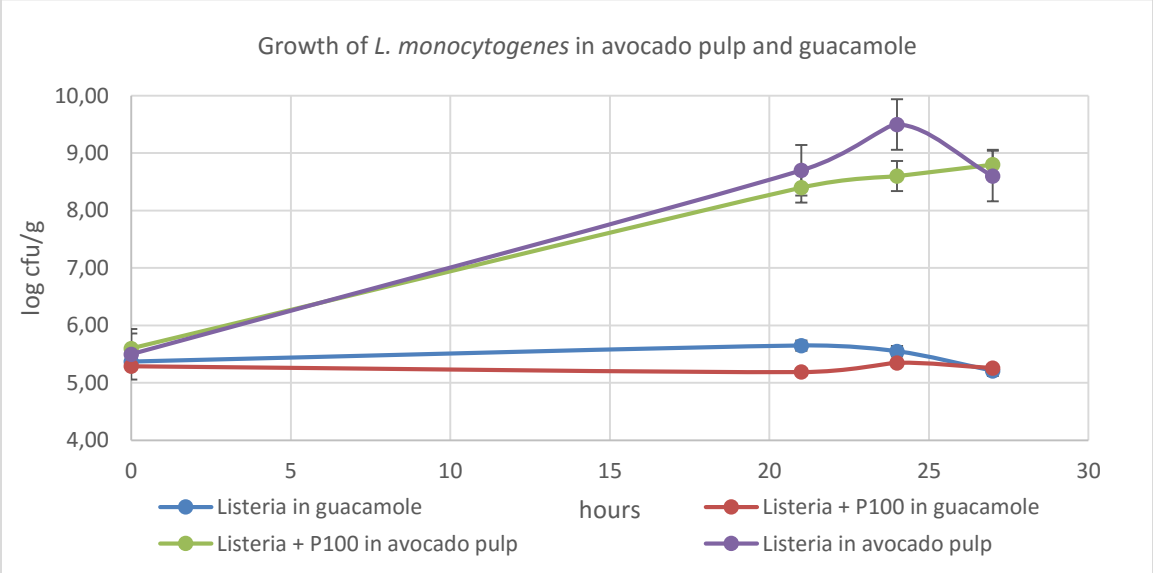

Figure 3. Growth of *L. monocytogenes* in guacamole in the absence (blue) and presence (red) of Listex™ P100. Growth of *L. monocytogenes* in avocado pulp in the absence (purple) and presence (green) of Listex™ P100. Error bars indicate standard deviation. Significant difference ($P < 0.05$).

log units after 21 h and then spontaneously decreased with 0.44 log units to a final concentration of 10^5 cfu/g (Fig. 3). Growth of *L. monocytogenes* in avocado has previously been measured by Iturriaga, Arvizu-Medrano, & Escartín (2002), who also reported increase in avocado pulp, but not in guacamole. It is reasonable to assert that avocado pulp poses a greater risk for transferring *L. monocytogenes* to consumers, than the final product, guacamole.

There was no significant difference between the control and phage treated samples in both the guacamole and the avocado pulp. The matrix of these food products probably does not allow for mobility of phage or bacteria, which limits the effectiveness of the phages greatly. The most promising results of bacteriophage biocontrol of *L. monocytogenes* was found in liquid products tested, specifically chocolate milk (Guenther et al., 2009). Results from concentrations of *L. monocytogenes* on the surface of other food products were also more promising than that of the avocado pulp and the guacamole. Clearly, the food matrix of guacamole is not suited for bacteriophage biocontrol.

4. Conclusion

The use of Listex™ P100 to control *L. monocytogenes* in various circumstances has been positively reviewed. However, the results presented here indicates that the P100 phage might not be useful to control the *L. monocytogenes* strains in this specific facility. Too few of the strains isolated were susceptible to the phage product and there was no phage activity against the bacteria in the avocado pulp and guacamole. The high percentage of resistant bacterial strains to this specific phage is unusual and further work should be done to identify the resistance mechanisms in these specific strains. These results should not, however, be seen as evidence that the bacteriophages cannot be used as biocontrol agents, since significant reductions in *L. monocytogenes* can be accomplished under certain circumstances. This should rather emphasise the complexity of biocontrol of bacterial strains in a food processing facility. A bacteriophage product with a broader host range, for example a phage cocktail, may work well in the processing environment to minimise transfer of bacterial cells to the final product. It is, however, important to do

preliminary tests to determine if the targeted bacterial strains are susceptible to the phage intended for biocontrol, as well as to determine the activity of the phage against the bacteria in the specific food matrix in relation to the specific chemico-physical characteristics of the food compared to model systems.

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Supplementary Table *Listeria monocytogenes* strains resistant to phage P100

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
1	104(194/10)	Product	2010	-	*	*
2	118(197/10)	Product	2010	-	*	*
3	140(202/10)	Product	2010	Lysogenic	*	*
4	141.193(16)	Product	2010	-	1/2c	*
5	142(193/10)	Product	2010	-	*	*
6	205.10(3132)	Product	2010	-	1/2a	*
7	Ap 12074 159/09 (16)	Product	2009	-	*	*
8	B13	Waste water	2012	-	*	*
9	C4	Waste water	2012	-	*	*
10	D2	Waste water	2012	-	*	*
11	L01	Product	2008	-	1/2c	I
12	L02	Product	2008	-	1/2c	I
13	L05	Product	2008	-	1/2a	II
14	L07	Product	2008	Lysogenic	1/2a	II
15	L08	Product	2008	-	1/2c	II
16	L10	Product	2008	Lysogenic	1/2a	II
17	L11	Product	2008	-	*	II
18	L12	Product	2008	-	*	II
19	L13	Product	2008	-	1/2a	II
20	L14	Product	2008	-	1/2a	II
21	L15	Product	2008	Lysogenic	1/2c	II

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
22	L19	Product	2008	-	1/2a	II
23	L21	Product	2008	-	1/2a	II
24	L22	Product	2008	-	1/2a	II
25	L23	Product	2008	Lysogenic	1/2a	II
26	L25	Product	2008	-	ND	II
27	L27	Product	2008	-	1/2a	II
28	L28	Product	2008	Lysogenic	1/2a	II
29	L29	Product	2008	-	1/2c	II
30	L30	Product	2008	-	1/2c	II
31	L31	Product	2008	-	*	II
32	L32	Product	2008	-	1/2a	II
33	L34	Fruit swab	2008	-	1/2a	II
34	L35	Fruit swab	2008	-	1/2a	II
35	L36	Fruit swab	2008	Lysogenic	1/2a	II
36	L38	Drain	2008	-	1/2c	I
37	L40	Product	2008	-	1/2c	II
38	LS-28	Processing environment	2013	-	*	*
39	LS-07	Processing environment	2013	-	*	*
40	M05	Unknown	2009	-	*	*
41	M08	Unknown	2009	-	1/2c	*
42	M11	Product	2009	-	*	II

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
43	M12	Unknown	2009	-	*	*
44	M13	Unknown	2009	-	*Listeria*	*
45	M14	Buckets	2009	-	1/2a	II
46	M16	Unknown	2009	-	1/2a	*
47	M17	Unknown	2009	-	*	*
48	M19	Product	2009	-	1/2c	I
49	M21	Platform bottom	2009	-	*	*
50	M22	Floor store room	2009	-	*	*
51	M23	Bucket	2009	-	*	I
52	M24	Product	2009	-	*	I
53	M25	Buckets	2009	-	*	I
54	M26	Platform bottom	2009	-	*	I
55	M27	Floor blast freezer	2009	Lysogenic	*	II
56	M29	Floor blast freezer	2009	-	*	II
57	M30	Unknown	2009	-	1/2c	*
58	M36	Platform bottom	2009	-	1/2c	II
59	M38	Table	2009	-	ND	II
60	M44	Product	2009	-	1/2a	II
61	M46	Product	2009	-	1/2a	II
62	M51	Store room floor	2009	-	1/2a	*
63	M53	Unknown	2009	-	1/2a	*

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
64	M54	Unknown	2009	-	1/2a	*
65	M61	Unknown	2009	-	1/2a	*
66	M66	Unknown	2009	-	1/2a	*
67	M81	Unknown	2009	Lysogenic	1/2a	*
68	M86	Unknown	2009	-	1/2c	*
69	M89	Unknown	2009	-	1/2c	*
70	M93	Unknown	2009	-	1/2a	*
71	M94	Unknown	2009	-	1/2a	*
72	M96	Unknown	2009	-	1/2a	*
73	M98	Unknown	2009	-	1/2c	*
74	P104	Product	2010	-	1/2a	*
75	T001/10	Drain	2010	-	1/2c	*
76	T002/10	Floor	2010	-	1/2a	*
77	T003/10	Floor	2010	-	*Listeria*	*
78	T004/10	Table leg	2010	-	1/2a	*
79	T009/10	Drain	2010	-	1/2c	*
80	T016	Black hosepipes	2010	-	1/2c	*
81	T019	Unknown	2010	-	1/2a	*
82	T042	Floor 3A	2010	-	*	*
83	T043	Floor bucket washing	2010	-	*	*
84	T044	Floor 2A	2010	-	*	*

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
85	T045	Tables underneath	2010	-	*	*
86	T046	Boot captive floor	2010	-	*	*
87	T050	Tables	2010	-	*	*
88	T052	Scooping hands	2010	-	*	*
89	T054	Waste bins	2010	-	1/2c	*
90	T060	Tables underneath	2010	Lysogenic	*	*
91	T061	Boot captive floor	2010	-	1/2a	*
92	T062	Waste bins	2010	-	*	I
93	T064	Cryovac hood selection	2010	-	*	*
94	T066	Hand wash basins	2010	-	*Listeria*	*
95	T067	Scooping hands	2010	-	*	I
96	T068	Unknown	2010	-	1/2a	I
97	T069	Tables underneath	2010	-	*	*
98	T070	Fruit conveyor controls	2010	-	1/2c	*
99	T071	Floor 1A	2010	-	1/2a	*
100	T072	Printing conveyor	2010	-	*	*
101	T073	Cryovac inside door	2010	-	1/2a	*
102	T074	Hand spray bottles	2010	-	*	*
103	T075	Bucket stand (mixing)	2010	-	*	*
104	T078	Waste remover's hands	2010	-	*	*
105	T080	Bucket stand scale	2010	-	1/2a	*

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
106	T081	Hand wash basins	2010	-	*	I
107	T083	Cutting hands	2010	-	*	*
108	T084	Cleaner	2010	-	1/2a	*
109	T085	Bucket stand (mixing)	2010	-	1/2a	*
110	T086	Tubfiller green strips	2010	-	1/2a	*
111	T087	Tables underneath	2010	-	1/2a	*
112	T088	Bucket stand scale	2010	-	1/2a	*
113	T089	Waste bins	2010	-	*	*
114	T090	Store room table	2010	-	ND	*
115	T091	Hand spray bottles	2010	-	1/2a	*
116	T092	Printing conveyor	2010	-	*	*
117	T094	Hand spray bottles	2010	-	*Listeria*	*
118	T095	Waste remover's hands	2010	-	ND	*
119	T096	Scale	2010	Lysogenic	1/2c	*
120	T097	Apron	2010	-	ND	*
121	T099	Blast freezer operators	2010	-	*	*
122	T100	Waste bins below tables	2010	-	1/2a	*
123	T102	Table underneath	2010	-	*Listeria*	*
124	T103	black hosepipes	2010	-	*	*
125	T104	Floor 4A	2010	-	1/2a	*
126	T106	Floor 1A	2010	-	*Listeria*	*

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
127	T107	Tubfiller electric box	2010	-	1/2a	*
128	T108	Waste bins below tables	2010	-	*	*
129	T112	Apron	2010	-	1/2a	*
130	T118	Blast freezer operators	2010	-	1/2a	*
131	T120	Waste bins below tables	2010	-	1/2a	*
132	T121	Table underneath	2010	-	*	*
133	T122	Floor 1A	2010	-	1/2a	*
134	T123	Waste bins below tables	2010	-	*	*
135	T124	Apron	2010	-	1/2a	*
136	T127	Unknown	2010	-	1/2c	*
137	T128	Tubfiller green strips	2010	-	Lis	*
138	T129	Waste bins below tables	2010	-	1/2a	*
139	T134	Tables underneath	2010	-	1/2a	*
140	T136	Blast freezer operators	2010	-	1/2a	*
141	T137	Black hosepipes	2010	-	*	*
142	T140	Labelling hands	2010	-	1/2a	*
143	T141	Tubfiller electric box	2010	-	1/2c	*
144	T143	Scale	2010	-	Lis	*
145	T144	Apron	2010	-	*	*
146	T146	Blast freezer operators	2010	-	*	*
147	T147	Mixer's controls	2010	-	1/2c	*

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
148	T149	Tubfiller green strips	2010	-	1/2a	*
149	T150	Vacuum sealer underneath	2010	-	*	*
150	T152	Tubfiller green strips	2010	-	*	IV
151	T153	Tubfiller electric box	2010	-	*	IV
152	T156	Buckets on tables (with avocado)	2010	-	*	*
153	T157	Buckets on tables (with avocado)	2010	-	*	*
154	T158	Lids on tables (with avocado)	2010	-	*	*
155	T159	Floor 1A	2010	-	1/2a	*
156	T160	Lids on tables (with avocado)	2010	-	1/2a	*
157	T164	Waste remover's hands	2010	-	*	*
158	T165	Floor 3A	2010	-	1/2a	*
159	T166	Floor 1A	2010	-	*	*
160	T167	Conveyor labeling	2010	-	*	*
161	T168	Floor bucket washing	2010	-	*	*
162	T169	Door handle	2010	-	*	*
163	T170	Floor 3A	2010	-	1/2a	*
164	T172	Waste bins below table	2010	-	*	*
165	T175	Waste	2010	-	1/2a	*
166	T176	Floor 1A	2010	-	*	*
167	T177	Waste bins below table	2010	-	*	*
168	T178	Waste remover's hands	2010	-	1/2a	*

Supplementary Table 1 *Listeria monocytogenes* strains resistant to phage P100 continued

Number	Strain	Source	Date	P100	Serotype	[a]PFGE
169	T179	Floor 1A	2010	-	1/2a	*
170	T180	Floor 3A	2010	-	1/2a	*
171	T185	Blast freezer operators	2010	-	ND	V
172	T187	Floor 1A	2010	-	1/2c	*
173	T189	Blast freezer operators	2010	-	*	*
174	T191	Waste bins below table	2010	Lysogenic	*	*
175	T192	Floor 5A	2010	-	*	*
176	NCTC 7973	N/A	N/A	-	1/2a	V

[a]Results from Strydom, Bester, Cameron, Franz, & Witthuhn, 2013
* = not tested
1/2a = 1/2a (3a)
1/2c = 1/2c (3c)
Listeria = only *prs* gene was amplified during multiplex PCR (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004)
IP = indistinguishable profile
N/A – not applicable
ND = not determined
Lysogenic = spontaneous lysogeny in strain

Chapter 5

Successful management of *Listeria* spp. in an avocado processing facility

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Successful management of *Listeria* spp. in an avocado processing facility

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ABSTRACT

Listeria monocytogenes can grow and multiply in various food matrices and cause severe human illness. Apart from the influence on consumer health, *L. monocytogenes* contamination of ready-to-eat (RTE) food products causes major economic losses due to product recalls. Control of foodborne pathogens in RTE food products is a challenge, specifically in foods that cannot undergo a heat-treatment during processing. The aim of this study was to develop control strategies for the management of *L. monocytogenes* in an avocado processing facility, additional to a quality control system. An in-house monitoring system (IMS) was established to test specifically for *Listeria* spp. in the final products and processing environment, including floors, equipment, work areas and personnel. Guacamole and environmental samples were collected and tested on-site for *Listeria* with the ISO 11290-1 method. Based on the prevalence of *Listeria*, the facility introduced new strategies in processing to counter cross contamination. Results from the 2014 guacamole production season showed almost complete eradication of *Listeria* spp. in final products (0.17%, n = 1170) and the processing facility (0.79%, n = 1520). This is a major achievement since the highest incidence of *Listeria* spp. over a period of five years was measured at 11.39% (n = 948) in the final product during the 2013 season and 13.44% (n = 1927) in the processing facility in 2011. These results indicate that successful management of *Listeria* spp. in an avocado processing facility can be accomplished with in-house monitoring of the listerial population and subsequent adjustments to the processing system.

1. Introduction

Listeria monocytogenes is a food-borne pathogen that can cause listeriosis in immune compromised individuals. The rate of *L. monocytogenes* infection is not as high as other food-borne pathogens, but the mortality rate of listeriosis is one of the highest in the USA (Farber & Peterkin, 1991; Scallan et al., 2011). Consequently, some countries have strict regulations for food products concerning *L. monocytogenes* contamination. The USA has a strict zero tolerance policy (no viable cells detected in a 25 g sample) for *L. monocytogenes* in ready-to-eat (RTE) food products (9 C.F.R. § 430). In Europe the concentration of *L. monocytogenes* should be kept below 100 cfu/g in food products and absent in 25 g samples of RTE food products that can support the growth of the bacteria or is intended for infants or special medical purposes (European Commission, 2005). South African legislation require <100 cfu/g *L. monocytogenes* in canned and processed meat (SANS 885:2011).

The regulations regarding *L. monocytogenes* have a major impact on the food industry and in 2005 the cost of product recalls due to *L. monocytogenes* contamination in the USA was estimated between US$ 0.1–2.4 billion (Ivanek, Gröhn, Tauer, & Wiedmann, 2005). Additionally the annual cost of illness due to *L. monocytogenes* infection in the USA was estimated at 2.6 billion US$ (Hoffmann, Batz, & Morris, 2012). These are major economic losses and it clearly shows that food manufacturers need assistance in the management of these pathogens to protect themselves, as well as the health of the consumers.

Control of food pathogens is very difficult, especially in RTE food products such as guacamole, that do not require a heat treatment (Arvizi-Medrano, Iturriaga, & Escartin, 2001; Ratani et al., 2012; Rodríguez-Garcia, González-Romero, & Fernández-Escartín, 2011). The ubiquitous nature of *L. monocytogenes*, as well as the ability to grow at very low temperatures, high salt concentrations and in low

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pH environments make contamination of the food processing line possible at any point (Farber & Peterkin, 1991). Cross contamination has been suggested to be the main source of *L. monocytogenes* isolated from food products (Chen, Wu, Zhang, Wang, & Yan, 2013; Jamali, Chai, & Thong, 2013; Parisi et al., 2013). Additionally, persistence of *L. monocytogenes* in food processing facilities has been attributed to harbourage sites, rather than unique adaptation abilities that other pathogens do not exhibit (Carpentier & Cerf, 2011). This suggests that management strategies of *L. monocytogenes* should have a strong focus on prevention of cross contamination inside a facility, as well as careful monitoring of areas which can serve as harbourage sites for these bacteria. The first hurdle of defence is usually quality control and good manufacturing practices (GMPs), which include a strict cleaning regime. However, one of the biggest problems food manufacturers face is the unhygienic design of processing equipment and premises that makes cleaning difficult or even impossible (Carpentier & Cerf, 2011). Guacamole is an example of a RTE food product of which microbiological control depends solely on GMPs and temperature control. Processing of avocado fruits is extremely difficult since no heat can be used and conveyance of the avocado pulp is difficult (Arvizi-Medrano et al., 2001).

Parisi et al. (2013) stated that a facility-based approach which includes constant monitoring is necessary to control *L. monocytogenes*. The aim of this study is to describe an in-house monitoring system (IMS) during which the final product, as well as the food processing environment of a South African avocado processing facility were monitored on-site for the presence of *Listeria*. The *Listeria* prevalence was recorded over a period of five years and strategies additional to HACCP were introduced to increase control of these bacteria.

2. Materials and methods

2.1. Production of guacamole

The avocado processing facility is operational on a seasonal basis from the first week in May to mid-September, depending on availability of avocado fruit. On arrival, green fruit is sorted, washed over a brush roller conveyor with sprayers and sanitised by submersion in a water bath with a sanitizer. The green fruit is then incubated in ripening rooms between seven and ten days (11–22 °C) until ripe and ready for processing. Ripe fruit is sanitised again, by the same method, and directly transported to the high risk area, designated as the Hygiene Box (Fig. 1). Here, the avocado pulp is manually separated from the pips and skin, and transferred to buckets to a final weight of 20 kg per bucket. Ten buckets are then added simultaneously to the Blender (Scott Ribbon Blender, USA)

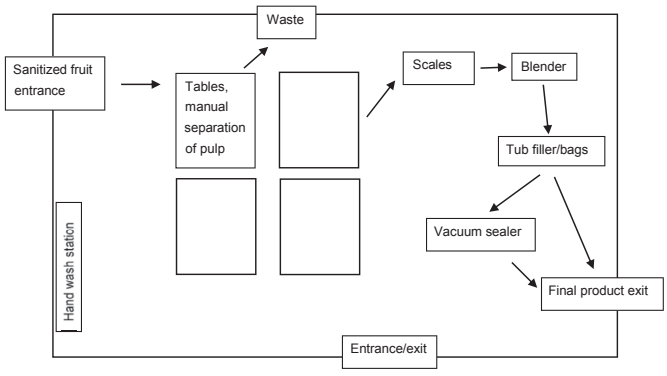

Fig. 1. Schematic layout of the Hygiene Box. Arrows indicate the flow of production.

which is used to mix the avocado pulp with dry ingredients such as spices and herbs. If there is cold damage (hardened pieces in the avocado pulp) or callus cells made by insects, the pulp has to be screened by the desinewing machine (Baarder, Germany) to remove these calluses. The guacamole is then either packaged in polyethylene terephthalate tubs and flushed with nitrogen by the tub filler (Molenaar, South Africa) or in polyethylene bags and vacuum sealed. The final product leaves the Hygiene Box by conveyer belt for date coding and freezing. All products are stored at − 18 °C and have a shelf life of 6 days at 4 °C.

2.2. In-house monitoring system for Listeria

The aim of the IMS was to monitor the presence *Listeria* in the facility by identifying harbourage sites and using those results to more effectively sanitize the facility. The IMS was implemented by testing the final products and the processing environment for the presence of *Listeria* (ISO 11290-1, 1996), as well as determining total plate counts (TPC) (ISO 4833, 2003) on the avocado fruit. Since the tests were performed by the facility (therefore in-house monitoring) more samples were tested for *Listeria*, including environmental samples which would otherwise not have been monitored. Additionally, preliminary results were available to processing managers within 48 h.

The final product were tested daily by taking 10 samples (25 g each) in total, representing all the batches produced on that specific day (Table 1). The guacamole samples were diluted in FSO (250 mL) and inoculated in ½ Fraser broth. All samples that changed colour after 24 h were inoculated in Fraser broth and incubated for 24 h. Presumptive positive *Listeria* samples (broth changed colour to black) were streaked onto *Listeria* Selective Agar (Oxoid), Brilliance *Listeria* Agar (Oxoid) and RAPID'L.mono Agar (Bio-Rad). Based on the colony formation on the differential media, samples were recorded as either *Listeria* spp. (all *Listeria* species excluding *L. monocytogenes*) or *L. monocytogenes*.

Contamination levels of *L. monocytogenes* in the product samples were only tested during 2011 and 2012 with ISO 11290-2 (1996). These levels were found to be very low and the facility ceased these tests due to time and economic restraints. However, if it is within a facility's budget, the data obtained from the contamination levels will be very valuable, especially in facilities with higher contamination levels.

In order to evaluate the presence of *Listeria* spp. in the processing environment of the facility, specific objects and areas were categorized as food contact, cross contamination or red areas according to the risk they carry for contamination of the final product. Workers who handle avocado pulp were classified in the food contact group, whereas the washing station in the Hygiene Box was categorized as cross contamination, since there can be indirect contact with the final product through workers. The floors and waste areas were classified as red areas, carrying the highest prevalence of *Listeria*. Depending on the processing schedule, specific objects inside these areas were tested at least three times a week during processing (52 samples per week), as well as post cleaning (38 samples per week). For example, the hands of workers who scoops the pulp out of the skin and the hands of the workers who cuts the avocado were tested during processing, but not post cleaning when they were not actively working. There are some variations in specific objects or areas that were tested, since it was possible to adapt the testing sites based on data from the previous year to focus on problem areas. Additionally, testing were also influenced by external factors in processing such as breakdown of a machine. All samples were taken with sterile swabs (prepared by the facility) on a 25 cm^2 area and were tested similarly to the guacamole samples (Table 2).

Table 1
Prevalence of *Listeria* spp. in product samples tested with ISO 11290-1.

Season	Product samples (n)	*Listeria* spp. other than *L. monocytogenes* (%)	*L. monocytogenes* (%)	Total *Listeria* in the product (%)
2011	718	4.74^{aa}	0.28^{a}	4.99^{aaa}
2012	679	5.60^{aa}	4.71^{b}	10.31^{bbb}
2013	948	3.05^{bb}	8.33^{c}	11.39^{bbb}
2014	1170	0^{cc}	0.17^{d}	0.17^{ccc}

Significant differences are indicated between seasons ($P < 0.05$).

Table 2
Prevalence of *Listeria* spp. in processing environment samples tested with ISO 11290-1.

Season	Processing (n)	*Listeria* spp. detected during processing (%)	Post cleaning (n)	*Listeria* spp. detected post cleaning (%)	Total samples tested (n)	Total *Listeria* found in the facility (%)
2010	1256	17.12^{a}	1003	7.58^{aa}	2259	12.88^{aaa}
2011	982	19.04^{a}	945	7.62^{aa}	1927	13.44^{aaa}
2012	613	4.57^{b}	509	2.16^{bb}	1122	3.48^{bbb}
2013	540	12.96^{c}	467	2.14^{bb}	1007	7.94^{ccc}
2014	942	0.64^{d}	578	1.04^{cc}	1520	0.79^{ddd}

Significant differences are indicated between seasons ($P < 0.05$).

The whole avocado fruit were also tested for the microbial load since this was seen as a possible source of *Listeria* contamination. No *Listeria* cells have been identified from samples taken from the skin of the whole fruit and, therefore, the total plate counts (TPC) on the fruit were determined. Each day that the facility received fruit, 16 green fruits were swabbed (25 cm^2 area) after sanitization to determine the microbial load of cleaned fruit entering the facility. These fruits included different cultivars and were produced on different farms. After incubation in the ripening rooms and a second sanitation, the TPC of the ripe fruit were again determined by swabbing a total of 28 fruits during the day (Table 3).

2.3. Statistical analysis

The *Listeria* data is proportional and a Normal distribution was used to determine if there were significant differences between sample proportions at a 95% level of significance. Additionally, specific data points of product and environmental samples were tested during the first and second half of the 2013 season for significant differences (Table 4). Total plate counts from fruit samples were analysed with the assumption that the data followed a Poisson distribution and due to the large sample sizes a t-test was done to compare average TPC from different years. The differences between data points were tested using a 95% level of significance. All calculations were done in Microsoft Excel.

3. Results and discussion

Based on the results from the *Listeria* surveillance tests, several additional control measures and strategic changes in processing were developed to manage the contamination problem (Table 5).

3.1. Listeria spp. in the final product

Enumeration of the listerial cells found in the final product showed almost all of the samples had concentrations of less than 3 cfu/g which is well below the EU regulation. None of the samples had a concentration level higher than 100 cfu/g and only 0.1% of samples had concentrations between 3 and 100 cfu/g in 2012. Determination of contamination levels of *L. monocytogenes* in the product samples were, therefore, ceased after the 2012 season.

The highest incidence of all *Listeria* spp. in the final product, was recorded in 2013 (11.39%; n = 948) after a significant increase since 2011, whereas the lowest prevalence was in 2014 (Fig. 2, Table 1) ($P < 0.05$). Specifically in 2014, only two product samples tested positive for *Listeria* (in this case *L. monocytogenes*) throughout the whole season (0.17%, n = 1170). This is remarkable since the *L. monocytogenes* prevalence increased significantly from 0.28% (n = 718) in 2011 to 4.71% (n = 679) in 2012 and finally 8.33% (n = 948) in 2013 ($P < 0.05$). The prevalence of *L. monocytogenes* strains in the final product, therefore, seemed unaffected by changes made in the facility. However, *Listeria* spp. other than *L. monocytogenes*, remained constant between 2011 and 2012 and decreased in 2013, until none were identified in product samples, tested during 2014 (Fig. 2, Table 1). This indicates that for *Listeria* spp. other than *L. monocytogenes*, changes inside the facility had an effect after 2012 (Table 5). One such change was to lower the ambient temperature inside the facility from 20° to 16–14 °C. Another change in the facility was to restrict movement of workers who were responsible for cleaning the Hygiene Box. These workers had contact with high risk areas, such as the floors and were not allowed to touch any food contact object such as the tables and buckets intended for transport of avocado pulp. Likewise, workers who had food contact were not allowed to touch waste disposal or

Table 3
Total plate counts on fruit detected with ISO 4833 by swabbing a 25 cm^2 area.

Season	Green fruit (n)	Average TPC on green fruit (cfu/25 cm^2)	Ripe fruit (n)	Average TPC on ripe fruit (cfu/25 cm^2)	Total fruit samples (n)	Average TPC of green and ripe fruit (cfu/25 cm^2)
2010	2286	253.83^{aa}	2003	149.14^{aaa}	4289	201^{a}
2011	613	25.21^{bb}	2613	9.26^{bbb}	3226	17^{b}
2012	1269	15.87^{bb}	3041	9.56^{bbb}	4310	13^{b}
2013	698	57.52^{cc}	2374	55.19^{ccc}	3072	56^{c}
2014	982	9.07^{dd}	2681	4.85^{ddd}	3663	7^{d}
Total	5848		12,712		18,560	

Significant differences are indicated between seasons ($P < 0.05$).

Table 4
Percentage positive samples taken in the facility environment during processing in the season of 2013.

Area		Weeks 18–29 (%)	Weeks 30–38 (%)
Food contact	Table[a*]	24.07	0
	Used buckets inside[b*]	25	0
	Filling hands[c]	7.40	0
	Scooping hands[d]	12.5	0
	Cutting hands[e]	11.11	0
	Tub filler pistons/nozzles	28.57	14.28
	Mixing hands[f]	25	0
Cross contamination areas	Hand soap container	12.5	0
	Hand wash basin	37.03	22.22
	Hand spray bottles	4.76	0
	Scale	64.28	28.57
	Aprons[*]	62.5	24.07
	Vacuum sealer	12.5	0
	Hanging hosepipe[g]	8.33	0
	Table below scale	25	0
	Tub holder[h]	12.5	0
	Drain pipe from hand wash basin	NT	33.33
Red areas	Floor 1A	0	25
	Floor 4A[*]	62.5	0
	Bucket (washing floor)	83.33	44.44

NT = not tested.
[*] Significant difference in positive samples between weeks 18–29 and 30–38 ($P < 0.05$).
[a] Table: tables where avocados are manually cut and pulp is separated from waste.
[b] Used buckets inside: buckets used to transport avocado pulp from tables to the blender.
[c] Filling hands: hands of workers who fill the buckets with avocado pulp.
[d] Scooping hands: hands of workers who scoop avocado pulp out of the avocado skin.
[e] Cutting hands: hands of workers who cut avocados open.
[f] Mixing hands: hands of workers who add avocado pulp to blender for mixing.
[g] Hanging hosepipe: A hosepipe that is hanging from the ceiling and used during cleaning.
[h] Tub holder: Part of the tub filler, where the plastic tubs are situated during filling with guacamole.

Table 5
Strategic changes made in the operational procedures of the facility based on the results of the IMS.

New policy or protocol	Date	Description
Fruit receiving	2009	Previously fruit were washed in a hot water bath (82–90 °C) but this made the fruit difficult to handle and influenced the quality of the avocado pulp. Fruit receiving was therefore changed to include a wash step before sanitation, during which the fruit was washed with a detergent and rinsed with clean water.
Environmental *Listeria* tests	2009	The environment of the processing facility was tested to identify areas or harbourage sites of *Listeria* spp. and to focus cleaning of the facility.
Microbial Testing Program	2010	The facility was divided into three main testing areas namely, food contact, cross contamination and red areas. Surfaces, equipment and workers falling in these areas were tested three times a week during processing and after cleaning.
Identification of the Hygiene Box	2010	The area where avocado pulp is handled was classified as a high care area and named the Hygiene Box. To control cross contamination the Hygiene Box was physically separated from the rest of the facility, first only by a line and later by a low wall.
Ambient temperature of the Hygiene Box	2012	The ambient temperature of the facility was lowered from 20 °C to 16–14 °C.
Colour codes for workers inside the Hygiene Box	2012	Cleaners in the Hygiene Box got red uniforms to visually separate them from personnel who handle the avocado pulp (white uniforms).
Construction of a new canteen	2013	This involved new changing and washing rooms, as well as a new entrance to the Hygiene Box, separated from the entrance to the red area. These new rooms and entrances decreased contamination from outside and enhanced control over the Hygiene Box.
Aprons	Mid-season 2013	After high cross contamination results in 2013 all the workers in the Hygiene Box were supplied with disposable aprons which was changed every 2 h. Previously they had aprons that were washed every 2 h at the washing basin in the Hygiene Box.
Fruit receiving supervisor	Mid-season 2013	A supervisor was stationed outside the facility at fruit receiving in order to maintain strict sanitation protocols, especially during peak processing when there is pressure to process fruit as soon as possible.
New sanitation protocols	2014	Bigger baths were constructed for the sanitation of the fruits on entering the facility and the Hygiene Box. This enabled a double sanitation period (10 min).
Walls of the Hygiene Box	2014	The Hygiene Box was completely enclosed with only one opening coming directly from the high risk boot captive and openings for the conveyer belts and waste disposal window.

cleaning items. However, the *L. monocytogenes* population was not influenced by these changes and significantly increased in the product ($P < 0.05$). It is, therefore, possible that some *L. monocytogenes* strains introduced into the facility in 2011, were deposited in a harbourage site in the food contact or cross contamination areas, from where the strains could continuously contaminate the avocado pulp.

Interestingly, weekly data of the total amount of samples that tested positive for *Listeria* spp. in 2013, showed a gradual decrease from week 30, just over halfway through the season. There is a significant difference between the total amount of samples that tested positive for *L. monocytogenes* from weeks 18–29 (12.70%, n = 488) and from weeks 30–38 (3.70%, n = 460) ($P < 0.05$). From week 35 in the 2013 season to the end of the season (4 weeks in

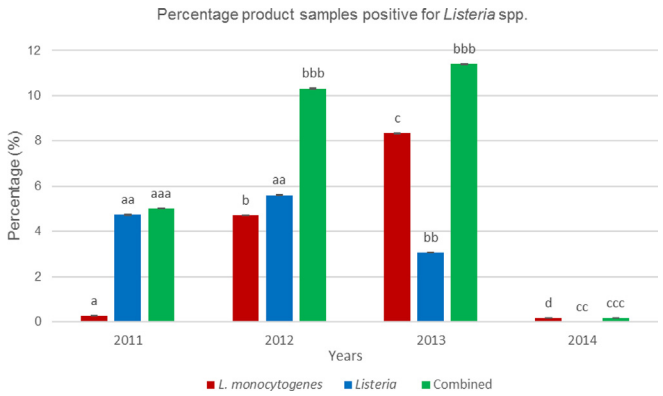

Fig. 2. Percentage product samples positive for *Listeria* spp. *Listeria* include all *Listeria* spp. except *L. monocytogenes*. Combined includes all *Listeria* spp. Significant difference is indicated between years ($P < 0.05$).

total) no samples tested positive for either *L. monocytogenes* or any other *Listeria* spp. This indicated that the changes made in the facility in the 2013 season had a significant effect on the entire *Listeria* population, including *L. monocytogenes*. These changes also had an influence on the *Listeria* spp. prevalent in the processing facility.

3.2. *Listeria spp. in the environment*

Problem areas inside the facility were easily identified with the results from the IMS. Objects that always tested positive for *Listeria* from seasons 2010 to 2013 included the tables, buckets and worker hands in the food contact areas (Table 6). In the cross contamination areas the hand wash basin and hand spray bottles were always contaminated with *Listeria*, whereas the aprons showed high prevalence of *Listeria* in 2013 as 43% (during the whole season) of samples (n = 34) tested positive. In the red areas the floors often tested positive for *L. monocytogenes*, especially the areas designated as F1A and F4A, which always tested positive for *Listeria* from seasons 2010 to 2013. F1A and F4A are located in places where the avocado pulp can easily be spread to the floor and, therefore, these areas can act as harbourage sites for listerial cells. Floors and drains have previously been reported to be major sites of *Listeria* contamination in different processing plants (Barancelli et al., 2014; Fallah, Saei-Dehkordi, & Mahzounieh, 2013; Jami, Ghanbari, Zunabovic, Domig, & Kneifel, 2014). Additionally, equipment and personnel have also been implicated in *L. monocytogenes* contamination, emphasizing the need for close monitoring of the bacterial population in a specific facility to prevent cross contamination of the final product (Strydom & Witthuhn, 2015).

The highest incidence of *Listeria* in the facility was in 2011 (which did not significantly differ from 2010) at 13.44% (n = 1927) and the lowest in 2014 (0.79%, n = 1520) (Fig. 3, Table 2). Data from the environmental swabs showed a significant decrease in the percentage swabs that tested positive for *Listeria* from 2011 to 2012, but a significant increase from 2012 to 2013 and finally a decrease from 2013 to 2014 ($P < 0.05$). During 2012, the results from the IMS were immediately reported to the supervisors who were then able to inform all the personnel in the facility about problem areas. Additionally, all the electrical boxes were removed from the Hygiene Box to decrease the amount of workers that have to enter this high care area. Personnel working in the Hygiene Box were also given colour coded uniforms to separate cleaners (red uniforms) from workers who handle the avocado pulp (white uniforms) (Table 4). To avoid cross contamination the cleaners were prohibited from touching any area in the Hygiene Box where the avocados are handled. The decrease in *Listeria* contamination from in the 2012 season can, therefore, possibly be attributed to avoiding cross contamination, as well as cleaning problem areas immediately after they were identified with the IMS.

Nevertheless, these results could not be sustained and a major increase in *Listeria* prevalence was seen in 2013 from the second week of the season. The increase was observed only during processing as post cleaning results did not differ significantly from the 2012 season. The cleaning regimes was, therefore, sufficient to keep the contamination levels (post cleaning) low, but there seemed to be an influx of listerial cells or an increase in cross contamination during processing that had a significant effect on the amount of environmental samples that tested positive for *Listeria* spp. ($P < 0.05$). The weekly data for 2013 (Fig. 4) indicate that until almost mid-season the processing results were very high and then decreased rapidly from week 29 together with a slighter, but still significant decrease in the post cleaning results ($P < 0.05$). Even more important, this decrease in *Listeria* counts were maintained during the whole 2014 season and reflected in the product samples of 2014. Clearly control strategies applied during the 2013 season had a major influence on the *Listeria* prevalence in the facility, as well as contamination of the final products.

These control strategies included a supervisor at fruit receiving whose responsibility it was to assure that the incoming fruit were submitted to the total sanitation period since pressure to process as much fruit as possible during a shift becomes more when more fruit is delivered to the facility. A new entrance to the Hygiene Box, separate from the entrance to other parts of the facility, were installed, enabling more control over contamination through workers from outside the facility. Additionally, the use of disposable aprons as opposed to aprons that were washed during processing at the wash basin in the Hygiene Box, were initiated (Table 5). These aprons were a critical cross contamination point because they had contact with the table surfaces where avocado pulp was separated from the waste, as well as the washing station that was used by the workers in the Hygiene Box. A likely route of contamination inside the Hygiene Box would have started at a harbourage site such as the hand wash station where workers wash their hands and plastic aprons before these were changed to disposable aprons. The 'washed' plastic aprons would carry listerial cells over to the tables where avocado pulp is separated from the waste. Contaminated pulp carries listerial cells to machinery such as the Baarder and tub filler, as well as the scale table where the pulp is manually allocated in plastic bags and then vacuum sealed. If the original harbourage site is not sufficiently sanitized and the cross contamination vehicle, in this case the aprons, not removed from the processing line, the bacteria will persist in the facility.

Weekly results of the positive samples taken during processing in the season of 2013 show a decrease from week 29 (Fig. 4). When the season of 2013 is divided in two and the amount of samples tested from weeks 18–29 are compared to weeks 30–38, a significant decrease can be seen ($P < 0.05$) (Table 4). In the first half of the season the highest positive samples were isolated from tables, the tub filler, worker hands, the hand soap container, the hand wash basin, the scale, aprons, floors and waste buckets (Table 2). These are all located throughout the whole facility, including the food contact areas in the Hygiene Box. In the second half of the season only the tub filler, hand wash basin, drain pipe leading from the hand wash basin, scale and aprons still tested positive for *Listeria* spp., as well as the floor and buckets for washing the floor in the red areas. In 2014 only samples taken from the red areas were positive for *Listeria* spp. (Table 6). The aprons showed a significant decrease in *Listeria* prevalence from the first half of the 2013 season to the second half ($P < 0.05$) (Table 4). Additionally, no *Listeria* was found on either the aprons or in the washing station during the 2014 season, supporting the suggestion that they were important cross

Table 6
Noteworthy environmental samples that tested positive for *Listeria* spp.

Object/area	2010 (%)		2011 (%)		2012 (%)		2013 (%)		2014 (%)	
	Processing	Post cleaning	Processing	Post cleaning	Processing	Post cleaning	Processing	Post cleaning	Processing	Post cleaning
Table[a]	12.06	*	*	*	4.1	*	11.76	0	0	0
Filling hands[b]	6.25	*	3.23	*	0	*	5.88	*	0	*
Scooping hands[c]	14	*	*	*	2.17	*	6.25	*	0	*
Cutting hands[d]	12.12	*	*	*	0	*	3.13	*	0	*
Tub filler pistons/nozzles	*	0	15.4	0	0	0	22.22	0	0	0
Tub filler holding tank	14.3	0	*	0	*	0	*	*	*	0
Tub filler controls	14.29	5.26	*	5.56	0	0	*	0	*	0
Cleaned buckets inside	4.94	0	50	0	0	2.44	0	2.70	0	0
Mixing hands[e]	12.5	*	12.5	*	0	*	15.79	*	0	*
Hand soap container	7.14	0	0	0	0	3.57	5.88	0	0	0
Hand wash basin	12.76	1.54	13.95	1.72	5.56	0	28.57	0	0	0
Hand spray bottles	11.29	1.89	11.54	2.04	5.56	3.03	3.03	2.44	0	0
Scale	25	0	25	0	0	0	43.75	0	0	0
Bucket stand at scale	27.58	*	32	*	0	*	0	*	*	*
Aprons	44.44	2.94	44.4	3.2	0	*	42.85	3.45	0	0
Vacuum sealer	*	7.14	*	7.69	0	15.38	3.23	0	0	0
Supervisor's hands	6.67	*	*	*	0	*	0	*	0	*
Hanging hosepipe[f]	0	0	0	0	0	0	6.06	0	0	0
Table below scale	*	13.33	*	8.3	*	0	11.11	0	0	0
Blender controls	22.22	0	22.2	0	*	0	*	0	*	0
Floor 1	52.38	17.65	53.3	14.29	50	0	20	8.33	50	6.25
Floor 2	26.3	22.2	33.3	18.18	0	0	*	0	12.5	10.53
Floor 3	50	9.09	62.5	5	50	20	*	0	50	0
Floor 4	46.15	22.22	66.7	20	100	25	37.5	50	0	0
Floor 5	43.75	5	50	5.26	50	0	*	*	0	9.52
Bucket (washing floor)	*	25	*	21.43	80	28.57	60	9.09	*	*
Waste bin	54.35	2.77	60	3.45	*	0	*	0	*	*
Floor (BT)	50	15.15	*	17.24	0	0	0	0	7.14	0

*: not tested.
BT: Boot captive: room where shoes are exchanged for sanitized boots used only in the facility.
[a] Table: Tables where avocados are manually cut and pulp is separated from waste.
[b] Filling hands: Hands of workers who fill the buckets with avocado pulp.
[c] Scooping hands: Hands of workers who scoop avocado pulp out of the avocado skin.
[d] Cutting hands: Hands of workers who cut avocados open.
[e] Mixing hands: Hands of workers who add avocado pulp to blender for mixing.
[f] Hanging hosepipe: A hosepipe that is hanging from the ceiling and used during cleaning.

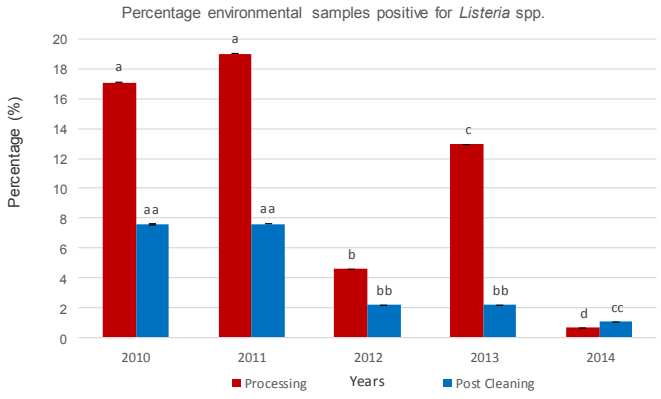

Fig. 3. Percentage environmental samples positive for *Listeria* spp. Samples were taken during processing and post cleaning. Significant difference is indicated between years ($P < 0.05$).

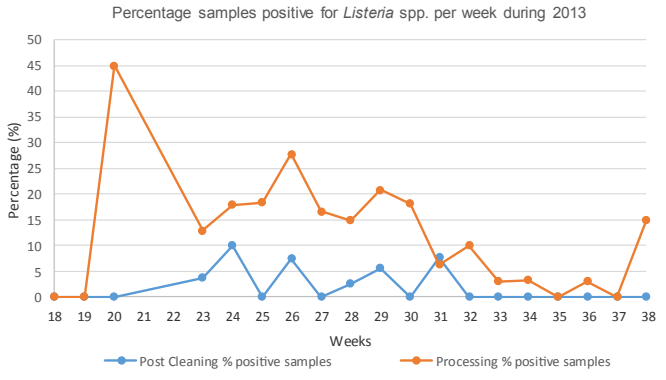

Fig. 4. Percentage samples positive for *Listeria* spp. taken during processing and post cleaning in the season of 2013. Weeks 18–29 had significantly more positive samples than weeks 30–38 ($P < 0.05$).

contamination problems during the 2013 season (Table 6).

3.3. Total plate counts on avocado fruit

From 2010 to 2011 there was a significant decrease in the average TPC (green and ripe fruit) of 201 cfu/25 cm^2 to 17 cfu/25 cm^2 on the fruit ($P < 0.05$) (Fig. 5, Table 3). From 2011 the TPC stayed constant throughout the season in 2012 (13 cfu/25 cm^2) but increased in 2013 to 56 cfu/25 cm^2 and then decreased again in 2014 to 7 cfu/25 cm^2, which were the lowest counts yet. These results suggests that the TPC on the fruit seems to be directly influenced by sanitation protocols used. The counts stayed constant in 2012, but when a different sanitizer was used in 2013, the TPC increased. Switching back to the previous sanitizer, the counts dropped in 2014 to the lowest count observed over the five years. The new sanitation baths, which were installed in 2014 and allowed for a longer sanitation period, might also have a significant influence on the TPC. Additionally, it is possible that the fruit receiving supervisor had an influence on the TPC since he had to specifically

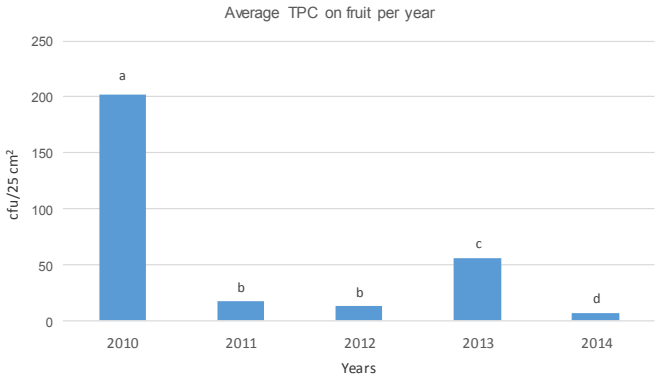

Fig. 5. Total plate counts detected on whole fruit per year. Bars indicate the average TPC of green and ripe fruit. Significant differences are indicated between years.

monitor the sanitation process of the green fruit (Table 5).

3.4. Combined analysis

Comparing the results of the fruit and environmental swabs, as well as the final product samples, it is clearly shown that the amount of *Listeria* found in the product and environment of the facility does not correlate with the TPC on the fruit. In fact, only the 2013 season showed a significant increase in *Listeria* prevalence in the product and environment, as well as the TPC on the whole fruit. Since swabs taken from the whole avocado fruit have never tested positive for *Listeria*, it is impossible to confirm this as the source of contamination. However, it cannot be ruled out since over 10,000 fruits are processed per day and it is very difficult to test a reasonable sample size that would represent that amount of fruit during the season. The facility tests at the peak of a season 44 fruits per day from both the green and ripe fruit which amounts to only about 0.44% of fruit processed on that specific day. This is a good illustration of how the IMS should be adapted to what is not only necessary for individual facilities, but also feasible.

In a previous study, typing of 170 *L. monocytogenes* strains isolated from this facility showed that it was not possible to molecularly identify the source of contamination (Strydom, Bester, Cameron, Franz, & Witthuhn, 2013) and that cross contamination played a role in the prevalence in this facility. The results presented here corroborates cross contamination as a problem in this facility and the fluctuation in *Listeria* spp. during five years sheds some light on the routes of contamination. Transfer of listerial cells between aprons and the hand wash station in the Hygiene Box ceased when the workers started to use disposable aprons. Additionally, contamination of the scale and hands of workers also decreased. Surveillance of a pig slaughterhouse and processing facility also reported that reduced contamination pathways was probably the most important factor in reduction of persistent *L. monocytogenes* strains. Although complete eradication was not achieved after 3 years in the slaughterhouse, there was a decrease of products samples that tested positive for *L. monocytogenes* (Ortiz et al., 2010).

The most important observation is that with the results of the IMS, the specific problem areas can be identified almost immediately, enabling focused cleaning and problem solving, which clearly results in successful management of *Listeria*. This provides evidence supporting literature where GMP were proposed to be the most important prophylactic measures to avoid *L. monocytogenes* contamination in cold meat cuts (Nesbakken & Caugantb, 1996). However, quality control systems such as HACCP is only as effective as the education and discipline of the workers that have to implement it. For this specific facility it took two seasons before the IMS was implemented (additional to the HACCP system) and only in another four seasons almost complete eradication of *Listeria* was accomplished. During this period the Quality Control manager had to educate herself, as well as supervisors and workers on *Listeria*, sample collection and microbiological testing. Additionally, personnel had to be educated on principles of bacterial cross contamination and different cleaning regimes. One example is to encourage minimal use of water during cleaning and explaining why hose pipes that cause water spray will transfer bacterial cells from the floor to tables or machinery. This particular facility clearly managed and almost eradicated their *Listeria* contamination problem by implementing an IMS, educating their workers and adjusting their processing based on the results from the IMS.

4. Conclusion

The success of this company in controlling *Listeria* spp. can be attributed to many different factors, from working closely with researchers, as well as related industry partners, to dealing with the unique problems that the facility was facing. The results of the *Listeria* counts of 2014 show the possibility of control over these pathogens, something that is believed to be unmanageable. Since a major problem in the facility was cross contamination, the key was to understand when and why a change was effective. However, the source of contamination is also an important factor to consider when dealing with *Listeria* and it would certainly be preferable to successfully identify the origin of contamination. The IMS should, therefore, be dynamic and adapted to the processing conditions and unique problems that individual facilities deal with. Contamination of any facility is an environmental problem and can occur from any source – whether it is a worker who forgot to clean his boots before entering a high risk area or entrance of bacterial cells with raw material and subsequent colonisation of a harbourage site. Therefore, it is important to constantly monitor the processing environment, and not only the final product in order to control contamination. It is clear that understanding the routes of cross contamination in a food processing facility will enable food manufacturers to effectively apply HACCP protocols and enhance quality control. The IMS results provides contamination information on site that can be used to make new strategic changes which in turn aid in the management of *Listeria* spp.

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Chapter 6

Conclusion and recommendations

The difficulty in controlling *L. monocytogenes* in food products and food processing environments have been attributed to characteristics such as biofilm formation and the ability to grow at refrigeration temperatures. Other opinions are that cross contamination and harbourage sites inside processing facilities play a major role in the persistence of these bacteria in food products. What is clear is that quality control systems are not sufficient to limit the persistence of *L. monocytogenes* in order to adhere to the microbial standards set for food products. Therefore, additional methods must be explored to aid in the control of these pathogens. In this thesis two approaches in controlling *L. monocytogenes* strains in a local avocado processing facility were investigated.

Bacteriophage biocontrol of pathogenic bacteria has gained interest in the last decade and *L. monocytogenes* is one of the most studied Gram+ bacteria as a target for biocontrol. The first commercial phage product awarded Generally Regarded As Safe (GRAS) status was a *Listeria*-targeted product, Listex^{TM}. Our first approach was, therefore, to assess if the phage product Listex^{TM} can be used for biocontrol of the *L. monocytogenes* strains isolated from the avocado processing facility. Host range analysis indicated that only 26.7% of the *L. monocytogenes* strains were susceptible to this bacteriophage. This was far lower than expected since higher percentages are reported in literature. Additionally, it was not possible to isolate a novel lytic bacteriophage from the facility. Although other biocontrol reports are very promising, reviewers warn that practical implementation of a bacteriophage treatment will be more complex. Results of this study indicate that successful bacteriophage treatment is not guaranteed when a commercial product such as Listex^{TM} P100 is implemented in a specific food processing facility.

The second approach to control *L. monocytogenes* in the avocado processing facility was to define and implement an In-house Monitoring System additional to the quality control program. The monitoring system consisted of regular monitoring of the *Listeria* population in the processing environment, as well as in the final products. The

data obtained were used to modify processing conditions to counter cross contamination in the facility. With this approach, very successful results were obtained and *L. monocytogenes* was almost eradicated from the facility in the 2014 season.

All the results in this thesis indicate that the cause of *L. monocytogenes* in the facility was due to cross contamination. Therefore, when the processing conditions were changed to counter cross contamination, a drastic decrease in the prevalence of *L. monocytogenes* was observed. Although bacteriophage biocontrol with Listex^{TM} P100 was not effective in this facility, it cannot be concluded that this will be the case for other facilities. Control of *L*. *monocytogenes,* however, will only be effective if the processing conditions counter cross contamination and transfer of bacterial cells from low risk areas to high risk areas, where the food product is exposed.

Every food processing facility can be seen as an unique ecosystem of resident microorganisms, influenced by the processing protocols of that specific facility. Control of pathogenic bacteria in a specific food processing facility should, therefore be implemented as a unique system, in a specific facility. Food manufacturers should use quality control programs as baseline instructions to which more specific control measures are added. It is recommended that analysis of the targeted bacteria in a facility should be done before additional control strategies are implemented. This will provide valuable data on prevalence, such as harbourage sites and genetic variability in the resident population. Control measures, additional to those called for in quality control programs, should be monitored.

Bacteriophage biocontrol, however promising, should be closely monitored in food processing facilities. Firstly, to ensure that the desired effect is accomplished and secondly, to determine if resistant bacterial strains are surviving in which the biocontrol would actually select for resistant strains that would populate and the facility. Research regarding the use of bacteriophages as biocontrol agents should be expanded to whole bacterial populations. Furthermore, activity of phages should be specifically tested in food products under circumstances representing real life conditions.

Appendix A

Author Contributions

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1. Chapter 2: ***Listeria monocytogenes*: A Target for Bacteriophage Biocontrol**

Strydom, A., & Witthuhn, C. R. (2015). *Listeria monocytogenes* : A Target for Bacteriophage Biocontrol. *Comprehensive Reviews in Food Science and Food Safety*, *14*, 649–704.

AS: research, writing and editing of the manuscript

CRW: editing

Peer review: editing, suggest extension of *L. monocytogenes* in the food industry

2. Chapter 3: **Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE**

Strydom, A., Bester, I. M., Cameron, M., Franz, C. M. A. P., & Witthuhn, R. C. (2013). Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE. *Food Control*, *31*(2), 274–279.

AS: main experimental work and analysis of the data, writing and editing of the manuscript

IMB: initial PCR-RFLP and PFGE analyses (2007-2009)

MC: editing

CMAPF: PFGE data analysis

RCW: editing

Peer review: editing

3. Chapter 5: **Successful management of *Listeria* spp. in an avocado processing facility**

Strydom, A., Vorster, R. Gouws, P. A. & Witthuhn, R. C. (2015). Successful management of *Listeria* spp. in an avocado processing facility. *Food Control*, *62*, 208–215.

AS: sample collection, data analysis, writing and editing of the manuscript

RV: sample collection, editing

PAG: editing

RCW: editing

Peer review: editing

Appendix B

List of *L. monocytogenes* strains

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE[a] number	PFGE[a] group	Serotype[b]	P100[b]
1	L01	2008	Product	1	I	1/2 c	-
2	L02	2008	Product	2	I	1/2 c	-
3	L04	2008	Product	3	I	N/T	x
4	L05	2008	Unknown	4	II	1/2 a	-
5	L07	2008	Product	5	II	1/2 a	Lysogenic
6	L08	2008	Product	6	II	1/2 c	-
7	L10	2008	Product	8	II	1/2 a	Lysogenic
8	L11	2008	Product	9	II	N/T	-
9	L12	2008	Product	10	II	N/T	-
10	L13	2008	Product	11	II	1/2 a	-
11	L14	2008	Product	12	II	1/2 a	-
12	L15	2008	Product	13	II	1/2 c	Lysogenic
13	L17	2008	Ribbon Blender	15	II	1/2 a	x
14	L19	2008	Product	17	II	1/2 a	-
15	L21	2008	Product	19	II	1/2 a	-
16	L22	2008	Product	20	II	1/2 a	-
17	L23	2008	Product	21	II	1/2 a	Lysogenic
18	L25	2008	Product	22	II	4	-
19	L27	2008	Product	24	II	1/2 a	-
20	L28	2008	Product	25	II	1/2 a	Lysogenic
21	L29	2008	Product	26	II	1/2 c	-
22	L30	2008	Product	27	II	1/2 c	-
23	L31	2008	Product	28	II	N/T	-
24	L32	2008	Product	29	II	1/2 a	-
25	L34	2008	Fruit swab	31	II	1/2 a	-
26	L35	2008	Fruit swab	32	II	1/2 a	-
27	L36	2008	Fruit swab	33	II	1/2 a	Lysogenic
28	L37	2008	Product	34	II	1/2 a	x
29	L38	2008	Drain	35	I	1/2 c	-
30	L40	2008	Product	37	II	1/2 c	-
31	L43	2008	Product	40	II	1/2 a	Unknown
32	#5 IMB	2009	Drain	43	II	1/2 a	x
33	#8 IMB	2009	Unknown	N/A	N/T	4	x
34	104(194/10)	2009	Product	N/A	N/T	N/T	-
35	118(197/10)	2009	Product	N/A	N/T	N/T	-
36	140(202/10)	2009	Product	N/A	N/T	N/T	Lysogenic
37	141.193(16)	2009	Product	N/A	N/T	1/2 c	-
38	142(193/10)	2009	Product	N/A	N/T	N/T	-
39	205.10(3132)	2009	Product	N/A	N/T	1/2 a	-
40	Ap 12074 159/09 (16)	2009	Product	N/A	N/T	N/T	-

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE number	PFGE group	Serotype	P100
41	Ap 12074 160.09(19)	2009	Product	N/A	N/T	1/2 c	x
42	Ls-20	2009	Unknown	N/A	N/T	N/T	x
43	Ls-20	2009	Unknown	N/A	N/T	N/T	x
44	Ls-27	2009	Unknown	N/A	N/T	N/T	x
45	Ls-28	2009	Unknown	N/A	N/T	N/T	-
46	Ls-7	2009	Unknown	N/A	N/T	N/T	-
47	M01	2009	Unknown	N/A	N/T	1/2 a	x
48	M02	2009	Unknown	N/A	N/T	1/2 a	x
49	M03	2009	Unknown	N/A	N/T	N/T	x
50	M04	2009	Unknown	N/A	N/T	N/T	x
51	M05	2009	Unknown	N/A	N/T	N/T	-
52	M06	2009	Unknown	N/A	N/T	1/2 a	x
53	M07	2009	Unknown	N/A	N/T	*Listeria*	x
54	M08	2009	Unknown	N/A	N/T	1/2 c	-
55	M10	2009	Unknown	N/A	N/T	1/2 c	x
56	M11	2009	product	46	II	N/T	-
57	M12	2009	Unknown	N/A	N/T	N/T	-
58	M13	2009	Unknown	N/A	N/T	N/T	-
59	M14	2009	platform buckets	47	II	1/2 a	-
60	M15	2009	Unknown	N/A	N/T	1/2 c	x
61	M16	2009	Unknown	N/A	N/T	1/2 a	-
62	M17	2009	Unknown	N/A	N/T	N/T	-
63	M18	2009	Unknown	ND	N/T	N/T	x
64	M19	2009	product	48	I	1/2 c	-
65	M20	2009	Unknown	N/A	N/T	1/2 c	x
66	M21	2009	Platform bottom	N/A	N/T	N/T	-
67	M22	2009	Floor store room	N/A	N/T	N/T	-
68	M23	2009	platform bottom bucket	49	I	N/T	-
69	M24	2009	product	50	I	N/T	-
70	M25	2009	platform buckets	51	I	N/T	-
71	M26	2009	platform bottom	52	I	N/T	-
72	M27	2009	floor blast freezer	53	II	N/T	Lysogenic
73	M28	2009	Floor store room	54	II	N/T	x
74	M29	2009	floor blast freezer	55	II	N/T	-
75	M30	2009	Unknown	N/A	N/T	N/T	-
76	M36	2009	platform bottom	57	II	1/2 a	-
77	M38	2009	table	58	II	N/T	-
78	M43	2009	Unknown	N/A	N/T	1/2 c	x
79	M44	2009	product	59	II	1/2 a	-

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE number	PFGE group	Serotype	P100
80	M45	2009	Unknown	N/A	N/T	1/2 c	x
81	M46	2009	product	60	II	1/2 a	-
82	M48	2009	Unknown	N/A	N/T	1/2 c	x
83	M51	2009	store room floor	N/A	N/T	1/2 a	-
84	M53	2009	Unknown	N/A	N/T	1/2 a	-
85	M54	2009	Unknown	N/A	N/T	1/2 a	-
86	M60	2009	Unknown	N/A	N/T	1/2 c	x
87	M61	2009	Unknown	N/A	N/T	1/2 a	-
88	M63	2009	Unknown	N/A	N/T	N/T	x
89	M64	2009	table	63	II	1/2 a	x
90	M65	2009	Unknown	N/A	N/T	1/2 a	x
91	M65	2009	product	64	II	1/2 a	x
92	M66	2009	Unknown	N/A	N/T	1/2 a	-
93	M67	2009	Unknown	N/A	N/T	1/2 c	x
94	M73	2009	Unknown	N/A	N/T	1/2 a	x
95	M75	2009	Unknown	N/A	N/T	1/2 a	x
96	M77	2009	Unknown	N/A	N/T	*Listeria*	x
97	M81	2009	Unknown	N/A	N/T	1/2 a	Lysogenic
98	M84	2009	Unknown	N/A	N/T	N/T	x
99	M86	2009	Unknown	N/A	N/T	1/2 c	-
100	M89	2009	Unknown	N/A	N/T	1/2 c	-
101	M93	2009	Unknown	N/A	N/T	1/2 a	-
102	M94	2009	Unknown	N/A	N/T	1/2 a	-
103	M96	2009	Unknown	N/A	N/T	1/2 a	-
104	M98	2009	Unknown	N/A	N/T	1/2 c	-
105	P104	2010	Product	N/A	N/T	1/2 a	-
106	T001/10	2010	drain	N/A	N/T	1/2 c	-
107	T002/10	2010	Floor	N/A	N/T	1/2 a	-
108	T003/10	2010	Floor	N/A	N/T	*Listeria*	-
109	T004/10	2010	Table leg	N/A	N/T	1/2 a	-
110	T005/10	2010	Tables's leg	N/A	N/T	1/2 c	x
111	T006/10	2010	Boot captive floor low risk area	N/A	N/T	1/2 c	x
112	T008/10	2010	drain boot captive	N/A	N/T	1/2 c	x
113	T009/10	2010	drain	N/A	N/T	1/2 a	-
114	T010/10	2010	drain	N/A	N/T	1/2 a	x
115	T011/10	2010	drain boot captive high risk area	N/A	N/T	1/2 c	x
116	T012/10	2010	tubfiller machine roller	N/A	N/T	1/2 a	x

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE number	PFGE group	Serotype	P100
117	T013	2010	floor below tables	N/A	N/T	N/T	x
118	T014	2010	hose pipe	N/A	N/T	1/2 c	x
119	T015	2010	green hose pipe	N/A	N/T	N/T	x
120	T016	2010	black hosepipes	N/A	N/T	1/2 c	-
121	T019	2010	Unknown	N/A	N/T	1/2 a	-
122	T040	2010	Floor 4A	N/A	N/T	N/T	x
123	T041	2010	Floor 2A	N/A	N/T	N/T	x
124	T042	2010	Floor 3A	N/A	N/T	N/T	-
125	T043	2010	Floor bucket washing	N/A	N/T	N/T	-
126	T044	2010	Floor 2A	N/A	N/T	N/T	-
127	T045	2010	Tables underneath	N/A	N/T	N/T	-
128	T046	2010	Boot captive floor high risk area	N/A	N/T	N/T	-
129	T049	2010	Hand spray bottles	N/A	N/T	N/T	x
130	T050	2010	Tables	N/A	N/T	N/T	-
131	T052	2010	Scooping hands	N/A	N/T	N/T	-
132	T053	2010	Washed clean bucket	N/A	N/T	1/2 c	x
133	T054	2010	waste bins	N/A	N/T	1/2 c	-
134	T058	2010	hand wash basins	83	V	1/2 c	x
135	T060	2010	Tables underneath	N/A	N/T	N/T	Lysogenic
136	T061	2010	Boot captive floor high risk area	N/A	N/T	1/2 a	-
137	T062	2010	waste bins	84	I	N/T	-
138	T064	2010	cryovac hood selection	N/A	N/T	N/T	-
139	T065	2010	Floor 2A	N/A	N/T	*Listeria*	x
140	T066	2010	hand wash basins	N/A	N/T	*Listeria*	-
141	T067	2010	Scooping hands	86	I	N/T	-
142	T068	2010	Unknown	72	I	1/2 a	-
143	T069	2010	Tables underneath	N/A	N/T	N/T	-
144	T070	2010	fruit conveyor controls	N/A	N/T	N/T	-
145	T071 room	2010	floor 1A	N/A	N/T	1/2 a	-
146	T072	2010	printing conveyor	N/A	N/T	N/T	-
147	T073	2010	cryovac inside door	N/A	N/T	1/2 a	-
148	T074	2010	hand spray bottles	N/A	N/T	N/T	-
149	T075	2010	bucket stand (mixing)	N/A	N/T	N/T	-

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE number	PFGE group	Serotype	P100
150	T078	2010	waste remover's hands	N/A	N/T	N/T	-
151	T079	2010	blender controls	N/A	N/T	N/T	x
152	T080	2010	bucket stand scale	N/A	N/T	1/2 a	-
153	T081	2010	hand wash basins	89	I	N/T	-
154	T083	2010	cutting hands	N/A	N/T	N/T	-
155	T084	2010	cleaner (spoons and knives)	N/A	N/T	1/2 a	-
156	T085	2010	bucket stand (mixing)	N/A	N/T	1/2 a	-
157	T086	2010	Tubfiller green strips	N/A	N/T	1/2 a	-
158	T087	2010	Tables underneath	N/A	N/T	1/2 a	-
159	T088	2010	bucket stand scale	N/A	N/T	1/2 a	-
160	T089	2010	waste bins	N/A	N/T	N/T	-
161	T090	2010	store room table and measuring equipment	N/A	N/T	N/T	-
162	T091	2010	hand spray bottles	N/A	N/T	N/T	-
163	T092	2010	printing conveyor	N/A	N/T	N/T	-
164	T094	2010	hand spray botlles	N/A	N/T	*Listeria*	-
165	T095	2010	waste removers hands	N/A	N/T	N/T	-
166	T096	2010	scale	N/A	N/T	1/2 c	Lysogenic
167	T097	2010	Apron	N/A	N/T	N/T	-
168	T098	2010	bucket stand (mixing)	N/A	N/T	1/2 c	x
169	T099	2010	Blast freezer operators	N/A	N/T	N/T	-
170	T100	2010	Waste bins below tables	N/A	N/T	1/2 a	-
171	T102	2010	Table underneath	N/A	N/T	*Listeria*	-
172	T103	2010	black hosepipes	N/A	N/T	N/T	-
173	T104	2010	Floor 4A	N/A	N/T	1/2 a	-
174	T106	2010	Floor 1A	N/A	N/T	*Listeria*	-
175	T107	2010	Tubfiller electric box	N/A	N/T	1/2 a	-
176	T108	2010	Waste bins below tables	N/A	N/T	N/T	-
177	T112	2010	Apron	N/A	N/T	1/2 a	-
178	T118	2010	Blast freezer operators	N/A	N/T	N/T	-
179	T120	2010	Waste bins below tables	N/A	N/T	1/2 a	-
180	T121	2010	Table underneath	N/A	N/T	N/T	-
181	T122	2010	Floor 1A	N/A	N/T	1/2 a	-

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE number	PFGE group	Serotype	P100
182	T123	2010	Waste bins below tables	N/A	N/T	N/T	-
183	T124	2010	Apron	N/A	N/T	1/2 a	-
184	T125	2010	Bucket standing (mixing)	N/A	N/T	1/2 c	x
185	T126	2010	Scale	N/A	N/T	1/2 c	x
186	T127	2010	Unknown	N/A	N/T	N/T	-
187	T128	2010	Tubfiller green strips	N/A	N/T	*Listeria*	-
188	T129	2010	Waste bins below tables	N/A	N/T	1/2 a	-
189	T130	2010	Unknown	N/A	N/T	1/2 a	x
190	T134	2010	Tables underneath	N/A	N/T	1/2 a	-
191	T135	2010	Scale	N/A	N/T	N/T	x
192	T136	2010	Blast freezer operators	N/A	N/T	N/T	-
193	T137	2010	black hosepipes	N/A	N/T	N/T	-
194	T140	2010	Labelling hands	N/A	N/T	1/2 a	-
195	T141	2010	Tubfiller electric box underneath	N/A	N/T	N/T	-
196	T142	2010	Waste bins below tables	N/A	N/T	N/T	x
197	T143	2010	Scale	N/A	N/T	N/T	-
198	T144	2010	Apron	N/A	N/T	N/T	-
199	T146	2010	Blast freezer operators	N/A	N/T	N/T	-
200	T147	2010	mixer's controls	N/A	N/T	N/T	-
201	T149	2010	Tubfiller green strips	N/A	N/T	1/2 a	-
202	T150	2010	vacuum sealer underneath	N/A	N/T	N/T	-
203	T151	2010	tubfiller controls	N/A	N/T	1/2 a	x
204	T152	2010	Tubfiller green strips	99	IV	N/T	-
205	T153	2010	Tubfiller electric box underneath	95	IV	N/T	-
206	T156	2010	buckets on tables (with avo)	N/A	N/T	N/T	-
207	T157	2010	buckets on tables (with avo)	N/A	N/T	N/T	-
208	T158	2010	lids on tables (with avo)	N/A	N/T	N/T	-
209	T159	2010	Floor 1A	N/A	N/T	1/2 a	-
210	T160	2010	lids on tables (with avo)	N/A	N/T	N/T	-
211	T161	2010	Apron	N/A	N/T	N/T	x
212	T162	2010	buckets on tables (with avo)	N/A	N/T	N/T	x
213	T164	2010	waste remover's hands	N/A	N/T	N/T	-
214	T165	2010	Floor 3A	N/A	N/T	1/2 a	-
215	T166	2010	Floor 1A	N/A	N/T	N/T	-

***Listeria monocytogenes* strains and characteristics**

Number	Strain	Date	Source	PFGE number	PFGE group	Serotype	P100
216	T167	2010	Conveyor labeling	N/A	N/T	N/T	-
217	T168	2010	Floor bucket washing	N/A	N/T	N/T	-
218	T169	2010	Door handle (weiging room)	N/A	N/T	N/T	-
219	T170	2010	Floor 3A	N/A	N/T	1/2 a	-
220	T172	2010	Waste bins below table	N/A	N/T	N/T	-
221	T175	2010	waste	N/A	N/T	1/2 a	-
222	T176	2010	Floor 1A	N/A	N/T	N/T	-
223	T177	2010	Waste bins below table	N/A	N/T	N/T	-
224	T178	2010	waste remover's hands	N/A	N/T	1/2 a	-
225	T179	2010	Floor 1A	N/A	N/T	1/2 a	-
226	T180	2010	Floor 3A	N/A	N/T	N/T	-
227	T182	2010	Waste bins below table	N/A	N/T	N/T	x
228	T185	2010	Blast freezer operators	97	V	N/T	-
229	T187	2010	Floor 1A	N/A	N/T	N/T	-
230	T188	2010	Paper towel container (boot captive)	N/A	N/T	1/2 a	x
231	T189	2010	Blast freezer operators	N/A	N/T	N/T	-
232	T191	2010	waste bins below table	N/A	N/T	N/T	Lysogenic
233	T192	2010	Floor 5A	N/A	N/T	N/T	-
234	T196	2010	Floor bucket washing	N/A	N/T	1/2 a	x
235	Zesty	2010	product	N/A	N/T	N/T	x
236	B13	2012	Waste water	N/A	N/T	N/T	-
237	B51	2012	Waste water	N/A	N/T	N/T	x
238	C4	2012	Waste water	N/A	N/T	N/T	-
239	D2	2012	Waste water	N/A	N/T	N/T	-
240	E1	2012	Waste water	N/A	N/T	N/T	x
241	F3	2012	Waste water	N/A	N/T	N/T	x
242	Type	N/A	N/A	65	V	1/2 a	-

a: Chapter 3
b: Chapter 4
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N: negative
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