# Survival and proliferation of *Listeria monocytogenes* in a South African ready-to-eat food factory

by

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# SUMMARY

*Listeria monocytogenes* is a foodborne pathogen that has the ability to survive within a wide range of conditions found within food processing environments. It is the cause of a potentially life-threatening infection, listeriosis. Its presence is of major concern within ready-to-eat food processing environments and food products. Since no further processing or heat treatment is required by the consumer, post production cross contamination thereof should be minimised. Considering the lack of information about *L. monocytogenes* in ready-to-eat (RTE) foods in the South African context, the aim of this study was to study the survival and proliferation thereof in a RTE food factory, situated in the Western Cape, South Africa. Presumptive positive samples in the form of inoculated Rapid'*L.mono* plates (n=434) were collected from the factory's *Listeria* management plan. Visual inspection for characteristic black colonies, provided 64 presumptive positive *L. monocytogenes* species. Polymerase chain reaction protocol was optimised for amplification of target genes *iap* (*Listeria* spp.) and *lmo2334* (*L. monocytogenes*), to differentiate positive species. The Rapid'*L.mono* method was also evaluated for enrichment bias that cause false negatives for *L. monocytogenes* in the presence of *L. innocua*. The method was found to be sufficient for detection of *L. monocytogenes*, if the CFU.g$^{-1}$ of both species were the same prior to enrichment. Isolates were subtyped through automated *Eco*RI ribotyping which was conducted using DuPont RiboPrinter® and identified as, DuPont ID 1038, DuPont ID 1041, DuPont ID 1042, and DuPont ID 18596. These strains were previously implicated in human listeriosis cases and international product recalls. DuPont ID 20243, that was isolated from the RTE factory, has not yet been logged on the global Food Microbe Tracker database. From the 29 ribotypes obtained, nine different DuPont ID's were assigned, which was indicative of the variety of contamination sources within the RTE factory, on par with similar studies conducted. Lineage assignments of *L. monocytogenes* could be made using the DuPont ID's and the RTE factory studied was found to host both lineage I and II strains. The cluster analysis revealed contaminated work boots, trolleys and crates to be possible contamination mechanisms. The response of *L. monocytogenes* biofilms, cultivated under flow conditions, to sanitisers used in the factory environment was evaluated. A protocol was developed using the $CO_2$ evolution measurement system (CEMS) to evaluate the effect of four sanitisers used by the RTE food factory on *L. monocytogenes* biofilms. In a novel approach, it was found, that even though no bactericidal effect occurred by either sanitiser, the QAC free sanitiser resulted in the best eradication of the biofilm. Peracetic acid and QAC based chemicals had no effect on the biofilm, as recovery of *L. monocytogenes* was observed after multiple treatments. The RTE factory was advised to use QAC free chemical sanitisers currently available to manage biofilms, specifically in drains. This study not only created more awareness regarding the complexities of *L. monocytogenes* in the RTE food factory, but also laid the groundwork for further study into the survival and proliferation of *L. monocytogenes* in the RTE environment.

# OPSOMMING

*Listeria monocytogenes* is ‘n voedselverwante patogeen wat die vermoë besit om in ‘n wye reeks toestande gevind in voedselverwerkingsomgewings, te oorleef. Dit is die oorsaak van ‘n potensieël lewensgevaarlike infeksie, listeriose. Die teenwoordigheid daarvan is van groot kommer binne gereed-om-te-eet (RTE) verwerkingsomgewings en voedselprodukte. Aangesien geen verdere verwerking of hittebehandeling benodig word deur die verbruiker nie, moet na-produksie kruiskontaminasie daarvan geminimiseer word. Aangesien daar ‘n tekort aan inligting rakende *L. monocytogenes* in RTE voedselprodukte in die Suid-Afrikaanse konteks is, is die doel van hierdie studie om die oorlewing en verspreiding daarvan in ‘n RTE voedselfabriek in die Wes-Kaap van Suid-Afrika, te ondersoek. Vermoedelike positiewe monsters in die vorm van geïnokuleerde Rapid’*L.mono* plate (n=434) is ingesamel deur middel van die fabriek se *Listeria* bestuursplan. Visuele inspeksie van die kenmerkende swart kolonies het 64 vermoedelike *L. monocytogenes* spesies verskaf. ‘n Polimerase kettingreaksie is geoptimiseer vir die amplifikasie van teikengene iap (*Listeria* spp.) en lmo2334 (*L. monocytogenes*), om positiewe monsters te onderskei. Die Rapid’*L.mono* metode is ook geëvalueer vir verrykingspartydigheid wat vals negatiewes vir *L. monocytogenes* in die teenwoordighied van *L. innocua* veroorsaak. Daar is gevind dat die metode voldoende is vir die opsporing van *L. monocytogenes* mits die KVE.$g^{-1}$ van beide spesies dieselfde was voor verryking. Isolate was gesubtipeer deur *EcoR*I ribotipering wat gedoen is deur die gebruik van die DuPont RiboPrinter. Die isolate is geïdentifiseer as DuPont ID 1038, DuPont ID 1041, DuPont ID 1042, en DuPont ID 18596. Hierdie stamme was voorheen geïmpliseer in menslike listeriose gevalle asook internasionale voedselherroepings. DuPont ID 20243 wat geïsoleer is in OF uit die RTE fabriek is nog nie van tevore aangemeld op die globale “Food Microbe Tracker” databasis nie. Van die 29 ribotipes verkry, was nege verskillende DuPont ID’s aangewys. Hierdie is aanduidend van die verskeidenheid van kontaminasiebronne binne-in die RTE fabriek en dit is in lyn met soortgelyke studies. Linie toekennings van *L. monocytogenes* kon gemaak word deur gebruik te maak van DuPont ID’s. Daar is gevind dat die fabriek wat bestudeer is, beide linie I en II stamme huisves. Die groepsanalise het gewys dat gekontamineerde werksskoene, trollies en kratte moontlike kontaminasiemeganismes was. Die reaksie van *L. monocytogenes* biofilms, gekweek onder vloeikondisies, teenoor saniteermiddels wat in díe fabrieksomgewing gebruik word, is geëvalueer. ‘n Protokol is ontwikkel, deur gebruik te maak van die $CO_2$ Evolusie Metingsisteem (CEMS), om die effek van vier saniteermiddels, gebruik in die RTE voedselfabriek, te evalueer. As eerste van sy soort, is gevind dat al was daar geen bakterieëdodende effek deur enige saniteermiddel nie, het die chemiese saniteermiddels wat geen kwaternêre ammonium samestelling (QAC) bevat het nie, die beste uitwissing van die biofilm veroorsaak. Perasynsuur en QAC-gebaseerde chemikalieë het geen effek op die biofilms gehad nie omdat herstel van *L. monocytogenes* gesien is na verskeie behandelings. Daar is aanbeveel dat die RTE fabriek

gebruik maak van die QAC-vrye chemiese saniteermiddels tans beskikbaar vir die bestuur van biofilms, spesifiek in die dreine. Hierdie studie het nie net meer bewusmaking aangaande die kompleksiteit van *L. monocytogenes* in die RTE voedselfabriek tot gevolg gehad nie, maar het ook die fondasie gelê vir verdere studies aangaande die oorlewing en verspreiding van *L. monocytogenes* in die RTE omgewing.

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“The woods are lovely, dark and deep,

But I have promises to keep,

And miles to go before I sleep,

And miles to go before I sleep”

- Robert Frost

**This thesis is dedicated to:**

My parents,

Herman & Brenda Ackermann

*“I stand tall, because I stand on the shoulders of giants”*

# CONTENTS

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of three research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion, recommendations and conclusions. Language, style and referencing format used are in accordance with the requirements of the International Journal of Food Science and Technology. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

# ABBREVIATIONS

| | |
|---|---|
| BEC | Biofilm eradication concentration |
| CAC | Codex Alimentarius Commission |
| CEMS | $CO_2$ evolution measurement system |
| CFU | colony forming units |
| EC | Epidemic clone |
| eDNA | Extracellular DNA |
| EFSA | European Food Safety Association |
| EPS | Extracellular polymeric substance |
| MPF | Minimally processed food |
| FBO | Food business operator |
| FCS | Food contact surfaces |
| FSIS | Food safety and inspection service |
| HR | High risk area |
| LOD | Level of detection |
| LR | Low risk area |
| MIC | Minimum inhibitory concentration |
| MPF | Minimally processed foods |
| NA | Nutrient Agar |
| NFCS | Non-food contact surfaces |
| OD | Optical density |
| PAA | Peracetic acid |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed field gel electrophoresis |
| POD | Probability of detection |
| RFLP | Restriction fragment length polymorphisms |
| rpm | revolutions per minute |

| | |
|---|---|
| rRNA | Ribosomal RNA |
| RTE | Ready to Eat |
| spp. | Species |
| QA | Quality assurance |
| QAC | Quaternary Ammonium compound |
| QFC | QAC Free chemical |
| USDA | United State Department of Agriculture |

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# CHAPTER 1

# INTRODUCTION

In 2016, 80% of food recall cases in the United States of America were due to *Listeria monocytogenes* contamination (Anonymous, 2017). The total food recalls due to similar reasons in South Africa, were unknown. As a saprotrophic pathogen, *L. monocytogenes* has the ability to survive and proliferate in various conditions associated with food processing environments (Dhama *et al*., 2015; Lokerse *et al*., 2016). *L. monocytogenes* causes a potentially fatal infection, listeriosis. In healthy individuals, it can manifest as self-limiting febrile gastroenteritis. However, vulnerable groups such as pregnant woman, young children, the elderly and immunocompromised individuals are at risk of potentially fatal invasive listeriosis that can cause spontaneous abortions, neo-and peri-natal infections, meningitis and septicaemia (Meloni *et al*., 2009; Dhama *et al*., 2015).

The main vehicle of infection is through the consumption of contaminated food and ready to eat (RTE) products and it is therefore the responsibility of Food Business Operators to ensure that food products are microbiologically safe. However, information regarding product recalls and illness incidences due to contaminated food products are not available to the public in South Africa, with the exception of occasional and fleeting news reports (Scholtz. 2017).

When considering the socio-economic context of South Africa, food safety and the regulation thereof should gain more attention. In 2012, the national estimate for HIV prevalence among the citizens of South Africa was 12%, which showed a statistically significant increase from the 10.6% in 2008 (Shisan *et al.*, 2014). Therefore, in 2012 approximately 6 422 179 people lived with compromised immune systems, which inherently means a large part of the population was susceptible to a fatal listeriosis infection.

Due to pre-and post-production handling conditions, RTE foods are known for their risk of *L. monocytogenes* contamination (Vongkamjan *et al.*, 2013; Nyarko & Donnelly, 2015). RTE foods are defined as "…any food (including beverages) which is normally consumed in its raw state or any food handled, processes, mixed, cooked, or otherwise prepared into a form in which it is normally consumed without further processing" (Foodstuffs, Cosmetics and Disinfectants Act and Regulations, 2010). The microbiological risks associated with RTE foods are due to the fact that no heating or further processing by the consumer is required prior to consumption. Information regarding the South African RTE food sector is extremely limited. The lack of public access to existing documents are reflected in a single market entry report "Ready to eat food industry in South Africa: Analysis of Growth, Trends and Progress (2017-2022)" (Anonymous, 2016), that can only be purchased at a high cost.

The Listeria genus contains genetically heterogenous species (Nyarko & Donnelly, 2015), with only a small fraction of the specie subtypes being associated with food related listeriosis. Yet, a

large amount of resources in the food industry are directed towards the eradication and control of *Listeria* in its processing environments. Testing and detection of foodborne pathogens is a crucial element in risk management and long term control in the food chain (Dalmasso *et al.*, 2014).

The focus of this study was on a RTE food factory situated in the Western Cape, South Africa which was faced with a very familiar challenge: managing the presence and subsequent cross contamination of *L. monocytogenes* in the processing environment. However, without a thorough understanding of the sources, sub-types, contamination mechanisms and effect of sanitation practices, no pro-active management steps could be taken. It was therefore the aim of this study to examine the survival and proliferation of *L. monocytogenes* microflora of this RTE food factory.

Contamination of RTE food products with *L. monocytogenes* will lead to food recalls and possible fatal infection for the South African consumer. Contamination can only be prohibited if the factors driving the contamination as well as the source thereof is identified, as stated in the objectives of this study.

The first objective was to isolate and positively identify *L. monocytogenes* isolates from the factory environment and food products, using conventional and multiplex PCR. Also, to evaluate the reliability of Rapid'*L.mono* chromogenic agar for the routine detection of *L. monocytogenes* in the presence of *L. innocua.*

The second objective was to subtype the isolates from the RTE food factory, through automated ribotyping to examine potential contamination sources and establish contamination mechanism and trends. Furthermore, to compare ribotype data with other similar studies as well as an international database, in order to gain a global perspective of *L. monocytogenes* in food and human clinical isolates.

The final objective was to study the response of *L. monocytogenes* biofilms, cultivated under flow conditions, to sanitisers currently used within the RTE food factory. By achieving this objective, recommendations could be made to adapt the use of current sanitsers for a greater antimicrobial effect.

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# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

The presence of *Listeria monocytogenes* in the food processing environment has been the subject of various research efforts in the last 20 years. Not only as a model gram-positive micro-organism but also as an intelligent pathogen that survives despite numerous control and management efforts. With the increased emergence of antimicrobial resistance in food related pathogens, *L. monocytogenes* has gained increased attention in ready-to-eat (RTE) food products. This review aims to reflect the recent global surge of research into *L. monocytogenes* and the factors related to its presence in the food chain and also the lack of information and research regarding this pathogen within the South African RTE food industry.

The detection of *Listeria* spp. in the food chain and processing environment is not only crucial to ensure that safe foods are provided to the consumer, but it is also a tool that assists in identifying conditions that support the growth and persistence of *Listeria monocytogenes* (Dalmasso & Jordan, 2012; Orsi & Wiedmann, 2016). With the rapid increase in discovery of new *Listeria* spp. (Orsi & Wiedmann, 2016) the need to continually evaluate, improve and expand the current detection methods have become evident (Barre *et al.*, 2016). This refers to, amongst others, the revision of the ISO 11290-1:1996 & 11290-2:1996, to include all *Listeria* spp. (Barre *et al.*, 2016). In 2017, the updated ISO 11290-1:2017 and 11290-2:2017 were made available (Anonymous, 2017a; 2017b). It further refers to the acceleration of research into more rapid detection and identification methods for *L. monocytogenes* and the movement toward whole genome sequencing and metagenomic analysis (Bryant *et al.*, 2014).

## 2.2 Food Safety

Concerns for food safety have increased in recent years with the growing trends of minimally processed food (MPF) (Law *et al.*, 2015a; Wang & Salazar, 2015). Bansal *et al.* (2015) divided minimally processed foods into two groups: plant sourced MPF and animal based MPF. The authors described RTE foods as a combination of these two categories. RTE food requires minimal or no processing by the consumer after it has been produced by the Food Business Operator (FBO) (Foodstuffs, Cosmetics and Disinfectants Act and Regulations, 2010). Thus, there is a lack of a microbial control for post-production contamination, increasing the concern for consumer safety.

These concerns were reflected in research as a study conducted by Vongkamjan *et al.* (2016) found that 7.5% of 200 RTE products were contaminated with *L. monocytogenes*, which is alarmingly high considering *L. monocytogenes'* lowered Probability of Detection (POD) in the presence of

competing *Listeria* spp. (Zitz *et al.*, 2011). Yu and Jiang, (2014) found 6.2% of 954 RTE food products in China to be positive for *L. monocytogenes* with 30.5% of isolates displaying resistance to the antibiotic cefotaxime. A similar study that aimed to detect the prevalence of various food related pathogens in 7 regions in China, found 1.43% of food products tested, positive for *L. monocytogenes* (Yang *et al.*, 2016). Studies with similar scopes have not been conducted in South Africa, only broad based hygiene studies on RTE food street vendors (Mosupye & Von Holy, 2000), roadside cafeterias and retail outlets in the Eastern Cape (Nyenje *et al.*, 2012) have been done.

International regulatory bodies include United States Department of Agriculture (USDA); Food Safety and Inspection Service (FSIS); Food and Drug Association (FDA) (Zunabovic *et al.*, 2011), European Food Safety Authority (EFSA) and its institutions and agencies (Anonymous, 2016). In South Africa, the main stakeholder in food safety policing is the Department of Health, subsequently compelling the food industry to apply international standards and regulations and even in some cases private food safety standards.

In the light of a rapidly growing global market and the food industry's effort to regulate and control food safety, Fagotto (2014) explores the roles that private food safety standards have on these efforts. These private standards come as a response to the need for flexible and relevant frameworks that decrease food safety risks to the consumers, something that public and government regulations have, in some cases, failed to do. Nevertheless, when private standards attempt to supplement government regulations, the issue of transparency and accountability comes into question, since these standards are enforced by third parties (Fagotto, 2014). This trend and its effect is of importance since many major stakeholders within the South African food industry has and will turn to private standards to regulate their food products.

For the global community of producers, consumers and food regulators nationally and internationally, the Codex Alimentarius Commission (CAC) has served as a reference point and guideline for acceptable food safety practices (Luber, 2011). The CAC "Guidelines on the Application of General Principles of Food Hygiene to the Control of *L. monocytogenes* in Ready-To-Eat Foods" and its three annexes aim to reduce the probability of *L. monocytogenes* contamination in RTE food products (Luber, 2011; Anonymous, 2012). This is done by outlining procedures based on risk assessment and subsequent control measures from primary production to consumption. In RTE foods where there is opportunity for growth of *L. monocytogenes* after production, the microbial limit is stated as "Absent in 25g" or "<0.04 CFU.$g^{-1}$" (Anonymous, 2012).

To illustrate the effect and burden that *L. monocytogenes* poses to the global community, De Noordhout *et al.* (2014) established, through systematic and meta-analysis, that in 2010 alone 23 150 illnesses and 5 463 deaths occurred because of listeriosis. It should be noted that these conclusions were made without sufficient data from developing countries since this type of data is still unavailable, as in the case for South Africa (De Noordhout *et al.*, 2014).

## 2.3 *Listeria monocytogenes*

### 2.3.1 The evolution of the genus

To date the *Listeria* genus comprises of 17 species. Orsi and Wiedmann (2016) divide the genus into two distinct groups as these groups relate to *L. monocytogenes*. *Listeria* sensu strictu (*L. monocytogenes, L. seeligeri, L. ivanovii, L. marthii, L. welshimeri* and *L. innocua*) contain all species described before 1985 with the exception of *L. marthii* (described in 2010). *Listeria* sensu lato (*L. grayi, L. fleischmannii, L. cornellensis, L. floridensis, L. aquatica, L. weihenstephanensis, L. newyorkensis, L. rocourtiae, L. grandensis, L. riparia,* and *L. booriae)* contain all species discovered after 2009, with the exception of *L. grayi* (described in 1966) (Orsi & Wiedmann, 2016).

In addition a pattern has emerged where the *Listeria* genus has evolved from facultative pathogen to obligate saprotroph, with the evident disappearance of various virulence factors (Bryant *et al.*, 2014). Whole genome sequencing has revealed that through limited gene acquisition and/or limited gene loss during speciation, this transition took place (Nyarko & Donnelly, 2015). This pattern is further confirmed by *L. monocytogenes* and *L. ivanovii* still being the only pathogenic species, even throughout the discovery and describing of new species.

### 2.3.2 Characterisation of *Listeria monocytogenes*

As a facultative pathogenic saprotroph, forming part of *Firmicutes* group (Den Bakker *et al.*, 2012), *L. monocytogenes* is known as the main pathogenic species of the *Listeria* genus, accounting for human and ruminant illness (Orsi *et al.*, 2011; FDA, 2012; Orsi & Wiedmann, 2016). It is characterised as gram-positive, catalase-positive, oxidase negative, facultative anaerobic, non-spore forming bacillus (Goldfine and Shen, 2007) as well as a low G+C content bacteria (36-42%) (Bécavin *et al.*, 2014). Similar and related low G+C genus include *Clostridium*, *Bacillus*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Khelef *et al*., 2006).

The well adapted saprotrophic nature of *L. monocytogenes* makes it ubiquitous in the environment. It is found in soil, rivers, decaying plant matter and various food products such as meat, fresh produce and dairy (Dhama *et al.*, 2015; Lokerse *et al.*, 2016) as well as other RTE food products (Ferreira *et al.*, 2014). Its ubiquitous nature can be accounted for by the extensive amount of genes in its genome, dedicated towards regulators and transport proteins (Vivant *et al.*, 2013). This comprehensive regulatory capacity is reflected by 7% of the genome being dedicated to regulatory proteins (Buchrieser, Rusniok, Kunst, Cossart, & Glaser, sited by Zunabovic *et al.*, 2011)

Temperature is one of the environmental factors that favours the survival ability of *L. monocytogenes* the most, since this bacteria can grow at temperatures as low as 1°C (Morganti *et al.*, 2015), with tumbling mobility at 25°C and optimal growth at 30 - 37°C (Goldfine & Shen, 2007). In addition to the wide temperature range, it also survives extreme pH of 4.7 - 9.2 (Ferreira *et al.*, 2014), even 9.6 (Zunabovic *et al.*, 2011) and salinity levels of up to 11% (although this is dependent

on other external environmental factors (Caly *et al.*, 2009; Bergholz *et al.*, 2012). These flexible parameters for survival and growth are what allows *L. monocytogenes* to establish and survive in the dynamic RTE-food processing environment.

### 2.3.3 Lineages

The approach to sanitation and disinfection in the factory environment, is designed to target micro-organisms in terms of species. Chemical selection will be based on its ability to instil either a bacteriostatic or bactericidal effect. *Listeria* spp. contamination is considered as one scenario and treatment is then directed only at *Listeria* spp. However, it is now known that the different lineages of *Listeria monocytogenes*, display different adaptation mechanisms and resistance factors in response to processing factors in the RTE environment (Table 2.1).

**Table 2.1** Lineage related stress response adaptations in RTE processing environment

| RTE processing environment factors | Stress response adaptation |
|---|---|
| Temperature fluctuations | Reaction to processing temperatures (lineage specific) (Orsi *et al.*, 2011). |
| Compromise on optimal storage temperature | Ability to survive at refrigeration and room temperature. |
| Change in procedure | $\sigma^b$ (stress factor) expression in lineage II and survival in wide range of conditions (-0.5-9.3°C) refrigeration, pH 4.7-9.2 and salt concentration (10% wt/vol) (Ferreira *et al.*, 2014).<br>Biofilm formation and transfer coefficients (Hoelzer *et al.*, 2012). |
| Machine and equipment maintenance | Persistent *Listeria* strains are more likely to be isolated from the processing environment than from raw materials (Ferreira *et al.*, 2014). |

In theory, by establishing the dominant lineage strain present in the factory environment, sanitation protocols can be targeted at the specific lineages present, resulting in a more effective approach. Through molecular typing, lineage assignments can be made and better insight into the dynamics of the *L. monocytogenes* contamination scenario can be established. It should be emphasised that this is only a theoretical approach, as a practical application in an already dynamic and complex food processing environment, would not be sustainable.

The *L. monocytogenes* species can be divided into lineage groups. Through extensive phylogenetic and subtyping studies, four distinct groups have been identified, with lineage I and II representing the majority of strains (Orsi *et al.*, 2011; Da Silva & De Martinis, 2013). Lineage IV was previously classified as a subgroup of lineage III (IIIB), but through phylogenetic studies been characterised as a separate lineage (Ward *et al.*, 2008; Den Bakker *et al.*, 2012). In recent years Lineage IV has also been increasingly isolated, as in a study by Vongkamjan *et al.* (2016). However, due to lineage IV's relatedness to lineage III (IIIB) and its irrelevance to this study, it will not be included in further discussions (Table 2.2).

**Table 2.2** Description of lineage groups

| | Lineage | | |
|---|---|---|---|
| | I | II | III |
| Serotype | 1/2b, 3b, 3c, 4b[1] | 1/2a, 1/2c, 3a[1] | 4a, 4c[3] |
| Subgroups | ECI, ECII, ECIV[1] | ECIII[1] | IIIA, IIIB, IIIC[3] |
| High prevalence | Human listeriosis[1] | Food and environment[1] | Animal listeriosis[2] |

(Orsi *et al.*, 2011)[1](Milillo & Wiedmann, 2009)[2] (Liu *et al.*, 2006)[3]

Differentiation between lineage I and II is based on multiple single nucleotide polymorphisms as well as the absence and presence of genes within the genome (Nelson *et al.*, 2004; Paul *et al.*, 2014). A pan-genomic study has shown that there are 86 genes and 8 small regularity RNAs that are responsible for the differentiation between *L. monocytogenes* lineages, specifically in regard to stress resistance and usage of carbohydrates in both the environment and the host's intestinal tract (Deng *et al.,* 2010).

Lineage I is mainly recovered in human listeriosis cases and lineage II is represented in food and environmental isolates (Milillo & Wiedmann, 2009). Serotypes mainly associated with lineage I (4b and 1/2a) have intact, full length virulence factor internalin *A* (*inlA)*, whereas lineage II isolates feature premature stop codons in *inlA* (Orsi *et al.*, 2011). *InlA* forms part of a group of internalins that encode for proteins that are responsible for the invasion of the bacteria into cells such as human intestinal epithelial cells (Den Bakker *et al.*, 2010). Invasion into human cells is the main vehicle of pathogenesis of *L. monocytogenes* and therefore its ability to effectively invade host cells is directly correlated to its virulence. The overrepresentation of lineage I strains in human listeriosis cases can thus be attributed to their increased virulence (Orsi *et al.*, 2011; Vongkamjan *et al.*, 2016).

Contrarily, the overrepresentation of lineage II strains isolated from food and environmental samples is mainly due to their enhanced ability and fitness to overcome environmental stress conditions (Orsi *et al.*, 2011). Examples of such adaptations are a) increased biofilm forming ability under nutrient limited conditions; b) overexpression of stress factor, *sigB*; c) increased recombination rates under selective pressure; d) increased resistance to bactericidal agents (Orsi *et al.*, 2011).

Lineage III is not generally associated with human listeriosis although it is generally isolated from environmental samples (Wiedmann *et al.*, 1997; Liu *et al.*, 2006; Rosef *et al.*, 2012). By comparing *actA* and *sigB* gene sequences through phylogenetic analysis, *L. monocytogenes* lineage III is further subdivided into subgroups IIIA,IIIB and IIIC (Roberts *et al.*, 2006). Lineage III isolate's confinement to animal listeriosis cases are due to the lack of surface proteins transcribed by *inlAB* as well as other genes of unknown function (Goldfine & Shen, 2007). This lineage, together with lineage IV, is overrepresented in animal isolates (Roberts *et al.*, 2006; Da Silva & De Martinis, 2013) and therefore, due to its current underrepresentation in human and food isolates and its non-pathogenic nature (Camejo *et al.*, 2011), further discussion thereof within this context is currently irrelevant.

What lineage III, however, does contribute to this study is an indication that there are still unidentified genetic factors of lineage I and II that facilitate pathogenesis and environmental stress adaptations. This hypothesis is derived from the observation that although the main virulence factors, such as *prfA*, are conserved throughout the entire *L. monocytogenes* species, lineage III is still underrepresented in food and clinical isolates (Deng *et al.*, 2010).

### 2.3.4 Strain fitness

Studies attempting to establish a correlation between strains and their increased ability to adapt, survive and outcompete in an environment i.e. strain fitness, have shown varied outcomes and conclusions. Bruhn *et al.* (2005) demonstrated how lineage II strains outcompete lineage I strains during enrichment with University of Vermont selective enrichment. Therefore, proposing the possibility of increased strain fitness among *Listeria* lineages. In contradiction, Gorski *et al.* (2006) found variance in strain fitness, but could not correlate them to specific lineages.

Considering that correlations between strain fitness and the general processing environment has not yet been established, strain fitness is potentially dependent on specific environmental factors. In a critical review by Valderrama and Cutter (2013) it was hypothesised that there is a correlation between increased adaptation and survival abilities of certain serotypes in specific environmental conditions. This differential fitness is therefore likely to be present in food processing environments, possibly explaining the epidemiological variance of strains at food processing levels.

## 2.4 Virulence: from saprotroph to pathogen

*Listeria monocytogenes* has the ability to survive in the environment, through a saprophytic lifestyle, while still maintaining its pathogenic ability. This phenomenon is a crucial concept in regard to food and consumer safety (Xayarath & Freitag, 2012) considering that contaminated food products are the main vectors of infection (Freitag *et al.*, 2010). This maintenance of pathogenicity is achieved by regulation of the positive regulatory factor A (*prfA*), where a combination of environmental factors signals the activation of *L. monocytogenes'* virulence factors (De las Heras *et al.*, 2011). Transition

between saprotroph and pathogen demands that the bacteria react to changes in environmental factors such as lowered pH, other stresses caused by bile of host gut and reduction of available carbon (Fuchs *et al.*, 2012). Contrary to existing theories, temperature is not the main factor that switches on *prfA*. De las Heras *et al.* (2011) agreed that carbohydrate source is a key factor that induces transition to its pathogenic state. Sugar mediated repression is guided by the availability of sugars such as glucose, fructose and other β-glucosides. These sugars are known as PTS (phosphotransferase system) sugars and are abundant in nature, hence being the signal to *L. monocytogenes* to repress any virulence gene expression (De las Heras *et al.*, 2011). The availability of these PTS sugars' intercellular phosphate derivatives serves as a signal for the activation of virulence genes as *L. monocytogenes* now finds itself in a warm-bodied host. Another effect of the changing availability of sugars is that the bacteria switches its biochemical pathway from glycolysis to an oxidative pentose phosphate pathway (Xayarath & Freitag, 2012).

The regulation of *prfA* adds to the survival fitness of *Listeria* as it supresses the expression of genes and actions that aren't crucial to the survival of the bacteria in its particular extracellular environment, therefore saving energy by limiting wasteful production of virulence factors (De las Heras *et al.*, 2011; Xayarath & Freitag, 2012). In addition, up-regulation of genes for virulence must be accompanied by down-regulation of environmental survival factors such as mobility based factors. *Listeria monocytogenes'* extracellular mobility at 22-25°C reflects this phenomenon by its repression at 37°C (De las Heras *et al*., 2011).

Of the four sigma factors, *sigB* ($\sigma^B$) is the only stress related factor that is linked to virulence. Its contribution to pathogenesis is believed to be limited to the gastrointestinal phase of invasion as it increases the bacteria's tolerance of the unfavourable conditions of the intestinal tract (De las Heras et al., 2011). $\sigma^B$ regulates genes that transcribe for the known virulence factors of *L. monocytogenes* and as the same study by Sue *et al.* (2004) concludes, *Listeria* related foodborne infections are enabled by this factor.

## 2.5 Listeriosis

The most likely means of infection is through ingestion of contaminated food products (Ooi & Lorber, 2005). The severity of *L. monocytogenes* infection is dependent on the host and is classified as either non-invasive or invasive (Camejo *et al.*, 2011). In immunocompetent individuals, non-invasive *L. monocytogenes* infection manifests simply as febrile gastroenteritis which is self-limiting and in most cases does not warrant any antibiotic therapy (Ooi & Lorber, 2005). In the vulnerable population, which Meloni *et al.* (2009) refers to as YOPI (Young, Old, Pregnant and Immuno-compromised), invasive listeriosis can be fatal. In these cases it manifests as septicaemia, encephalitis, meningitis, stillbirth or perinatal infection (Dhama *et al.*, 2015). The incubation time of *L. monocytogenes* before onslaught of invasive listeriosis infection has been reported to differ between vulnerable groups. Bacteraemia cases, invasive listeriosis, central nervous system

involvement cases and pregnancy associated infections have median incubation times of 2 days, 8 days, 9 days and 27.5 days, respectively (Goulet *et al.*, 2013).

The pathogenesis of *L. monocytogenes* is grounded in its ability to bridge the intestinal, placental, and blood-brain barriers of the body (Bécavin *et al.*, 2014) and to avoid the effects of the host's immune response. Intestinal, hepatocytic and macrophage-like cells are all involved in the invasion pathway of the bacteria (Pricope *et al.*, 2013). Camejo *et al.* (2011) segments the invasion cycle into essentially five stages: adhesion and invasion of host cell, multiplication and motility inside host cells and intercellular spread within host body (Figure 2.1).

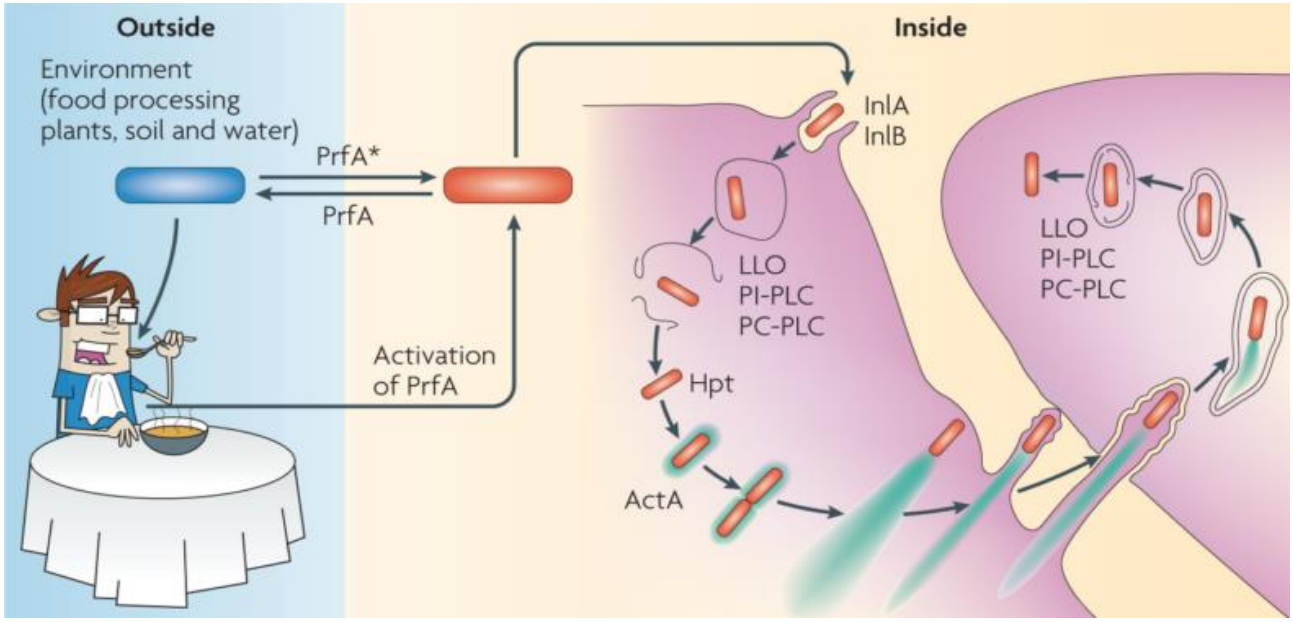


**Figure 2.1** The transition of *L. monocytogenes* from saprotroph to intracellular pathogen (Freitag *et al*., 2010).

There are 50 known virulence factors involved in the infection cycle of *L. monocytogenes* (Camejo *et al.*, 2011), however only the major virulence factors will be considered in this review as stated by the same authors. The major virulence factors (LLO, *InlA*, *InlB*, *ActA* and *PrfA*) contribute directly to the adhesion and invasion process of the bacteria where the other virulence associated proteins only play a secondary role in this process (Camejo *et al.*, 2011). Upon consumption of contaminated food by the host, the bacteria enters non-phagocytic cells such as the cells of the intestinal lining (epithelial cells) through utilisation of virulence factor internalins *(InlA* and *InlB*) (De las Heras *et al.*, 2011; Fuchs *et al.*, 2012). Invasion of the host cell is advanced by interaction of the leucine-rich repeat (LRR) domains with its surface ligands. *InlA* interacts with E-cadherin and *InlB* interacts with hepatocyte growth factor receptor tyrosine kinase C-Met simultaneously. *L. monocytogenes* cells enter the cell vacuole through a process similar to clathrin-mediated phagocytosis (De las Heras *et al.*, 2011). Escape from the phagocytic cell is mediated by the expression of virulence factors, listeriolysin (*Hly*) and phospholipase A (*PlcA*). This is a characteristic that is unique to *L. monocytogenes*, setting it apart from other facultative intracellular pathogens.

Once in the cytoplasm of the host cell, the bacteria are safe to grow and replicate, void of any attack from the host body's immune response (Fuchs *et al*., 2012). From here the bacteria is free to manifest infection in the blood, central nervous system and placenta (in the case of pregnancy) (Goldfine & Shen, 2007).

## 2.6 Detection methods

### 2.6.1 Traditional culturing and detection methods

Official methods available for the detection of *L. monocytogenes* are FDA, NGFIS, USDA-FSIS, Cold enrichment NKML method no. 136 and ISO 11290-1:1996 (Zunabovic *et al.*, 2011). Due to the scope of this study and its relevance to the South African food industry, only ISO approved and equivalent methods will be discussed and evaluated. Conventional identification and differentiation of and between *Listeria* spp. include Gram staining, motility observation, and biochemical reactions (catalase test and acid production from D-glucose test) (Law *et al.*, 2015a)

### 2.6.2 ISO 11290-1:2017

This method is the only ISO approved method for detection and enumeration of *L. monocytogenes* in environmental and food samples (Anonymous, 2017a). It contains a primary and secondary enrichment step with Fraser broth after which the enriched medium is streaked onto Agar Listeria Ottovani & Agovti (ALOA) agar as well as a second selective medium. The plates are incubated and only then after 96 h can the confirmation test for differentiation between *Listeria* spp. and *L. monocytogenes* be conducted (Zunabovic *et al.*, 2011). Although this method is deemed as accurate and reliable, it can become tedious, time consuming (Dalmasso *et al.*, 2014) and expensive as the sample numbers increase, as in a food manufacturing environment. Considering the high demands of a food industry laboratory and the high turnover of samples, there is a need for an alternative, simpler method. In addition, but outside the scope of this study, culture independent methods that inherently exclude enrichment steps which minimise sample-to-result time is progressively being used in routine analysis for detection of foodborne pathogens (Wang & Salazar, 2015).

Alternative methods, which are not ISO accredited can be allowed by the discretion of the governing body. Proprietary and alternative methods are validated and certified by third parties (AFNOR Certification, AOAC, NordVal) using the ISO 16140 method (Auvolat & Besse, 2016).

Standardised and approved methods for detection of *L. monocytogenes* in food and environmental samples contain in most part an enrichment step. Although *L. monocytogenes* is an ubiquitous bacteria, it is found in low numbers in the food processing environment and contaminated food products (Bruhn *et al.*, 2005), and for that reason an enrichment step is included in approved detection methods.

Culturing of enriched samples can be done on various approved *Listeria* selective agars. However, the selection of the agar should be done with the aim of the study in mind, since selected agar only allows culturing and others allow differentiation between pathogenic *Listeria* spp. and non-pathogenic *Listeria* spp. Chromogenic agar is the preferred method for *L. monocytogenes* detection due to its time efficiency, affordability, simplicity and high turnover capability (Gasanov *et al.*, 2005). Thus, it is also the chosen method for the majority of Food Business Operators (FBO) and consequently the method used in this study.

### 2.6.3 Rapid'*L.mono*® chromogenic agar

Rapid'*L.mono*® agar is a proprietary, commercial product manufactured and distributed by Bio-Rad Laboratories (USA). Detection and enumeration of *L. monocytogenes* using this agar is regarded as an alternative method and is AOAC-RI approved (N° 030406), NordVal approved and carries an NF Validation according to ISO 16140 (Anonymous, 2014).

Rapid'*L.mono*® is a chromogenic agar that relies on the phosphatidylinositol phospholipase C (PIPLC) activity of *L. monocytogenes* and *L. ivanovii* for detection and differentiation from other non-pathogenic *Listeria* spp. Further chromogenic differentiation is made between the two pathogenic species based on their ability to metabolise the added xylose. *L. ivanovii* has this ability and consequently forms black colonies with a yellow halo where *L. monocytogenes* will only form distinct black colonies (Lauer *et al.*, 2005). Non-pathogenic species show no PIPLC activity and will therefore form white colonies with no halo, with the exception of *L. welshimerii* which is the only species with xylose metabolism and therefore a white colony and yellow halo (Lauer *et al.*, 2005).

In contrast, the results and recommendations of a challenge study by Stessl *et al.* (2009) warrants further investigating into the reliability of chromogenic agar methods, in the light of competing microflora and enrichment bias.

### 2.6.4 Overcoming enrichment bias

Due to the zero-tolerance approach that regulatory bodies and the food industry have regarding *L. monocytogenes* in food products, it is crucial that the methods used to detect and qualify the presence thereof be unbiased and reliable. The ideal method would have a low Level of Detection (LOD) and a high Probability of Detection (POD).

*L. monocytogenes* occurs in low counts in naturally contaminated food products and the food processing environment (Bruhn *et al.*, 2005) and it is therefore crucial that the selected enrichment step and method ensure that a true representation of the *Listeria* spp. be detected.

Due to the heterogeneous microflora found in the food processing environment and the low counts of *L. monocytogenes*, the growth and interference of background microflora thus needs to be eliminated to ensure the precise detection of low counts of *L. monocytogenes* (Zitz *et al.*, 2011). Zitz *et al.* (2011), harnessed the ISO 11290-1:1996 method to evaluate enrichment bias toward

*L. monocytogenes* in the presence of *L. innocua.* A lowered detection ability was seen with low concentrations (≤1 to 5 CFU) of *L. monocytogenes* in the presence of *L. innocua.* Consequently, an increased POD with an increase in *L. monocytogenes* CFU.

Reduction of growth kinetics in mixed culture broths (Tryptic Soy Broth-Yeast (TSB-Y)) have been reported by Zilelidou *et al.* (2015), demonstrating the effect of strain competition. Suppression of lineage I strains have been reported by Bruhn *et al.* (2005) when enrichment was conducted in University of Vermont selective enrichment media. However, this may be due to the selective compounds in the enrichment medium and bias was not specifically strain related.

Overgrowth of other *Listeria* spp. was observed at the late exponential, early stationary phase largely due to the "Jameson effect" (Besse *et al.*, 2010). In essence this effect causes the suppression of all microorganisms within a matrix once the maximum population density has been reached (Ross *et al.*, 2000). For that reason, the species or strain that reaches its stationary phase first will halt the growth of any other species or strains present, consequently "masking" the presence of slower growing organisms.

Nevertheless, in a review by Zunabovic *et al.* (2011) it was concluded that significant true positives have been reported using only the half Fraser enrichment step. This step included in the Rapid'*L.mono* method is thus currently deemed as reliable for the detection of *L. monocytogenes* in contaminated food matrices.

### 2.6.5 Enumeration

The enumeration of *L. monocytogenes* in food products is of great importance to not only research related to predictive microbiology and risk assessments, but it is needed to support routine safety analysis in the food industry (Auvolat & Besse, 2016). Current enumerations methods for *L. monocytogenes* is described by the ISO 11290-2:2017 (Anonymous, 2017b). However, Auvolat and Besse (2016) reported that there has currently been no development of enumeration methods adequate and sensitive enough for food matrices. In contrast a study by Chen *et al.* (2017) found Rapid'*L.mono* as a reliable method for enumerating low levels of *L. monocytogenes* in ice cream samples.

## 2.7 Molecular typing and subtyping methods

Typing methods are used for the identification of an organism, such as pathogen detection. The application of a molecular method for further study and differentiation of a target organism, after it has been defined or identified, is a subtyping method. Some methods are suitable for both typing and subtyping (for example PCR), where other methods are not sensitive enough for subtyping and others too sensitive for only typing. Therefore, understanding all available methods are crucial to optimising the application thereof.

Different rapid molecular detection methods are based on one of the following principles: nucleic acid, biosensor or immunological (Law *et al.*, 2015a). Although biosensor and immunological based methods are suitable for detection of pathogens across a range of matrices, the application thereof in source tracking is limited. Nucleic acid based methods such as ribotyping, Pulsed Field Gel Electrophoresis (PFGE) and Polymerase Chain Reaction (PCR) are more suitable for typing and subtyping of pathogens. Further, nucleic acid sequence based amplification (NASBA), loop-mediated isothermal amplification (LAMP), DNA microarray sequencing and next generation sequencing (NGS) (Law *et al.*, 2015b) have emerged as rapid molecular detection methods.

### 2.7.1 Polymerase Chain Reaction

The current molecular methods that are at the disposal of researchers to type *L. monocytogenes* are PCR, multiplex PCR (mPCR), real-time/quantitative PCR (qPCR); where PCR as a method has been used to detect a variety of foodborne pathogens (Law *et al.*, 2015a). Due to the application of this study to the food industry in South Africa only the most basic and simple forms of molecular typing, including PCR and multiplex PCR, will be explored.

PCR methods are based on amplification of selected target genes for identification and typing of isolates. To amplify a selected target DNA sequence in conventional PCR, two primers (single stranded synthetic oligonucleotides) are used in a three-step process (denaturation, annealing and elongation) using a thermal cycler. The amplified DNA sequences are then separated and visualised using agarose gel electrophoresis (Law *et al.*, 2015b) and the appropriate imaging software. Multiplex PCR, is a more rapid method that entails the simultaneous amplification of multiple selected target genes (Wang & Salazar, 2015). It has the ability to identify five or more pathogens at the same time (Law *et al.*, 2015a). Chen and Knabel (2007) successfully utilised mPCR for differentiation of *Listeria* spp. and *L. monocytogenes* as well as epidemic clones thereof, simultaneously.

### 2.7.2 Ribotyping

Ribotyping has been used as a valuable tool in epidemiological studies but now that the process has been automated, ribotyping as a molecular detection method can be used in routine analysis, despite it still having less discriminatory power in comparison to other more conventional methods (Gasanov *et al.*, 2005). Pavlic and Griffiths (2009) better state that the discriminatory ability of specifically automated ribotyping is very dependent on the pathogen being investigated. The attraction of automated ribotyping, apart from the efficiency, is its ability to ensure standardisation (Lorber, 2014)

It is important to note that even though the method is called “ribotyping”, the assumption that the main source of the observed polymorphism is the ribosomal RNA is incorrect (Bouchet *et al.*, 2008). Since the main aim of ribotyping is to establish phylogenetic relationships between the organism being studied, the genes encoding for ribosomal RNA are investigated (Gasanov *et al.*, 2005).

Ribosomal RNA is highly conserved between species as they encode for the 5S, 16S and 23S sequences and thus variation in these sequences are inadequate to distinguish between species, let alone strains. Therefore, the polymorphisms observed by ribotyping are due to the restriction fragment length polymorphisms (RFLPs) from what is known as the neutral housekeeping genes, found in the flanking regions of the rRNA sequences. rRNA sequences are found to be so highly conserved they are seen as anchoring genes to the RFLP's observed during ribotyping. The gene sequences encoding for the neutral housekeeping genes evolve through point mutations caused by random genetic drift. They are thus not subject to diversifying Darwinian evolution (Bouchet *et al.*, 2008).

Ribotyping is a type of RFLP analysis because it is dependent on varying locations and number of ribosomal RNA (rRNA) gene sequences found in bacterial genomes. It is a rapid molecular detection technique that can identify and type bacteria to their strain level by analysis of band pattern or ribopattern differences. These bands originate when labelled rRNA is hybridised with DNA fragments obtained from the cleavage of total DNA by the selected endonuclease (Lorber, 2014). The main cause of variations in the ribopatterns are the variations in flanking sequences among the different strains. These variations in flanking sequences originate from point mutations in the housekeeping genes due to random genetic drift.

The process of ribotyping entails digesting and fragmenting genomic DNA with restriction enzymes like *Eco*RI, *Pvu*II and *Xho*I. A Southern blot is then conducted to detect the genes that code for rRNA (Jadhav *et al.*, 2012). Due to the use of the selected probes in combination with imaging techniques, distinct and unique ribopatterns are generated (Bouchet *et al.*, 2008).

Endonucleases used for ribotyping are *Eco*RI, *Pvu*II and *Xho*I (Jadhav *et al.*, 2012), with *Eco*RI being used more frequently (Pavlic & Griffiths, 2009). A study done by De Cesare *et al.* (2001) found that out of fifteen different restriction enzymes, these three restriction enzymes displayed the highest discriminatory power when typing *L. monocytogenes*. A dual enzyme strategy, using *Eco*RI and *Pvu*II, has shown acceptable *L. monocytogenes* strain differentiation (Jadhav *et al.*, 2012). However, considering that each endonuclease or combination thereof will produce a distinct set of bands (profile), comparisons between results from studies using different endonuclease combinations cannot be made (Pavlic & Griffiths, 2009). Since a fundamental aspect of this study is generating DuPont Identification Library Codes (DUP-IDs), *Eco*RI ribotyping has to be the method of choice (Jadhav *et al.*, 2012).

## 2.8 Automated ribotyping

### 2.8.1 DuPont Riboprinter®

The automated ribotyping, RiboPrinter® System by Qualicon Inc (Figure 2.2) conducts all processes associated with ribotyping automatically, with only 30 minutes of sample preparation taking place

manually (Anonymous, 2013a). Each sample that is processed is then assigned a unique DuPoint Identification Library Code (DUP-ID), that is added to the internal and external (optional) database. The DuPont Riboprinter® system has been the instrument of choice in similar studies conducted, due to its reproducibility, reliability and interlaboratory comparison ability (De Cesare *et al.*, 2001; Kabuki *et al.*, 2004; De Cesare *et al.*, 2007). As to the authors knowledge, no South African studies have utilised the RiboPrinter® as a means to sub-type *L. monocytogenes* strains isolated from food processing environments.

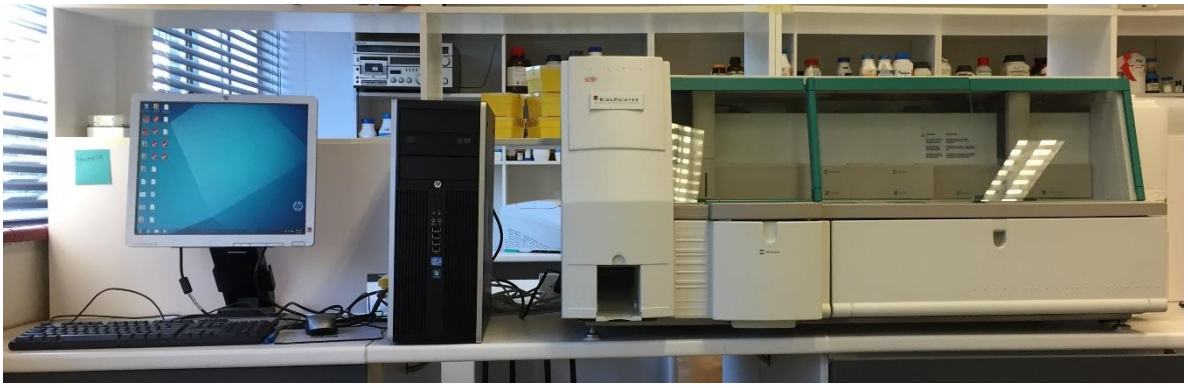

**Figure 2.2** DuPont RiboPrinter® used for automated ribotyping of isolates from RTE food factory.

### 2.8.2 Food Microbe Tracker

Food Microbe Tracker (previously known as PathogenTracker) is an online database that was created as part of Cornell University's "Food Safety Laboratory Bacterial Stains WWW Database Project" (Anonymous, 2013b). The aim of the database is to provide a public platform for researchers where bacterial strains and subtypes isolated during their research can be added and globally compared, to study the micro-organism strain biodiversity (Anonymous, 2013; Vangay *et al*., 2013). Other databases, such as MLST.net are limited in their scope and others, such as PulseNet, are not available to the public (Vangay *et al*., 2013).

Klaeboe *et al.* (2006) used the online database to determine whether the strains isolated during their study have been implicated in any human listeriosis cases and found that they hadn't. Additionally, Roberts *et al.* (2006) made the ribotyped fingerprints of the lineage III strains isolated during their study available on the database for comparison. Manuel *et al.* (2015) utilised Food Microbe Tracker to find available strains for the specific DuPont ID *L. monocytogenes* strain they used to study *inlA* alleles. Although these studies only reflect the use of Food Microbe Tracker for DuPont ID's and *L. monocytogenes* it should be noted that the database hosts various food-borne micro-organisms with a variety of band pattern data for source tracking and cross-referencing (Vangay *et al*., 2013).

## 2.9 Comparison of competing methods: Riboprinter vs PFGE

Ribotyping and pulsed field gel electrophoresis (PFGE) are both restriction fragment length polymorphism (RFLP) based methods. Therefore, no amplification of genes take place, but rather restriction enzymes are used to cleave DNA sequences at targeted nucleotides resulting in fragments. When comparing ribotyping and PFGE regarding their ability to indicate relations between separate bacterial isolates, ribotyping proves to be more reliable. The restriction enzymes used in PFGE analysis cut DNA sequences in larger units, much larger that the pieces produced by ribotyping (Wiedmann, 2002). With the PFGE RFLP recognition sites that are spread across the whole genome, genetic information regarding diversifying selection is seen. This information can be overwhelming, which can complicate the analysis and interpretation of the results regarding evolutionary relatedness. This obstacle is overcome by ribotyping due to its ability to readily interpret evolutionary relatedness (Bouchet *et al.*, 2008).

PFGE is still the method of choice, specifically in North America, due to its high discriminatory power, but only in regard to outbreak investigations (Pavlic & Griffiths, 2009). Numerous studies have shown PFGE to have higher discriminatory power in comparison to *Eco*RI ribotyping (Fugett *et al.*, 2007), however it is important to note that these studies are case specific without the long-term intent of interlaboratory comparison or collaborations. Also, when considering PFGE's sensitivity and dependence on laboratory specific environments and methods, interlaboratory comparison and reproducibility is difficult (Jadhav *et al.*, 2012). This indicates its suitability of case specific outbreak investigations. Large scale studies as well as industrial application of a rapid molecular detection method demand a highly standardised and automated method that does not require a specialised set of skills, hence the applicability of ribotyping (Wiedmann, 2002). When considering the requirements and demands of outbreak investigation in comparison to mapping in house flora for source tracking, automated ribotyping is more suitable for source tracking. This is because PFGE requires expensive equipment and reagents and is also a scares skill and time consuming process (Jadhav *et al.*, 2012). Pavlic and Griffiths (2009) support automated ribotyping as a suitable typing method for micro-organisms in a processing environment. That being said, both methods can complement each other by being utilised together as in a study by Vongkamjan *et al.* (2013).

## 2.10 Whole Genome Sequencing: A new approach to outbreak investigation

Subtyping methods have become fundamental in studying the genetic diversity and ecological distribution, specifically for *L. monocytogenes* (Orsi *et al.*, 2011). Even though molecular methods are still considered to be better than traditional phenotypic analysis, sub-par discriminatory power between strains in certain subgroups have been reported (Nyarko & Donnelly, 2015). Whole Genome Sequencing (WGS) is the next generation technique that will transform how food safety issues are approached and investigated, since sequence data can unlock even more information

regarding virulence and contamination source (Nyarko & Donnelly, 2015). Due to the long and varying incubation time of *L. monocytogenes* (Goulet *et al.*, 2013), it becomes increasingly difficult to determine the cause of sporadic outbreaks. In addition, serotyping for source tracking, is time consuming and has limited discriminatory ability. Thus, with incredible advances made in WGS, in addition to advances in computational ability, rapid and accurate outbreak investigations can be done (Datta & Burall, 2017).

## 2.11 Similar studies

Table 2.3 compares similar studies and their research areas as they relate to this study. It is aimed at providing a broad overview of research areas and combinations thereof that are still to be explored. It further aims to highlight the lack of research into *L. monocytogenes* contamination within the South African context, with ground breaking research mainly originating from more developed countries.

## 2.12 The study of Biofilms: Response to bactericidal factors

### 2.12.1 Biofilms and persistence

Biofilms are defined as bacterial communities that are enclosed in a self-produced matrix, an extracellular polymeric substance (EPS) containing polysaccharides and proteins (Pilchová *et al.*, 2014) that adheres to each other and/or other inert surfaces. These communities contain a homeostatic environment, circulatory system and metabolic cooperation (Costerton *et al.*, 1995). Lasa (2006) refers to biofilms as a microbial lifestyle and indeed it is an adapted mechanism of survival for pathogenic bacteria. The ability of *Listeria* to form mono/multi specie biofilms on food processing surfaces is not only relevant (Zunabovic *et al.*, 2011), but is of a major concern to the food industry. The main source of *Listeria* spp. contamination in RTE foods is said to occur post-production (Kerouanton *et al.*, 2010; Vongkamjan *et al.*, 2013; Nyarko & Donnelly, 2015). The most common post-production contamination mechanism in RTE foods is contaminated food contact surfaces, including machinery and other equipment (Hansen & Fonnesbech, 2011; Fouladynezhad *et al.*, 2013). However, the formation of biofilms in food processing environments are yet to be fully understood as a review by Cappitelli *et al.* (2014) explains.

The occurrence and formation of *Listeria* spp. biofilms in food processing environments has been a prominent research topic in recent years (Di Bonaventura *et al.*, 2008; Da Silva & De Martinis, 2013; Fouladynezhad *et al.*, 2013; Giaouris *et al.*, 2014; Dzieciol *et al.*, 2016; Puga *et al.*, 2016) and yet a comprehensive understanding of the intricate mechanism involved has not yet been reached. Nevertheless, the major mechanisms are being established and continuously investigated and expanded.

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**Table 2.3** Comparison of similar studies as they relate to the current study

| Study | South Africa | RTE food | *Eco*RI ribotyping | Source tracking | Lineage grouping | In-house environmental samples | Sanitary practices evaluation |
|---|---|---|---|---|---|---|---|
| Van Nierop *et al.* (2005) | x | | | | | | |
| Klaeboe *et al.* (2005) | | | x | | | | x |
| Klaeboe *et al.* (2006) | | | | x | | x | |
| Fugett *et al.* (2007) | | | | | x | | |
| Meloni *et al.* (2009) | | | x | x | | | |
| Klaeboe *et al.* (2010) | | | x | x | x | | |
| Odjadjare and Okoh (2010) | x | | | | | | |
| Rosef *et al.* (2012) | | | x | | | | |
| Dalmasso and Jordan (2012) | | | x | | | | x |
| Almeida *et al.* (2013) | | | | | | x | |
| Strydom *et al.* (2013) | x | | | | | | |
| Spanu *et al.* (2015) | | | x | | | | |
| Ruckerl *et al.* (2014) | | | | | | | x |
| Awofisayo-Okuyelu *et al.* (2016) | | | | x | | | |
| Lokerse *et al.* (2016) | | x | | | | | |

### 2.12.2 Biofilm formation and structure

In the case of complex and sophisticated *Listeria* spp. biofilms, the initial biofilm structure is critical. Therefore, studying the initial stages and early attachment mechanism of biofilms are important. A holistic understanding thereof can assist in development and improvement of prevention and control methods (Pilchová *et al.,* 2014). However, initial adhesion conditions and the state of early biofilms, have no significant influence on the biofilm's resistance ability to sanitisers, but rather resistance is increased in mature biofilms (Ibusquiza *et al.,* 2011)Consequently, it is important that the maturing of biofilms in a processing environment should be avoided. It can thus be said, that by preventing maturation of biofilms, increase in sanitation resistance can be avoided.

The physicochemical characteristics of the environments (pH, temperature, surface hydrophobicity) and bacterial mobility is the main determining factor in the case of adhesion of planktonic cells (Bonsaglia *et al.*, 2014). The characteristics of the surface is a large contributing factor to the attachment of biofilms. Biofilm attachment occurs on both hydrophilic surfaces (glass and stainless steel) and hydrophobic surfaces (polystyrene), although hydrophilic surfaces show increase formation rates (Bonsaglia *et al.*, 2014). Several other studies have looked at the attachment of *L. monocytogenes* biofilms to surfaces found in the food processing environment. Di Bonaventura *et al.* (2008) found increased biofilm formation on stainless steel and glass. In contrast Poimenidou *et al.* (2016) found that polystyrene was more favourable for biofilm formation. These contradictory findings highlight the influence that fluctuating environmental factors have on the formation of biofilms and that a universal approach to *L. monocytogenes* biofilms is futile.

The biofilm structure formation is highly dependent on the environmental conditions especially pertaining to static and flow conditions, since it influences the cell attachments phase of biofilm formation (Pilchová *et al.*, 2014). It has been reported that under flow conditions *L. monocytogenes* tends to produce thicker biofilms of increased volume that takes the form of a network of "knitted"-chains (elongated cells) that surround ball shaped microcolonies. In contrast, biofilms grown under static conditions form a homogenous layer of either rod cells or microcolonies or a heterogeneous combination thereof (Rieu *et al.*, 2008; Da Silva & De Martinis, 2013).

The construction of a biofilm can be divided into four fundamental phases. Initial attachment (reversible adhesion) by cells takes place through electrostatic forces, hydrophobic interactions and van der Waals forces. Irreversible adhesion occurs as the bacteria multiplies and extracellular polymeric substance (EPS) excretion initiates. During biofilm maturation, the biofilm structure's complexity increases and transport mechanisms for communication and nutrients are established. As a final phase, the biofilm disperses and attaches to other nearby surfaces and the cycles are repeated (Da Silva & De Martinis, 2013; Nguyen & Burrows, 2014; Pilchcová *et al.*, 2014; Colagiorgi *et al.*, 2017).

The EPS contains polysaccharides, extracellular DNA (eDNA) and proteins, where the polysaccharides and complex proteins function as a connection between the molecules on the bacterial membranes and the matrix (Azeredo *et al.*, 2017). eDNA is excreted by the cells in response to quorum sensing signals (Puga *et al.*, 2016). The intricacies and content of the *L. monocytogenes* biofilm EPS is still relatively unknown, with various studies attempting to define it. Harmsen *et al.* (2010) was the first to explore eDNA as the only fundamental component of a *Listeria* spp. biofilm matrix. The authors hypothesised that the function of eDNA in the biofilm's architecture is that of a structural nature as well as a source of energy and nutrition (Colagiorgi *et al.*, 2017). The study also attempted to show the role of eDNA in the adhesion and development of *L. monocytogenes* biofilms. It was concluded that adhesion ability of the biofilms were increased with longer eDNA strands and larger amounts of macromolecular component of the biofilm matrix.

Expression of selected genes and regulators further mediate adhesion. *Agr*, a peptide based quorum sensing accessory gene regulator (Nguyen & Burrows, 2014), has a significant function in early biofilm development, since deletion of this gene caused a major reduction in the biofilm formation, specifically adhesion, of *L. monocytogenes* (Rieu *et al.*, 2007, 2008). In addition, a study by Chen *et al.* (2008) found that the expression of both *InlA* and *InlB* (surface proteins encoded by *inlaA* gene) has an influence on the attachment of *L. monocytogenes* cells to glass surface.

### 2.12.3 The genetics of biofilms

The use of transposon mutagenesis has become a popular technique in identifying adaption mechanisms under various conditions as well as genes that are responsible for biofilm formation. Alonso *et al.* (2014), using a *Himar1 mariner* transposon, identified two new genes that contribute to biofilm formation *dltABCD* and *phoPR* and confirmed the importance of the flagellar motility genes during initial biofilm formation. Similarly, Piercey *et al.* (2016a) investigated novel genes responsible for biofilm formation at 15°C, by creating 14 mutants using random insertional mutagenesis and observing the biofilm forming ability thereof. The study identified 9 genes, previously not linked to biofilm formation that contributed to biofilm formation at a lowered temperature.

### 2.12.4 Biofilms, persistence and resistance

The factors that contribute to persistence in food processing environments have not been successfully defined, but it is possible that the ability of *Listeria* spp. to form robust biofilms may be a significant contributing factor (Pan *et al.*, 2006; Da Silva & De Martinis, 2013). It should be noted that in this context robust does not refer to the size of the biofilm but rather its ability to adhere and survive. This is because *L. monocytogenes* does not have the ability to construct thick biofilms. Gram *et al.* (2007) reported the surface adhering count of $10^4$ to $10^7$ CFU.cm$^{-2}$, which is less than the expected $10^9$ to $10^{12}$ CFU.cm$^{-2}$ of other biofilm forming micro-organisms. This is with the exception of hyperbiofilm (HB) formation. This is displayed in a study where mutant bacteria with no flagellum

based mobility showed HB phenotypes, thus concluding that attempts to control *L. monocytogenes* by limiting flagellar motility, may in actual fact escalate biofilm infestation (Todhanakasem & Young, 2008).

The formation of biofilms provides the micro-organism's with survival advantages that allows for adaptation to (sub)lethal and fluctuating environmental factors (Ferreira *et al.*, 2014; Giaouris *et al.*, 2014). Through the structure of the biofilm, protection is provided from ultra-violet rays, antimicrobial agents, sanitation agents and acids (Da Silva & De Martinis, 2013; Giaouris *et al.*, 2015). Further advantages are given by improved nutrient availability and enhanced genetic variation through horizontal gene transfer (Ferreira *et al.*, 2014). eDNA possibly serves as the source of genes for this transfer action within the matrix (Harmsen *et al.*, 2010).

Each individual bacterial species optimises its genetic, physiological and structural characteristics to survive in various niches (Todhanakasem & Young, 2008). Contrarily, Carpentier and Cerf (2011) suggested that the survival and persistence abilities of different *L. monocytogenes* strains do not differ, but persistence is rather dependant on the available harbourage sites. Orgaz *et al.* (2013) further supports this notion by highlighting the inconclusiveness of research regarding strain specific persistence abilities and proposes that persistence is rather related to biofilm recovery ability. Hard to clean areas easily facilitate the recovery of biofilms. The food processing environment subsequently provides a range of inert surfaces that serve as niches for biofilm formation (Da Silva & De Martinis, 2013). Biofilms are able to mature in areas that are deemed hard to clean, such as cracks and poorly designed machinery (Møretrø *et al.*, 2017), since the effect of cleaning and sanitation is limited (Orgaz *et al.*, 2011). Doijad *et al.* (2015) found cells aggregating around sutures on industrial surfaces.

Occurrence of bacterial contamination within a food processing environment can either be transient or persistent. The first refers to occasional isolation of a specific bacteria and the latter refers to the repeated isolation of a specific bacteria over a longer period (months and years) at the same sampling site (Orgaz *et al.*, 2013). Concrete and statistical parameters for determining persistence are ambiguous and further research using meta-analysis and risk assessment is required to universally define this concept (Ferreira *et al.*, 2014). Thus, attributing persistence to a specific strain is currently to the discretion of the study that is done. Studying the persistence ability of biofilms are complex since a bacteria's response to sanitation methods and recovery ability will differ in regard to its position and relationship to the heterogeneous matrix (Orgaz *et al.*, 2013).

Persistence can, therefore, not automatically be linked to resistance. Magalhaes *et al.* (2016) showed that even though persistent strains have better environmental stress adaptation traits, there was no correlation between persistence and resistance to commonly used sanitisers (benzalkonium chloride and hydrogen peroxide). In contradiction Poimenidou *et al.* (2016) found that a persistent

strain, that produced the most biofilms, had higher resistance to quaternary ammonium compounds (QAC's). The current contradictions within this field of study is indicative of the variability and complexity of *L. monocytogenes* biofilms within various processing environments.

## 2.13 The study of biofilms

The study of microorganisms in complex communities (sessile state) rather than single cells (planktonic state) was the controversial brainchild of Bill Costerton in 1987 (Costerton *et al.*, 1987; Lappin-Scott *et al.*, 2014). His ground-breaking research was based on the concept that most microorganisms are found in sessile or biofilm state since they attach to wetted surfaces and therefore they differ phenotypically from free floating planktonic cells. After the development of confocal microscopy to study biofilms, they were seen, for the first time, as more than just slime adhering to surfaces, but rather complex communities of microorganisms (Costerton *et al.*, 1995; Lappin-Scott *et al.*, 2014).

### 2.13.1 Comparison of fluid and static biofilm measurement systems/methods

Various techniques are available by which to study biofilms, but when considering the fluctuating dynamics of the food processing environment, methods to study these biofilms need to be reflective of the environment. Available methods have evolved to reflect just that, which is crucial in optimising research into biofilm control measures. A review by Azeredo *et al.* (2017) critically evaluated these current methods used to study biofilms. These include Microtiter plate, Calgary device, Biofilm ring test, Robbins device, Modified Robins device, Drip Flow Biofilm Reactor, Rotary Biofilm devices, Flow chamber and Microfluidics.

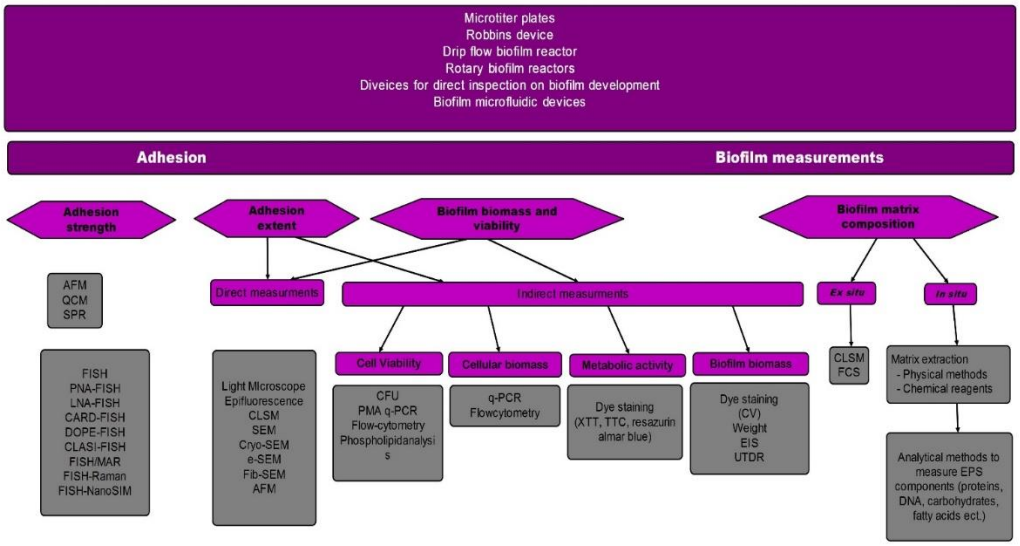


**Figure 2.3** Methods available for cultivation and characterisation of biofilms (adapted from Azeredo *et al.* (2017)).

These methods and instruments (Figure 2.3) are highly specialised and ideal to study specific factors regarding biofilm formation and behaviour. However, none of these are able to mimic factory environment conditions as accurately and efficiently as the $CO_2$ evolution measurement system (CEMS) does when considering the abovementioned demands of the factory environment. A review by Colagiorgi *et al.* (2017) reiterated the need for experimental systems that simulate environmental conditions found in food processing environments. These systems are required to accurately study *L. monocytogenes* and other foodborne pathogen biofilms.

In a demonstration of the effect of flow and static conditions, Todhanakasem and Young (2008) evaluated the formation of hyperbiofilm. They speculated that under flow conditions, rather than static conditions, more relevant information could be obtained. Finally, Dzieciol *et al*., (2016) concluded that control and management programs, specifically for *Listeria* spp. and specifically *L. monocytogenes,* that only monitor drain water is not sufficient, as *L. monocytogenes* is also present in drain biofilms. Studying their response to sanitation when cultivated under flow conditions, becomes imperative.

### 2.13.2 $CO_2$ Evolution Measurement System (CEMS)

Few studies have assessed the recovery of biofilms after sanitation procedures (Orgaz *et al.*, 2013; Olszewska *et al.*, 2016) and since persistence and resistance of micro-organisms in a biofilm can be attributed to resuscitation ability (Ferreira *et al.*, 2014), valuable observations that can improve current control procedures, are lost.

The theory, mathematical equations and system details of CEMS is outlined in great detail by Kroukamp and Wolfaardt (2009). The system set-up and preparation is described by Loots (2016) and demonstrated in Figure 2.4.

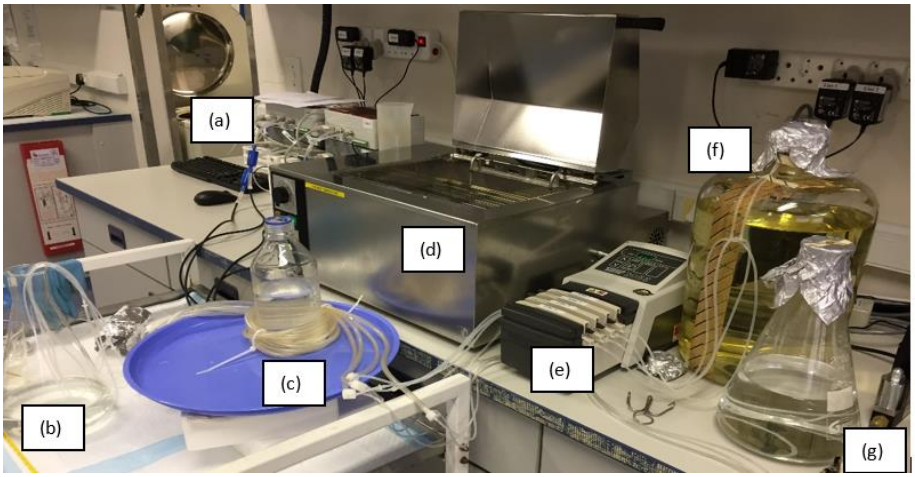


**Figure 2.4** CEMS system set-up (a) $CO_2$ analysers, (b) outflow and waste container, (c) four CEMS (to be inserted in (d) during study), (d) water bath, (e) peristaltic pump, (f) nutrient reservoir, (g) $CO_2$-free gas regulators ($CO_2$-free gas bottles not on figure).

CEMS is a silicone tube in which the nutrients and biofilm cultures flow (liquid phase), which is encased in a sealed Tygon® tube through which $CO_2$ - free air flows (gas phase) (Figure 2.5).

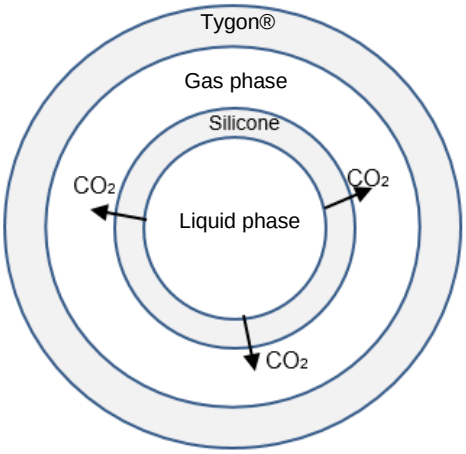


**Figure 2.5** Cross section of CEMS tubing to indicate transfer of $CO_2$ from bulk liquid phase (biofilm and nutrients) to gas phase ($CO_2$ - free air) (adapted from Kroukamp and Wolfaardt (2009)).

Biofilm metabolism as well as its response to environmental and chemical conditions is studied in real time by measuring gaseous $CO_2$ released by the biofilm during aerobic respiration. The $CO_2$ produced by the biofilm as it matures, diffuses through the inner silicone tube and is carried to the $CO_2$ analyser by a carrier gas (Kroukamp & Wolfaardt, 2009). This system has been used to evaluate biofilm responses to antimicrobial agents (Loots, 2016), shear force applied to *Pseudomonas* biofilms (Bester *et al.*, 2010) and observing cellulose activity of *Clostridium thermocellum* biofilms (Dumitrache *et al.*, 2013). No studies on *L. monocytogenes* in these systems have been conducted.

## 2.14 Sanitation

### 2.14.1 Sanitation in food processing environments

Sanitation is a duel process that consists of cleaning protocols, followed by disinfection. During the cleaning process detergents are used to remove all physical matter and organic compounds, this ensures optimal, unhindered disinfection (Walton *et al.*, 2008). ISO EN 13697 states that a disinfectant should provide a 4 log reduction of microbial count on both a clean and soiled surface (Gram *et al.*, 2007). Disinfectants used in food processing environments include lactic acid, quaternary ammonium containing chemicals, acetic acid, sodium hypochlorite (Da Silva & De Martinis, 2013), peracetic acid, hydrogen peroxide ($H_2O_2$), as well as other alternative, proprietary methods. Bacterial cells within a biofilm shows higher resistance to sanitation than cells in a planktonic state (Carpentier & Cerf, 2011; Piercey *et al.*, 2016b), since surfaces with newly deposited cells are easily disinfected (Carpentier & Cerf, 2011) . Therefore, decontaminating surfaces where

biofilms are present, is more challenging since the biofilm structure provides protection to the microbial community inside the biofilm (Renier *et al.*, 2011; Da Silva & De Martinis, 2013). In such cases, the biofilm eradication concentration (BEC) of a sanitiser should be implemented. The BEC, is the concentration of sanitation treatment where the biofilm is unable to resuscitate and grow. This is important since the survival of *L. monocytogenes,* despite sanitation effort, results in the construction of denser and more robust biofilms (Poimenidou *et al*., 2016).

The importance of correct sanitation practices is not only imperative in preventing increase of resistant bacteria but to also avoid increasing the virulence and in vitro proliferation as reported by Pricope *et al.* (2013). Deficient cleaning protocols are characterised by inadequate cleaning practises before disinfection protocols, insufficient disinfection of wet surfaces and inadequate sanitiser dosage and/or concentration application (Pricope *et al.*, 2013). The research field of antimicrobial resistance (AMR) in biofilms originated after studies on the effect of antimicrobial agents on biofilms and planktonic cells using the Robbins device. After it was seen that antimicrobial agents did not provide full growth inhibition or control, the concept of resistant biofilms emanated (Lappin-Scott *et al.*, 2014).

The misuse of quaternary ammonium compounds (QAC) in the food industry is one of the main factors that is leading to an increase in the incidence of antimicrobial resistance (Buffet-Bataillon *et al.*, 2017). There is an increasing amount of antimicrobially resistant *L. monocytogenes* strains that are being isolated from food processing environments. It can be attributed to sub-lethal sanitation practises that stimulate AMR stress responses and horizontal gene transfer and exchange of AMR genes in food processing environments (Allen *et al.*, 2016). This is of great concern, since the main vehicle of human listeriosis is through contaminated food products (Xayarath & Freitag, 2012).

Kremer *et al.* (2017) evaluated the clinical outcome of listeriosis patients and the presence of a benzalkonium chloride tolerance gene, e*mrC,* of the bacteria*.* It was found that the presence of the *emrC* gene increased the incidence of ST6 *L. monocytogenes* meningitis infection within the scope of the study. These types of studies show how crucial it is for the food industry to continuously evaluate (in regard to bacterial resistance) and correctly apply sanitation practises. The food industry should keep in mind not only the effect thereof on the processing environments microbial count, but the long-term safety of their consumers

### 2.14.2 Quaternary Ammonium compounds

Quaternary ammonium compounds are chemicals that comprise of a plain or substituted alkyl group, negative ion and nitrogen atoms where the structure can be shown as $N^{+}$ $R^{1}$ $R^{2}$ $R^{3}$ $R^{4}$ $X^{-}$ (Buffet-Bataillon *et al.*, 2017). As a cationic chemical, the mode of action of QAC entails the disruption of the lipid bilayer of bacterial cells that holds the bacterial cytoplasmic membrane. Lower concentration

levels of QAC instigates the loss of osmoregulatory abilities of bacterial cells. Intermediary concentrations disturb membrane functions such as respiration and transport of solutes. High concentrations lead to complete solubilisation of cell membranes leading to imminent cell death (Gilbert & Moore, 2005)

### 2.14.3 Peracetic acid

The antimicrobial activity of peracetic acid (PAA) is mainly attributed to its oxidising ability (Srey *et al.*, 2013), specifically oxidation of components of bacterial cells (Finnegan *et al.*, 2010). It is an environmentally friendly chemical since it decomposes into acetic acid and hydrogen peroxide (Srey *et al.*, 2013; Lee *et al.*, 2015). Lee *et al.* (2015) found that PAA treatment of *L. monocytogenes* biofilms were insufficient and recommended further treatment optimisation studies. More specifically, Poimenidou *et al.* (2016) found the minimum inhibitory concentration of PAA ($MIC_{PAA}$) of certain *L. monocytogenes* strains to be higher than the recommended PAA concentrations outlined in their study. These studies are indications of the resistance of *L. monocytogenes* to the bactericidal effects PAA.

### 2.14.4 Alternative methods

#### *2.14.4.1 Listeria phages*

The use of phage treatment as a bio-control method for *L. monocytogenes* in a processing environment should be approached with caution, since a study by Vongkamjan *et al.* (2013) found a rapid increase in mutations for phage resistance. The conclusions of this study, advocates for the further research to be done, before the widespread use of *Listeria* phages in sanitation practices. Similarly, in a South African context, Strydom and Witthuhn (2015) found *in vitro* tests of phages as biosanitiser to be successful, but recommended further investigation of it, in factory simulated environments.

#### *2.14.4.2 Enzymes*

Enzymatic treatment can be performed to obtain inhibition and dispersal of biofilms, as Nguyen and Burrows (2014) attempted to demonstrate. In essence, enzymes can be used to eradicate biofilms, since the major component of the EPS is proteins and polypeptides (Cappitelli *et al.*, 2014). These enzymes include bromelain, papain, DNase I and Protease A. It was found that Protease A could completely eradicate a biofilm on good grade stainless steel where DNase I only managed to reduce attachment to polystyrene (Nguyen & Burrows, 2014). Industrial application of these enzymatic treatments are currently not feasible to be used as bio-control methods within a large processing environment. This is part in due to the high financial cost thereof. However, as Nguyen and Burrows (2014) recommended, enzymatic treatment could be used as a complimentary asset to current sanitation practices.

#### *2.14.4.3 Amysin*

Amysin is a non-nisin bacteriocin that is isolated from Thai shrimp paste. It is intended to be used as a broad spectrum biopreservative in conjunction with nisin to prevent the growth of pathogens in food products. It proved to have a listericidal effect in sliced bologna sausage stored at 4°C for seven days. Further development and exportation of this biopreservative has potential to aid the food industry with overcoming the emerging resistance to nisin and in turn with control of *L. monocytogenes* in all its forms (Kaewklom *et al.*, 2013).

#### *2.14.4.4 Chitosan*

The antimicrobial activity of chitosan is dependent on factors including molecular weight and degree of acetylation (Raafat *et al*., 2008; Goy *et al*., 2009). Assainar and Nair (2014) even suggest factors such as pH, temperature, and other molecules (proteins, fats and other antimicrobial agents) that might interfere with chitosan's antimicrobial action. Many studies have attempted to establish the bactericidal or bacteriostatic mode of action of chitosan, but are only able to yield theories; concluding that the mechanism of chitosan is more complex than is currently understood. Goy et al. (2009) proposes that the mode of action that is most acceptable is the interaction that takes place between the positively charged chitin/chitosan molecules and the negatively charged membranes of the target organisms. This electrostatic interaction between chitosan and the microorganism is believed to be the most reasonable conclusion and explanation for chitosan's antimicrobial action. A review by Kong *et al.* (2010) concludes that in order for chitosan to be successfully used as an antimicrobial agent, its mechanism need to be established as well as the causes of resistance to these mechanisms. Due to an increase of antimicrobial resistance among pathogens, the need for safer and natural antimicrobial agents has increased. Among several natural antimicrobials, chitosan exhibits a higher rate and broader spectrum of antimicrobial activity.

## 2.15 Conclusion

The survival and proliferation of *L. monocytogenes* within the RTE food sector is of major concern to the safety of consumers. Control and management efforts are continuously developing as the complex and sophisticated adaptation mechanisms of *L. monocytogenes* are revealed through ground breaking research. This review found that together with increase in global research efforts to gain more in-depth understanding of *L. monocytogenes*, awareness of the long-term effect of incorrect sanitation practises and the power of next generation sequencing has increased. It was also found that although South Africa contributes to 33% of African research outputs pertaining to foodborne pathogens (Paudyal *et al.*, 2017) there is still a severe lack of research and information regarding *L. monocytogenes* and human listeriosis cases within the South African context.

Finally, *L. monocytogenes* is a well-adapted foodborne pathogen, that can cause fatal infection when contaminated food is consumed by vulnerable groups. Isolation and identification

methods and regulations are currently adequate in managing this pathogen. Rapid'*L.mono* is shown to be a reliable routine method that can be used by the food industry. Although these methods discussed are currently satisfactory, validation and improvement of these methods are required to ensure that they maintain their abilities to isolate and identify an adaptive and evolving pathogen such as *L. monocytogenes*. Current methods available for source tracking are competitive, however for source tracking and interlaboratory comparison, automated ribotyping is still regarded as the best method to use. Although many different methods are available to study biofilms and the effect of sanitation, in any form, studying them under conditions simulation factory environments is more beneficial. Currently, the CEMS system will serve as an excellent tool to accomplish this objective. By combining emerging discoveries of *L. monocytogenes* with current identification and subtyping techniques, studying its survival and proliferation in a RTE food factory will become possible.

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# CHAPTER 3

# ISOLATION AND IDENTIFICATION OF *LISTERIA MONOCYTOGENES* IN A SOUTH AFRICAN READY-TO-EAT FOOD FACTORY

## 3.1 Abstract

As an ubiquitous, facultative pathogenic saprotroph, the presence of *Listeria monocytogenes* in ready-to-eat (RTE) food processing environments and products is of major concern to consumer safety. This study aimed to isolate and positively identify *L. monocytogenes* isolates as well as validate current isolation methods used in the RTE food factory. In collaboration with the factory, 432 environmental and food samples, in the form of inoculated Rapid'*L.mono* plates were collected. Visual inspection for presumptive positive *L. monocytogenes* growth, identified 64 samples. A Multiplex PCR protocol for the amplification of genes, namely *iap* (*Listeria* spp.) and *lmo2234* (*L. monocytogenes*) using One*Taq* endonuclease could not be optimised. A singleplex assay PCR as confirmation test was developed. The occurrence of enrichment bias in half Fraser broth due to *L. innouca* and method specific and conventional streaking methods were evaluated. The current Rapid'*L.mono* method was found suitable for detection of *L. monocytogenes*, in artificially inoculated enrichment broth.

## 3.2 Introduction

The occurrence of *L. monocytogenes* in ready-to-eat (RTE) foods has gained increased attention due to the health risks associated with this emerging pathogen. *L. monocytogenes* is ubiquitous in the environment, mostly due to it being a facultative pathogenic saprotroph. Forming part of the *Listeria* genus, *L. monocytogenes* is the species type associated with foodborne human and ruminant illness (Orsi *et al.*, 2011). It is set apart from other foodborne pathogens by its ability to survive at temperatures ranging from 1ºC (Morganti *et al.*, 2015), with optimal growth at 30-37ºC (Goldfine & Shen, 2007), wide pH range of 4.7-9.2 (Ferreira *et al.*, 2014) and the presence of unique genes exclusive to *L. monocytogenes* used in host invasion and evasion namely *hlyA, iap, ActA* and *lmaA* (Law *et al.*, 2015b). *Listeria monocytogenes* is readily isolated from ready-to-eat (RTE) food processing environments as well as RTE products (Campdepadrós *et al.*, 2012; Dalmasso & Jordan, 2012; Jamali *et al.*, 2013; Välimaa *et al.*, 2015).

RTE foods are defined as "…any food (including beverages) which is normally consumed in its raw state or any food handled, processes, mixed, cooked, or otherwise prepared into a form in

which it is normally consumed without further processing" (Foodstuffs, Cosmetics and Disinfectants Act and Regulations, 2010). The concern and danger of contamination of RTE food products with *Listeria* thus lies in the fact that no further heat treatment is required before consumption. In addition, the RTE food matrix readily supports the growth of *L. monocytogenes* (Spanu *et al.*, 2014), due to storage conditions and individually processed components.

Routine detection and analysis of *L. monocytogenes* in the food chain is of great importance (Välimaa *et al.*, 2015). The Regulation (EC) No 2073/2005 on "Microbiological Criteria for Foodstuffs" specifically require RTE food operators to include sampling of the environment and processing equipment for *L. monocytogenes* as well as to conduct challenge testing for food products that support the growth of *L. monocytogenes* (Spanu *et al.*, 2014). Contaminated food products are of great concern to public health since it is causes a fatal infection known as listeriosis, with vulnerable groups with impaired T-cell immunity (old, young, pregnant, and immunocompromised individual) being the most at risk (FDA, 2012; Goulet *et al.*, 2013).

*Listeria* management plans are implemented by Food business operators (FBO) in an attempt to control and evade the contamination of food products and to ensure that contaminated products do not enter the food chain. A lack in necessary control measures will result in costly food product recalls or possible fatalities (De Noordhout *et al.*, 2014; Ferreira *et al.*, 2014)

The ISO 11290-1:2017 (Horizontal methods for detection and enumeration of *L. monocytogenes* and of *Listeria* spp. Part 1 Detection method) (Anonymous, 2017) method is widely used but is labour intensive with results only being obtained after 72 h. In a high turnover, result driven laboratory, a simple and rapid method is required to ensure punctual information regarding the microbial safety of food products. This would ensure that immediate precautions can be taken. Rapid'*L.mono* is a chromogenic agar that can detect the presence of *L. monocytogenes* as well as differentiate it from other non-pathogenic *Listeria* spp. As an alternative proprietary method, it is NF Validated (according to ISO 16140), NordVal approved and an AOAC-RI approved (Nº 030406) method that delivers reliable results within 48 h (Anonymous, 2014).

*L. monocytogenes* occurs in low numbers within the environment and food products (Bruhn *et al.*, 2005) and can potentially grow within a community of other *Listeria* spp. In order to detect its presence, an enrichment step is included in both ISO 11290:1 and Rapid'*L.mono* protocols. However, when considering that *L. monocytogenes* is rarely isolated alone from the processing environment (Besse *et al.*, 2010; Barre *et al.*, 2016; Vongkamjan *et al.*, 2016), enrichment bias can result in false negative detection of *L. monocytogenes*. This could compromise the reliability of *Listeria* management plans in RTE food environments. Subsequently, the overgrowth of *L. innocua* masking *L. monocytogenes* during enrichment has been the subject of many studies. Oravcova *et al.* (2008) found standard methods to be insufficient for the detection of $10^0$ colony forming units

(CFU) *L. monocytogenes* in the presence of $10^0$ CFU *L. innocua.* Besse *et al.* (2010) assigned the overgrowth of *L. innocua* over *L. monocytogenes* to their interaction during the late exponential phase. In conjunction, Zitz *et al.* (2011a) found similar overgrowth with selective enrichment of low *L. monocytogenes* CFUs similar to that found in naturally contaminated food products.

Polymerase Chain Reaction (PCR) is a molecular tool used routinely as a typing method (FDA, 2012). It entails the amplification of selected genes for the identification of microorganisms based on their genetic material. Multiplex PCR, is a more rapid method because it allows for the simultaneous amplification of more than one gene (Law *et al.*, 2015a), subsequently decreasing time and labour to obtain the same amount of results. This would be an ideal method, especially within a high turnover laboratory. It would be able to readily confirm *L. monocytogenes* and differentiate it from other *Listeria* isolates, since a multiplex PCR assay simultaneously screen for the presence of multiple genes.

A PCR protocol, using One*Taq* endonuclease, for confirmation of presumptive positive *L. monocytogenes* samples from the RTE food factory was developed. All *L. monocytogenes* isolates identified were used in the other research chapters. In addition, a comparative evaluation was conducted on the enrichment step of the Rapid'*L.mono* method, together with different streaking methods to determine its ability to detect *L. monocytogenes* in the presence of *L. innocua.* The isolation and positive identification of *L. monocytogenes* strains in this chapter will be the strains selected for the other parts of this study as well as validate the current methods utilised in the RTE food factory.

## 3.3 Materials and methods

### 3.3.1 Sampling method

Initially selected areas, identified as hotspots by technical staff, within the RTE factory environment were swabbed using sterile plain wooden applicator cotton tipped swabs (Copen Diagnostics Inc, USA). All samples were transported to the laboratory on ice and processed within 12 h of sampling. Enrichment and plating onto Rapid'*L.mono* chromogenic agar was done according to the protocol for alternative methods outlined by Anonymous (2014). However, to increase sample intake as well as probability of positive *L. monocytogenes* isolates, a different approach was taken. This entailed collecting presumptive positive *L. monocytogenes* strains on Rapid'*L.mono* agar plates from the factory's own quality control and *Listeria* management programmes.

A total of 434 inoculated Rapid'*L.mono* plates were collected for the duration of the study. Presumptive positive *L. monocytogenes* plates were identified by distinct growth of single black colonies with no halo (Law *et al.*, 2015a). After visual inspection, a total of 64 plates were selected as presumptive positive samples and submitted for further processing.

For the first months of 2016, all *Listeria* testing was conducted by an outsourced laboratory in Cape Town. Thus, during the year 2016, the majority of agar were collected from there. At the end of 2016 and for the duration of 2017, the factory implemented in house *Listeria* testing. The in-house sampling plan varied to a moderate degree during the duration of this study as Quality Assurance (QA) and laboratory managers changed.

### 3.3.2 Sample processing and glycerol stocks

Samples collected from the factory in-house food safety laboratory in the form of presumptive positive *Listeria monocytogenes* Rapid'*L.mono* agar plates were transported to the research laboratory and further processed. Presumptive positive colonies (black colony, no halo) were streaked out onto Nutrient Agar (NA) (Oxoid, USA) plates to ensure the isolation of a pure colony. The NA plates were incubated at 37ºC for 15 - 18 h. A single colony of *L. monocytogenes* from the NA overnight plate was inoculated into 10 mL Tryptic Soy Broth (TSB) (Merck, USA). The TSB was then incubated at 37ºC for 15 - 18h. Glycerol stocks of isolates were prepared by addition of 600 µL TSB overnight culture to 400 µL 50% glycerol. Stocks were stored at -20ºC until further use.

### 3.3.3 PCR

*3.3.3.1 Primer selection*

Table 3.1 contains the primers selected for Multiplex PCR of presumptive positive *L. monocytogenes* isolates. The *iap* gene was selected because it encodes for the P60 gene and the *lmo2234* gene was selected since it was identified as a specific molecular marker in L. monocytogenes, in a previous study (Chen & Knabel, 2007).

**Table 3.1** Primer sequences for multiplex PCR

| Primer set | Sequence ('5 – '3) | Product size (bp) | Source |
|---|---|---|---|
| *iap* | (F) ATGAATATGAAAAAAGCAAC<br>(R) TTATACGCGACCGAAGCCAAC | 1450 -1600 | (Chen & Knabel, 2007) |
| *lmo2234* | (F) TGTCCAGTTCCATTTTTAACT<br>(R) TTGTTGTTCTGCTGTACGA | 420 | (Chen & Knabel, 2007) |

*3.3.3.2 DNA extraction*

DNA extraction 1 (DC1)

Crude DNA extraction was conducted as described by Germishuys (2017). However, to optimise the DNA extraction method, *L. monocytogenes* colonies from Tryptic Soy Agar (TSA) (Merck, USA) plates were not added to the lysis buffer. This is due to the small size of *L. monocytogenes* colonies. Rather, 1 mL of overnight cultures in TSB was added to sterile 1.5 mL Eppendorf tubes and

centrifuged for 1 min at 3 500 rpm (approximately 1 000 x g), the supernatant was removed and step was repeated in the same tube to obtain white culture pellet in tube.

DNA extraction 2 (DC2)

The method described for DC1 was adapted by extending the boiling time of culture pellet suspended in 300 µL lysis buffer, by 2 min.

*3.3.3.3 Reaction mixture composition*

*Reaction mixture 1 (RM1)*

PCR reaction mixtures were prepared to a final volume of 25 µL. The reaction mixtures consisted of 1 1X One*Taq* standard reaction buffer (20 mM Tris-HCl, 1.8 mM $MgCl_2$, 22 mM $NH_4Cl$, 22 mM KCl, 0.06% IGEPAL CA-630 and 0.05% Tween 20) (New England BioLabs Inc), 1 unit OneTaq DNA polymerase (New England BioLabs Inc), 1 µL template DNA, 0.3 µM of selected primers (*iap* and *lmo2234*) (Inqaba Biotec), 200 µM for each dNTP (dATP, dCTP, dGTP and dTTP) (New England BioLabs Inc).

*Reaction mixture 2 (RM2)*

PCR reaction mixtures were prepared to a final volume of 25 µL. The reaction mixtures consisted of 1 1X One*Taq* standard reaction buffer (20 mM Tris-HCl, 1.8 mM $MgCl_2$, 22 mM $NH_4Cl$, 22 mM KCl, 0.06% IGEPAL CA-630 and 0.05% Tween 20) (New England BioLabs Inc), 1 unit OneTaq DNA polymerase (New England BioLabs Inc), 1 µL template DNA, 3 µM of selected primers (*iap* and *lmo2234*) (Inqaba Biotec), 200 µM for each dNTP (dATP, dCTP, dGTP and dTTP) (New England BioLabs Inc).

*Reaction mixture 3 (RM3)*

PCR reaction mixtures were prepared to a final volume of 25 µL. The reaction mixtures consisted of 1 1X One*Taq* standard reaction buffer (20 mM Tris-HCl, 1.8 mM $MgCl_2$, 22 mM $NH_4Cl$, 22 mM KCl, 0.06% IGEPAL CA-630 and 0.05% Tween 20) (New England BioLabs Inc), 1 unit OneTaq DNA polymerase (New England BioLabs Inc), 1 µL template DNA, 30 µM *lmo2234* primer and 35 µM *iap* primer (Inqaba Biotec), 300 µM for each dNTP (dATP, dCTP, dGTP and dTTP) (New England BioLabs Inc).

*Reaction mixture 4 (RM4)*

PCR reaction mixtures were prepared to a final volume of 25 µL. The reaction mixtures consisted of 1 1X One*Taq* standard reaction buffer (20 mM Tris-HCl, 1.8 mM $MgCl_2$, 22 mM $NH_4Cl$, 22 mM KCl, 0.06% IGEPAL CA-630 and 0.05% Tween 20) (New England BioLabs Inc), 1 unit OneTaq DNA polymerase (New England BioLabs Inc), 1 µL template DNA, 0.3 µM primer, *iap* and *lmo2234,* respectively (Inqaba Biotec), 200 µM for each dNTP (dATP, dCTP, dGTP and dTTP) (New England BioLabs Inc).

*3.3.3.4 PCR conditions*

*PCR condition 1 (PC1)*

The PCR conditions followed a cycle of an initial denaturation at 95ºC for 15 min, which was followed by 30 cycles of 94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min. The PCR cycle was ended with a final extension step of 72ºC for 8 min. PCR was completed using a Bio-Rad T100 Thermal Cycles (Bio-Rad, South Africa)

*PCR condition 2 (PC2)*

The PCR conditions included a cycle of initial denaturation at 95ºC for 15 min, which was followed by annealing of 15 cycles of 94ºC for 1 min, 55ºC for 30 sec, 51ºC for 30 sec and 72ºC for 1 min. Then a further 15 cycles of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min. The PCR cycle was ended with a final extension step of 72ºC for 8 min. PCR was completed using a Bio-Rad T100 Thermal Cycles (Bio-Rad, South Africa)

*PCR condition 3 (PC3)*

The PCR conditions included a cycle of initial denaturation at 95ºC for 15 min, which was followed by annealing of 15 cycles of 94ºC for 1 min, touchdown from 55ºC to 51ºC (3 cycles per temperature and 72ºC for 1 min. Then a further 15 cycles of 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min. The PCR cycle was ended with a final extension step of 72ºC for 8 min (Chen and Knabel, 2007). PCR was completed using a Bio-Rad T100 Thermal Cycles (Bio-Rad, South Africa)

*PCR condition 4 (PC4)*

The PCR conditions included a cycle of initial denaturation at 94ºC for 30 sec, which was followed by annealing of 30 cycles of 94ºC for 30 sec, 50ºC for 1 min, and 68ºC for 1 min. The PCR cycle was ended with a final extension step of 68ºC for 5 min. PCR was completed using a Bio-Rad T100 Thermal Cycles (Bio-Rad, South Africa)

*3.3.3.5 Gel electrophoresis visualisation conditions*

*Gel electrophoresis condition 1 (GC 1)*

Visualisation of PCR products was performed through gel electrophoresis, using 1.2% agarose gel, stained with GRGreen nucleic acid gel stain (Lab Supply Mall, InnoVita Inc). The gel underwent electrophoresis for 40 min at 80 V along with a 100 bp DNA Ladder (New England BioLabs Inc). Gel was visualised using the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) and its accompanying Image Lab Software (version 5.2.1).

*Gel electrophoresis condition 2 (GC 2)*

Visualisation of PCR products was performed through gel electrophoresis, using 1% agarose gel, stained with EZ Vision® IN-GEL solution (Amresco, LLC). The gel underwent electrophoresis for 60 min at 70 V along with a 100 bp DNA Ladder (New England BioLabs Inc). Gel was visualised using

the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) and its accompanying Image Lab Software (version 5.2.1).

**Table 3.2** Combinations of conditions for PCR optimisation trials

| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 |
|---|---|---|---|---|---|---|
| DNA extraction conditions (DC) | 1 | 1 | 1 | 2 | 2 | 2 |
| Reaction mixture (RM) | 1 | 1 | 2 | 3 | 2 | 4 |
| PCR condition (PC) | 1 | 2 | 3 | 4 | 4 | 4 |
| Gel electrophoresis condition (GC) | 1 | 2 | 2 | 2 | 2 | 2 |

### 3.3.4 Enrichment bias

*3.3.4.1 Validation of method*

Glycerol stocks of *Listeria inncoua* sample 133 (DuPont ID: 1006) and *L. monocytogenes* sample 135 (DuPont ID: 1042) were selected. Due to similar sources (Table 3.3), their growth fitness and behaviour is expected to be similar, resulting in a more reliable demonstration of growth behaviour. These samples were also selected as they would represent strains from this factory that were exposed to the factory's sanitation practises and environmental changes.

**Table 3.3** Isolates used for enrichment bias study

| Specie | Sample number | Source | DuPont ID |
|---|---|---|---|
| *Listeria innocua* | 133 | Drain defrost chiller | 1006 |
| *Listeria monocytogenes* | 135 | Drain pizza exit | 1042 |

Cultures of *L monocytogenes* and *L. innocua* were made by inoculating 10 mL TSB with a loopful of glycerol stock which was incubated at 37°C for 15 – 18h. These *Listeria* cultures were standardised to 0.1 OD using the formula: $c_1v_1=c_2v_2$. Standardisation of overnight culture was repeated five times and optical density (OD) of each was measured. Three dilution series were made from each standardised overnight culture and plated out on NA plates in duplicate. The agar plates were incubated overnight at 37ºC and counted.

Analysis of variance (ANOVA) was performed to determine whether there were significant differences between the CFU produced from a known concentration, due to standardisation *L. monocytogenes* and *L. inoccua,* as well as p-values of replications demonstrated in Figure 3.1 using Statistica, (Dell Inc. 2016, version 13).

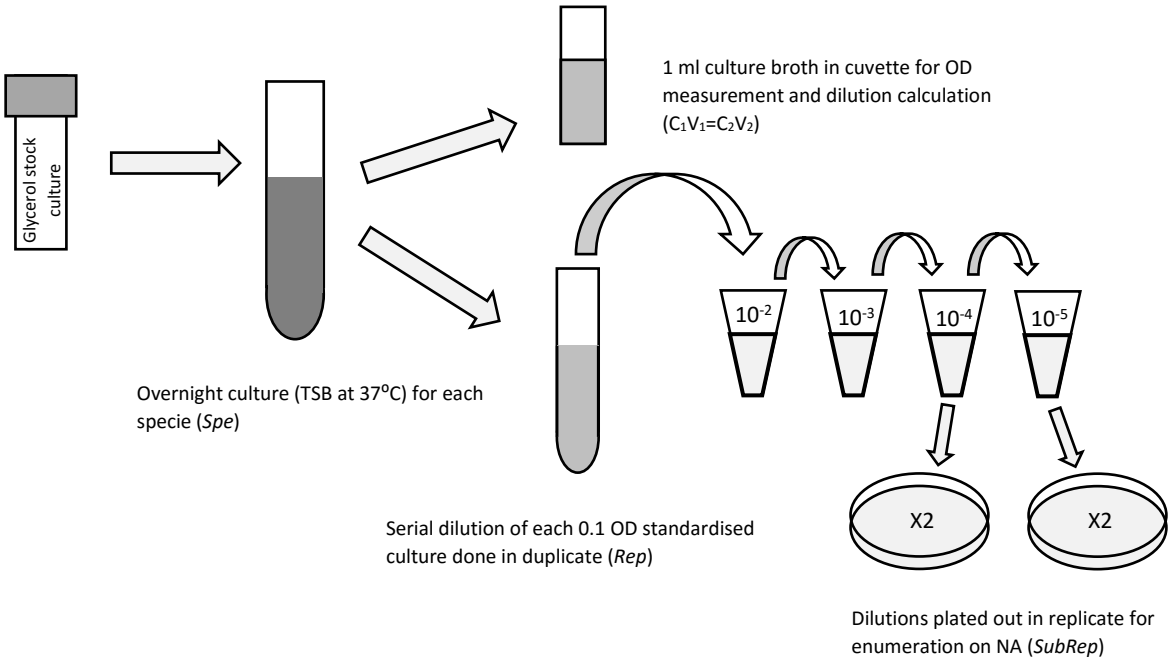


**Figure 3.1** Outline of protocol used for confirmation of equal CFU's present in a standardised 0.1 OD *L. monocytogenes* and *L. innocua* culture broth.

*3.3.4.2 Demonstration of enrichment bias on Rapid'L.mono plates*

Overnight cultures of *L. monocytogenes* and *L. innocua* were made by inoculation 10 mL TSB with a loopful of glycerol stock and incubated at 37 ºC for 15 - 18h (Figure 3.2).

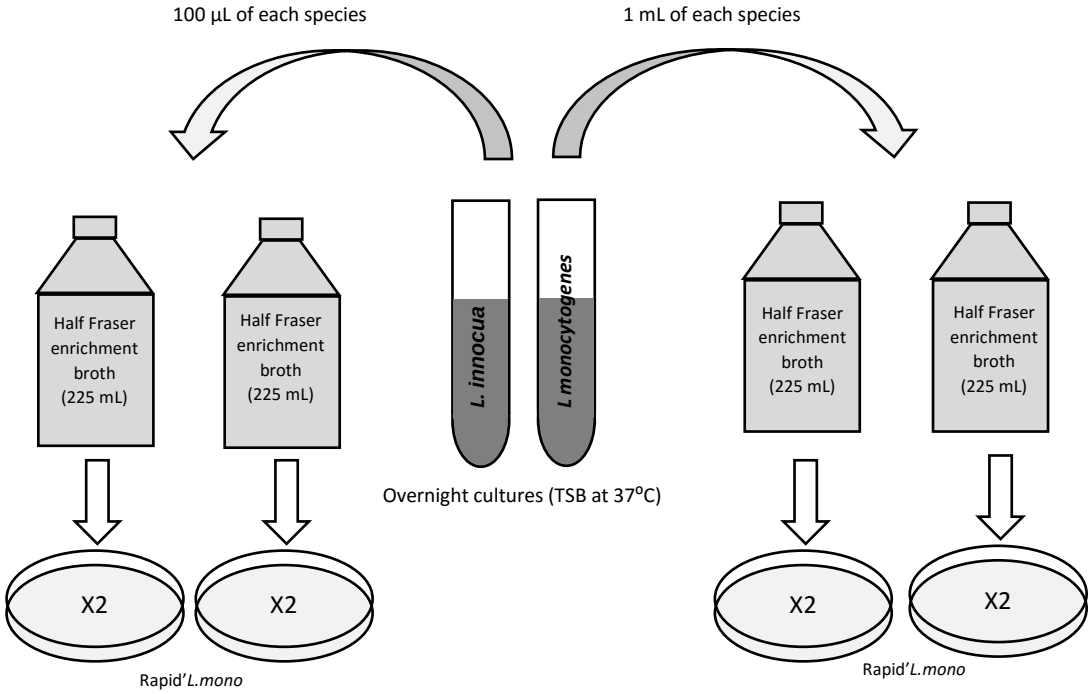


**Figure 3.2** Outline of protocol to study growth behaviour of co-inoculated half Fraser enrichment of *L. monocytogenes* and *L. innocua*.

Overnight cultures, as previously described were standardised to 0.1 OD. As outlined in Figure 3.2, 100 µL and 1 mL of each species was added to half Fraser enrichment broth (prepared according to manufacturer's instructions). The enrichment broth was incubated for 24 h at 37ºC. The enrichment broth was streaked out onto Rapid'*L.mono* agar in accordance with the experimental plan (Figure 3.2). Plates were incubated at 37ºC for 15 – 18h, after which it was inspected for growth behaviour of both *L. monocytogenes* and *L. innocua*.

## 3.4 Results and discussion

### 3.4.1 Multiplex PCR

*3.4.1.1 Extraction of DNA*

The crude extraction of DNA was optimised in 2 trials. DC1 produced only 79% viable DNA samples as in 21% of the samples no DNA was obtained. This could be attributed to the gram-positive nature of *Listeria* spp., which could reduce the release of DNA when compared to gram negative organism (Riffiani *et al.*, 2015) for which the original protocol was optimised for in Germishuys (2017). It was therefore decided in DC2 to increase the boiling time in the lysis buffer by 2 min to allow better conditions for complete extraction of DNA.

*3.4.1.2 Reaction mixture composition*

A total of 4 different reaction mixtures were used in an attempt to obtain a multiplex PCR result. RM1 confirmed the correct selection of primers as amplification was observed for *iap* and *lmo2234* at the expected regions of 1450-1600 bp and 420 bp, respectively (Table 3.1). However, amplification of two target gene sequences i.e *lmo2234* and *iap* was not seen as expected of multiplex PCR, as amplification preference was given to *lmo2234.* RM2, first attempted in trial 3, entailed a 10x increase in primers in an attempt to eliminate amplification preference, however a similar outcome to RM1 was obtained. Trial 4 included RM3 which saw a 5 µM increase in *iap* primer as well as 100 µM increase in dNTP's in an attempt to increase the probability of simultaneous *lmo2234* and *iap* amplification. RM3 only resulted in brighter amplicons, but still without the multiplex effect. RM4 was compiled for a single amplification and subsequently only contained one primer, *lmo2234* and *iap,* respectively. It was selected in trial 5 as the final reaction mixture for all confirmation tests to follow.

The positive control used was a *L. monocytogenes* strain previously isolated by Rip and Gouws (2009). As a confirmation step, it was ribotyped and confirmed as *L. monocytogenes* DuPont ID 19175.

*3.4.1.3 PCR conditions*

In order to have successful multiplex PCR amplification conditions, annealing temperatures that facilitate the amplification of all target genes present must be optimised. The initial PCR conditions (PC1-3) was adapted from Chen and Knabel (2007), which optimised a protocol for amplification of

multiple target genes, of which only two was the subject of this study. PC1 followed the Chen and Knabel (2007) protocol without application of touchdown annealing cycles. PC2 comprised of the lowest and highest annealing temperatures as described in section 3.3.3.4. PC3 incorporated the exact touchdown PCR conditions described by Chen and Knabel (2007). After these trails, the different PCR conditions still yielded a failed multiplex PCR (Figure 3.3). PC4 was subsequently developed specifically taking into account the reaction mixture that contained primers and One*Taq* endonuclease, since One*Taq* was not used by Chen and Knabel (2007) from which the protocols are based on.

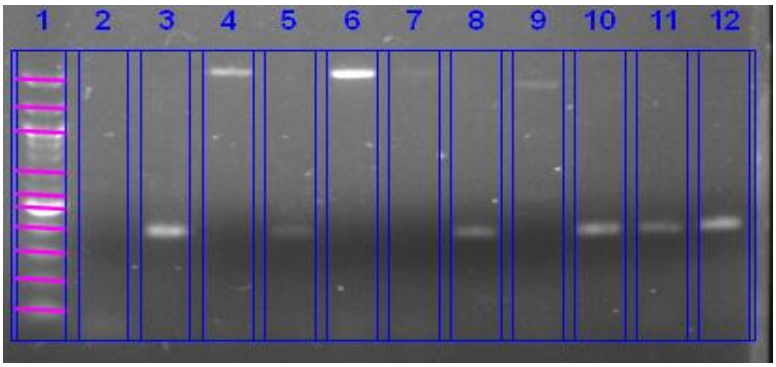


**Figure 3.3** Gel image demonstrating selective amplification of *iap (lane 4,5 and 9)* and *lmo2234 (lane 3,5,8 and 10-12),* negative control (lane 2) and positive control (lane 3).

It was advised, that One*Taq* would not facilitate multiplex amplification (A. Abera, 2017, Technical support manager, Inqaba Biotechnical Industries (Pty) Ltd, Hatfield, South Africa, personal communication, 30 January) and the results from the tailored PCR cycle (PC4), confirmed this. Multiplex amplification was not obtained, but excellent amplification of single target genes was obtained (Figure 3.4). Trial 6 thus included a single primer using PC4 to obtain a single PCR.

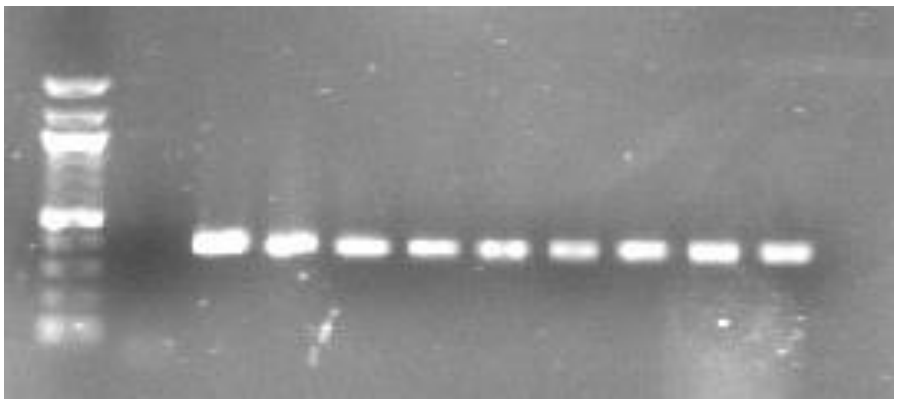

**Figure 3.4** Gel image demonstrating successful amplification of *lmo2234,* negative control (lane 2) and positive control (lane 3).

*3.4.1.4 Gel electrophoresis conditions*

Visualisation of PCR products was initially performed (GC1) through electrophoresis of 1.2% agarose gel for 40 min. This resulted in restricted movement of PCR amplicons and the 100 bp DNA ladder did not show sufficient separation. Thus, for the remainder of the trails GC2, electrophoresis of a 1% agarose gel for a 60 min run time, was utilised.

*3.4.1.5 Finalisation of methods*

Judging from the outcome of the tested reaction conditions, multiplex PCR amplification was unsuccessful. It was thus decided not to use multiplex PCR, but singleplex PCR. As mentioned in section 3.4.1.3, it was advised that One*Taq* would not be able to facilitate multiplex PCR gene amplification and another polymerase should be considered. This resulted in a more labour intensive protocol, but resulted in developing a method for confirmation of presumptive positive *L. monocytogenes* from enrichment media. In order for a multiplex PCR protocol to work, further optimisation should be done using different parameters and a more specific polymerase.

### 3.4.2 Enrichment bias

The basis for the development of a test to demonstrate possible enrichment bias was due to the growth behaviour of *L. monocytogenes* and *L. innocua* observed on Rapid'*L.mono* plates obtained from samples from the factory environment.

*3.4.2.1 Growth behaviour observed from factory samples*

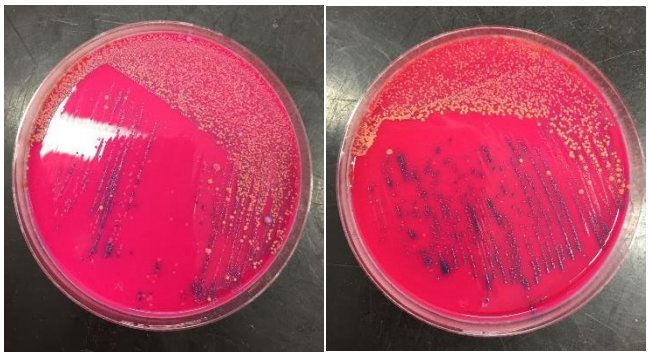

**Figure 3.5** Multi-specie *Listeria* on Rapid'*L.mono* plates obtained RTE food factory's *Listeria* management program.

Growth behaviour between the species of *Listeria* is demonstrated in Figure 3.5. The purple colour of the plates are only due to long term storage at refrigerated temperatures and is therefore not a reflection of microbial activity. These sample plates were not representative of all mixed species plates, but are discussed here due to the interesting multi specie growth behaviour observed. The Rapid'*L.mono* plates demonstrates that *L. monocytogenes* (black colony) can be present with other species, in this case *L. innocua* (white colony). This demonstrates the importance of testing for all

*Listeria* spp., since non-pathogenic *Listeria* spp. can be an indicator of the presence of *L. monocytogenes*. At the initial streaking site (top right corner on plates in Figure 3.5) an overgrowth of *L. innocua* was observed. It was thought to be due to enrichment bias, whereby *L. innocua* is known to mask the presence of *L. monocytogenes*. This observation formed the basis for development of a test, described below to determine whether L. innocu does outcompete L. monocytogenes under the same conditions. It can be seen that *L. monocytogenes (*black colony) can survive in the processing environment together with other species (Figure 3.5).

*3.4.2.2 Inoculum standardisation*

The standardisation protocol (section 3.3.4.1), utilising optical density showed no significant difference between standardised CFU.mL$^{-1}$ of both *L. monocytogenes* and *L. innocua* (*Spe*: p=0.678). In addition, there was no significant difference between the replications (*Rep*: p=0.292) and sub-replications (*SubRep*: p=0.856). This confirmed that if an enrichment broth was inoculated with standardised 0.1 OD culture of *L. monocytogenes* and *L. innocua* respectively, it would be inoculated with the same amount of CFU.mL$^{-1}$.

*3.4.2.3 Plating of co-inoculated half Fraser enrichment broth*

This test was done in an attempt to replicate the observed bacterial growth behaviour of sample plates seen in Figure 3.5.

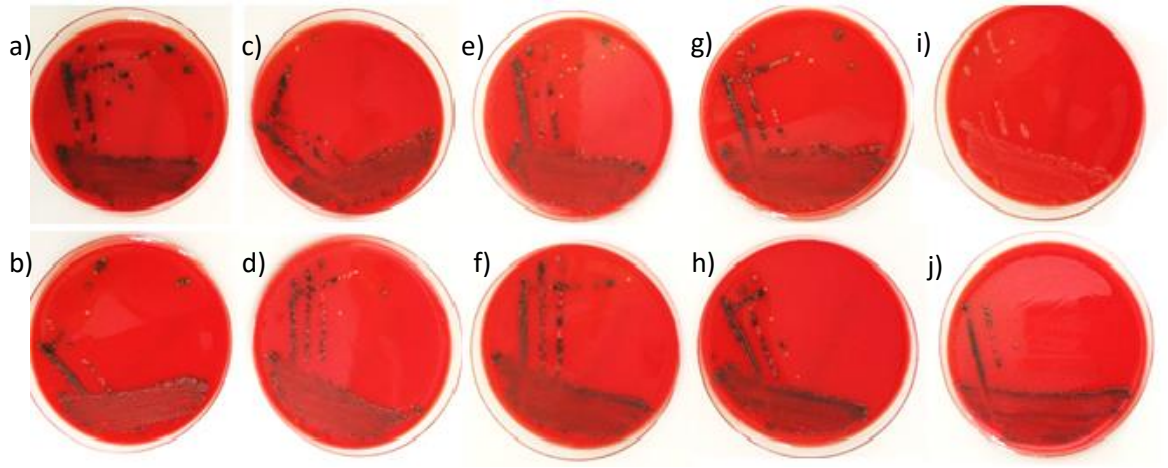


**Figure 3.6** Rapid'*L.mono* plates of simultaneous half Fraser enrichment of *L. monocytogenes* and L. *innocua*. a-d) had 100 µL of 0.1 OD as initial inoculation, e-f) 1 mL of O.1 OD of initial inoculation. i) *L. innocua* control, j) *L. monocytogenes* control.

The growth behaviour of the *Listeria* spp. (Figure 3.5) could not be replicated (Figure 3.6) using the experimental parameters. In this test no overgrowth of *L. innocua* was seen as expected. On the contrary, *L. monocytogenes* was the dominant species observed. This growth pattern occurred even though the enrichment broth was inoculated with the same amount (CFU) of both *L. monocytogenes* and *L. innocua*. This was a possible indication that enrichment bias in half Fraser

broth does not occur due to different growth rates of *L. monocytogenes* and *L. innocua*. The growth behaviour observed in Figure 3.5 could possibly indicate that *L. innocua* naturally occurs in larger numbers, and thus stunts the enrichment of *L. monocytogenes* through the Jameson effect (Besse *et al.*, 2010).

Additionally, it was seen that even though Rapid'*L.mono* plates a-d (Figure 3.6) were inoculated with 10x less CFU's, the amount of growth on the plates appear to be equal. This could be a further demonstration of the Jameson effect, where growth of a whole microbial community is halted once the dominant species reaches its stationary phase or where all the nutrient available has been depleted.

*3.4.2.4 Comparrison of different streaking methods for detection of L. monocytogenes*

The test aimed to evaluate the difference in detectability of *L. monocytogenes* (black colony) in the presence of *L. innocua* (white colony) using the of Rapid'*L.mono* manufacturer recommended streaking method (AFNOR Certified (EN ISO 16140)) (c,d,g,h) and single loop streaking method (a,b,e,f). *L. monocytogenes* was detectable using both methods, as evident by the black colonies present at each replication. The undetectability of white colonies at initially streaked region of plate c,d,g,h (Figure 3.7) could give the impression that no *L. innocua* was present.

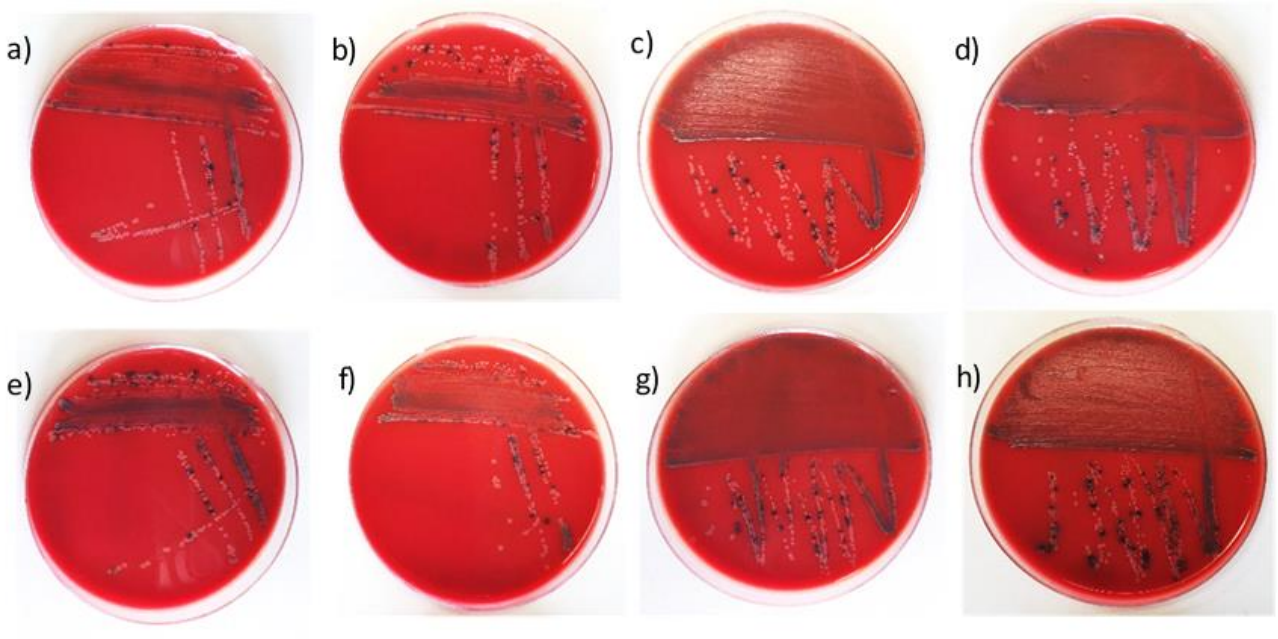


**Figure 3.7** Comparison of Rapid'*L.mono* manufacturer recommended streaking method (AFNOR Certified (EN ISO 16140)) (c,d,g,h) and single loop streaking method (a,b,e,f).

The results obtained from this test was contradictive from what was seen in the previous test (Figure 3.6). Here the presence of both species can be seen, with *L. innocua* even being seen in larger

quantities. It can thus be concluded that the enrichment step, similar to other culturing conditions, favours the growth of *L. innocua* over *L. monocytogenes*, but not to the extent that it masks the presence of *L. monocytogenes* (Zitz *et al.*, 2011b). This observation is limited to both species being present in the exact same quantities. If, as in the case of naturally contaminated samples, *L. innocua* is present in larger quantities, the overgrowth of *L. monocytogenes* might be observed more clearly as in Figure 3.5. Finally, the results obtained here were further evidence that *L. innocua* and *L. monocytogenes* can grow and proliferate together within various environments, enrichment and food matrices.

## 3.5 Conclusion

During this study, it was found that the use of Rapid'*L.mono* agar as a detection method within a *Listeria* management programme of a RTE food processing environment was a suitable and effective method. From the samples collected, 15% of the plates could be identified as presumptive positive for *L. monocytogenes*. This provided a good influx of samples to be verified and processed in order to achieve the other objectives set out in other chapters of this study.

Crude DNA extraction was investigated and optimised for PCR amplification of *L. monocytogenes* and *Listeria* spp. target genes. Optimum extraction was obtained after two trials, where it was found that additional boiling time enhanced the efficacy of DNA extraction method for the gram-positive bacterium. The aim of this study was set to develop and optimise a multiplex PCR protocol for differentiation between *L. monocytogenes* and *Listeria* spp genes. After three PCR trails, adapted from a previous study it was found that due to the use of a different endonuclease, a tailored PCR protocol was required. However, the results of trial 5 showed that OneT*aq* endonuclease would not facilitate multiplex PCR for the target genes. It was then decided to optimise individual amplification of *iap* (*Listeria* spp.) and *lmo2234* (*L. monocytogenes*) genes. Trail 6 yielded the required PCR amplification results and subsequently, rapid differentiation and confirmation of *L. monocytogenes* and *Listeria* spp.

After the observation of the growth behaviour of *L. monocytogenes* and *L. innouca* on samples obtained from the sampling plan, an experiment was designed to investigate whether enrichment bias was the cause thereof. Standardisation method of *L. monocytogenes* and *L. innouca* inoculums were optimised and the enrichment bias of half Fraser enrichment broth (Oxoid, USA) was evaluated. It was found that when the same quantity of *L. monocytogenes* and *L. innouca* are enriched together, there is no overgrowth of one organism over the other and thus no enrichment bias. Further studies can be conducted, using the optimised methods, to evaluate enrichment bias where levels of *L. monocytogenes* are less than *L. innocua*. This will be a better reflection of naturally contaminated food products.

In addition, a comparison was done between the manufacturer recommended streaking method and conventional streaking methods. The aim was to determine whether the streaking method could deliver false negative results when streaked on Rapid'*L.mono.* It was found to not have any effect on the results obtained and that both methods of streaking were sufficient for the detection of *L. monocytogenes*. The factors that contribute to the occurrence of enrichment bias found during the detection of *L. monocytogenes* in food products may thus be the food matrix and the dynamics of a naturally contaminated product. This warrants further investigation into these factors and how to overcome them.

To conclude, the isolation and identification of *L. monocytogenes* was obtained, through collaboration with the RTE food factory's *Listeria* management program and development of a PCR protocol. The Rapid'*L.mono* method, which includes half Fraser enrichment, was found to be reliable.

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Zitz, U., Zunabovic, M., Domig, K.J., Wilrich, P.T. & Kneifel, W. (2011b). Reduced detectability of *Listeria monocytogenes* in the presence of *Listeria innocua*. *Journal of food protection*, **74**, 1282–1287.

# CHAPTER 4

# AUTOMATED RIBOTYPING AND CLUSTER ANALYSIS OF *LISTERIA MONOCYTOGENES* ISOLATES FROM A SOUTH AFRICAN READY-TO-EAT FOOD FACTORY

## 4.1 Abstract

In this study 64 isolates from a ready-to-eat food factory were ribotyped using DuPont RiboPrinter®. Cluster analysis was conducted to evaluate the relatedness of strains from different sources, aiming to establish possible contamination routes and mechanisms. The geographical distribution of similar strains indicated that work boots, trolleys and crates were vectors for *L. monocytogenes* contamination. The Pearson correlation dendrogram also indicated the harbourage of strains and possible drain biofilms in both low and high-risk areas. Assigned DuPont ID's allowed for comparison of strains found with similar studies as well as comparison on an international database, Food Microbe Tracker. DuPont ID 1038, 1041, 1042 and 18596 found in the factory have been previously implicated in food recalls and clinal listeriosis cases. The *L. monocytogenes* contamination trends identified in this RTE factory, correlated with current global trends.

## 4.2 Introduction

Post production contamination of ready-to-eat (RTE) foods with *Listeria monocytogenes* is of major concern to the RTE food industry, as the products, per definition, require no further heating before consumption (Foodstuffs, Cosmetics and Disinfectants Act and Regulations, 2010). The main cause of post-production contamination is the contact of food products with contaminated food contact surfaces (FCS) (Fouladynezhad *et al.*, 2013; Vongkamjan *et al.*, 2013; Nyarko & Donnelly, 2015). The FCS are contaminated through cross contamination with the processing environment as well as other non-food contact surfaces. In order for a Food Business Operator (FBO) to manage *L. monocytogenes,* identifying sources that could harbour both planktonic cells and biofilms is essential. Source tracking should subsequently be implemented, for which ribotyping has been utilised successfully in various studies (Klaeboe *et al.*, 2005, 2006; Meloni *et al.*, 2009; Rosef *et al.*, 2012).

Ribotyping is a type of restriction fragment length polymorphism (RFLP) analysis because it is dependent on varying locations and number of ribosomal RNA (rRNA) gene sequences found in bacterial genomes. It is a rapid molecular detection technique that can identify and type bacteria to their strain level by analysis of band pattern or ribopattern differences (Lin *et al.*, 2014). These bands

originate when labelled rRNA is hybridised with DNA fragments obtained from the cleavage of total DNA by the selected endonuclease (Lorber, 2014). The main cause of variations in the ribopatterns are the variations in flanking sequences among the different strains. These variations in flanking sequences originate from point mutations in the housekeeping genes due to random genetic drift, not subject to Darwinian evolution (Bouchet *et al.*, 2008).

Automated ribotyping can be conducted by the DuPont RiboPrinter® Microbial Characterisation System by Qualicon Inc. (Welmington, DE). This instrument conducts all processes associated with ribotyping automatically, ensuring reproducibility and standardisation (Wiedmann, 2002; Pavlic & Griffiths, 2009). Each sample that was processed was then assigned a unique DuPont Identification Library Code related to the restriction enzyme used. The DuPont RiboPrinter® system has been the instrument of choice in similar studies conducted, due to its reproducibility, reliability and interlaboratory comparison ability. Kabuki *et al.* (2004) used automated ribotyping as a typing method to track *L. monocytogenes* in a fresh cheese processing area. Klaeboe *et al.* (2006) studied the ribotype diversity of *L. monocytogenes* in Norwegian salmon processing plants and De Cesare *et al.* (2007) utilised automated ribotyping for *L. monocytogenes* source tracking in the Taleggio cheese production plant.

The ability to compare ribotypes based on interlaboratory comparison is enhanced by assigned DuPont ID's using online databases that include Food Microbe Tracker (www.foodmicrobetracker.com). Since DuPont ID's are universal and standardised, more information about an isolate can be obtained from literature where further studies have been conducted, such as serotyping and lineage assignment (Meloni *et al.*, 2009; Klaeboe *et al.*, 2010; Rosef *et al.*, 2012). This allows the gathering and comparison of information that could not be obtained within the limits of a study, in order to make more informed conclusions.

Four distinct groups within *L. monocytogenes* species have been identified, based on phylogenetic and subtyping studies. These groups are known as lineage groups, with a majority of specie strains linked to lineage I (serotype 1/2b, 3b, 3c, 4b) and lineage II (1/2a, 1/2c, 3a) (Orsi *et al.*, 2011; Da Silva & De Martinis, 2013). Lineage I and II represent the majority of human listeriosis isolates and food and environmental isolates, respectively (Milillo & Wiedmann, 2009). It is of importance to know when implementing source tracking and risk management as lineage assignment is an indication of pathogenicity and environmental survival ability. Due to the underrepresentation of lineage III and IV in food and clinical isolates (Orsi *et al.*, 2011), these lineage types will be excluded from this study.

The aim of this study was to ribotype *L. monocytogenes* isolates from the RTE food factory in the Western Cape, as well as the company's Gauteng branch, using automated *Eco*RI ribotyping. The correlation and relatedness of the isolates and their sources using a Pearson correlated

dendrogram clustering the fingerprint data obtained from riboprinting, was created. Finally, the assigned DuPont ID was used to conduct an interlaboratory and interstudy comparison as a way to better understand the *L. monocytogenes* contamination mechanism.

## 4.3 Materials and methods

### 4.3.1 Selection of samples for Ribotyping

The selection of isolates to be ribotyped was not done with statistically significant parameters, but samples were rather selected based on a set of prioritised criteria that would support the aim and objectives of this study. In addition, due to the limited availability of positive *L. monocytogenes* samples and the high cost of automated ribotyping, samples were selected according to the following criteria, in descending priority:

a) Food isolates were selected due to the limited amount available;
b) Drain samples of cold storage and processing areas, since drain samples were a good indication of the current microflora present (Dzieciol *et al.*, 2016);
c) Other samples of non-food contact surfaces.

### 4.3.2 Sample preparation for automated ribotyping

Glycerol stock cultures were resuscitated by a loopful inoculation thereof in Tryptic Soy Broth (TSB) and incubation took place at 37°C for 15-18h. It was then streaked out onto Nutrient Agar (NA) and incubated for 15-18h at 37°C to obtain pure colonies. As instructed by the manufacturer's protocol, sterile colony picks were used to transfer samples to sample holder. Two separate single colonies per plate were picked up to ensure good quality riboprints, since a single colony as suggested by the manufacturer (Anonymous, 2013).

### 4.3.3 Automated ribotyping

Automated ribotyping was conducted in accordance with DuPont RiboPrinter® manufacturer's instructions for gram positive isolates. This entails suspending the pure colony picked picked from NA plate in 40 µL sample buffer using the handheld vortex provided. A 30 µL of cell suspension was transferred to sample carrier provided. The sample carrier was then placed in heating dock, for deactivation of cells. After heat treatment 5 µL Lysing Agent A and B were added to samples, respectively. System consumables and the sample carrier was manually loaded into the RiboPrinter® as guided by the system programme (Anonymous, 2013). The RiboPrinter® subsequently carries our cell lysis, DNA digestion, gel electrophoresis, DNA transfer to hybridisation membrane and finally Southern hybridisation with a chemiluminescent probe (Lin *et al.*, 2014).

### 4.3.4 Dendrogram construction for data analysis

A dendrogram was constructed using riboprint fingerprint data with a Pearson correlation using BioNumerics software package version 7.6.2 (Applied Maths, Sint-Martens-Latem, Belgium).

## 4.4 Results and discussion

Riboprint data obtained from automated ribotyping is processed by DuPont RiboPrinter® software. Figure 4.1 demonstrates the image output of two riboprint sample sets. Fingerprint and riboprint data that entail strain identification (genus and specie), correlation coefficients and DuPont ID could be obtained with set (a) and (b) yielded no results.

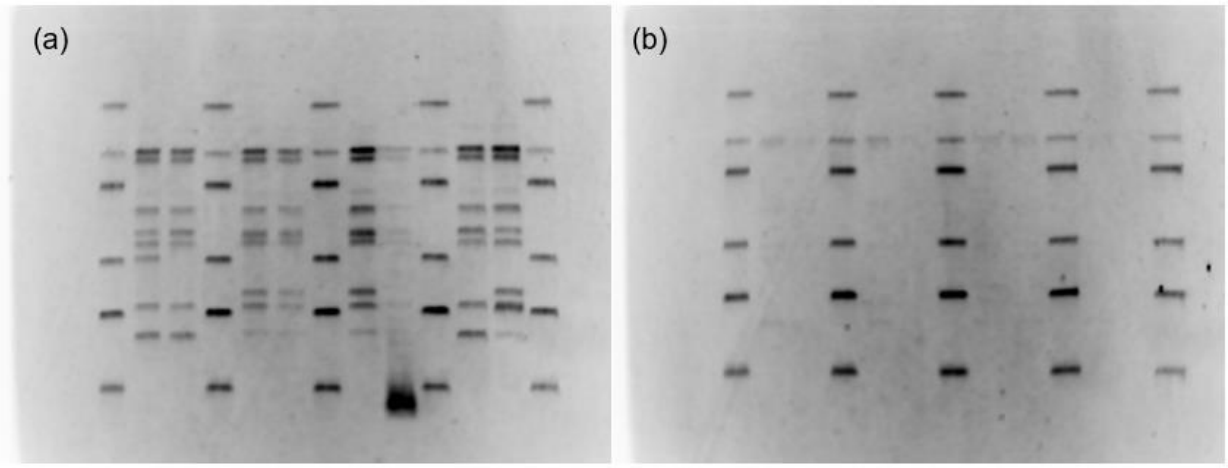


**Figure 4.1** Riboprinter images obtained (lane 1,4,7,10,13 are DuPont internal marker DNA) (a) sufficient number of cells inserted into RiboPrinter® (b) insufficient number of cells inserted into RiboPrinter®.

The clustering of similar DuPont ID's confirms their use as indicators of similar subtypes further supporting the use of automated ribotyping as a reliable method of molecular identification and source tracking (Figure 4.2). Additionally, a very definite distinction can be seen between clustering of *Listeria* spp. isolates and *Staphylococcus sciuri* isolates, which serves as confirmation of the reliability of the dendrogram. DuPont ID 1006 and 1017 were *L. innocua* isolates from 2016 and 2017, respectively. These samples were subtyped to obtain a positive control (DuPont ID 1006) for other tests as well as to confirm the differentiation ability and reliability of both the Riboprinter® and the dendrogram.

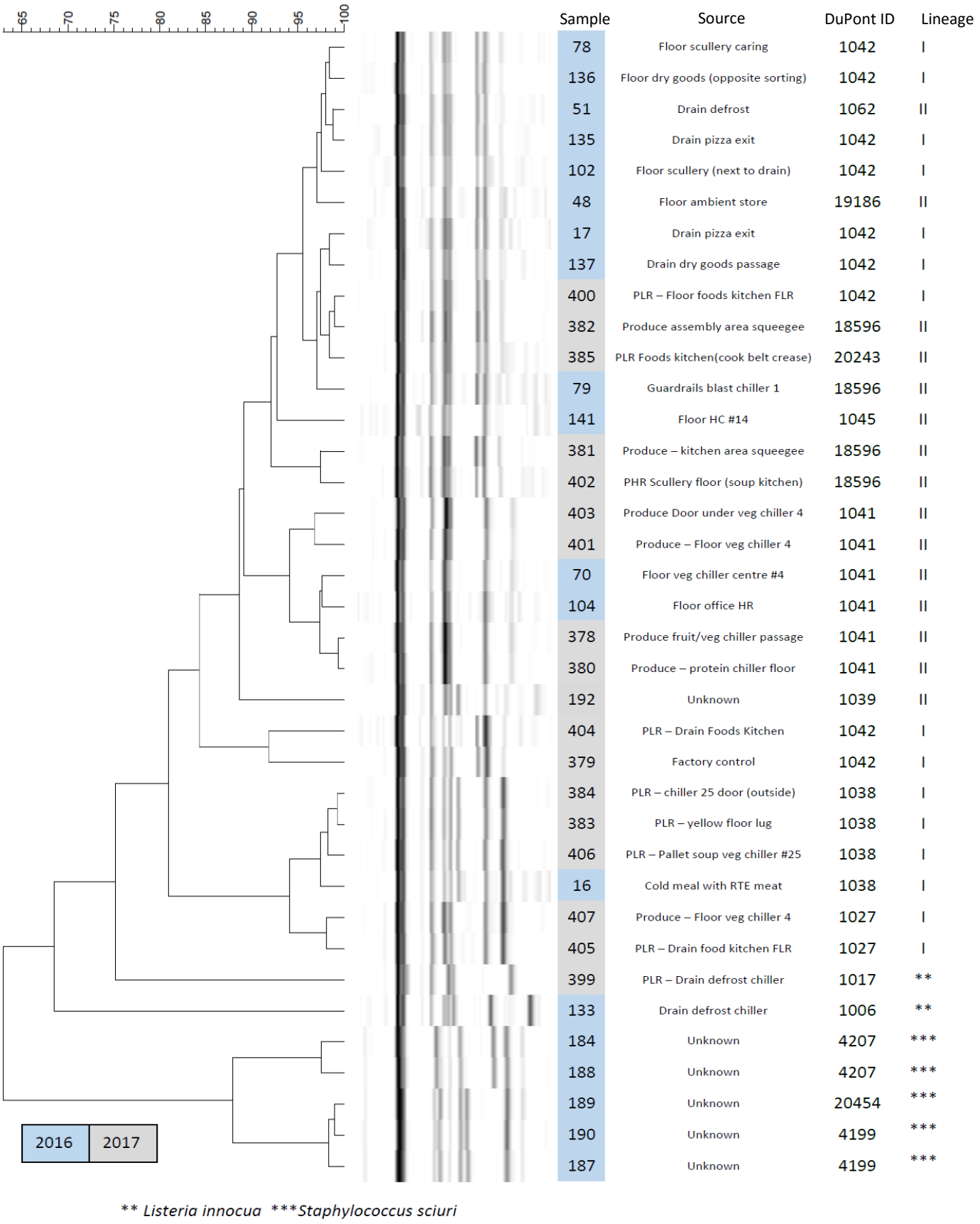


**Figure 4.2** Dendrogram of 37 ribotyped isolates obtained from RTE food processing environment from 2016 to 2017.

Within the 29 sub-typed *L. monocytogenes* isolates, 52% of the strains were identified as lineage I (serotypes 1/2b, 3b, 3c and 4b), where 87% thereof belonged to DuPont ID 1038 and 1042 (Table 2.1), a subtype related to various outbreaks of human listeriosis. Furthermore, 48% were identified as lineage II (serotypes 3a, 1/2a and 1/2c), with 71% thereof being represented by DuPont ID 1041 and 18596, a subtype related to sporadic human listeriosis outbreaks and product recalls. Rosef *et al.* (2012) found DuPont ID 1042 isolated from both human, environmental and food sources when studying the diversity of *L. monocytogenes* isolates in Norway. The study found no association between lineages and isolation locations, which is a similar trend observed in this study.

From the isolates, 5 of the 6 drain isolates were identified as lineage I (Figure 4.2). This could be due to this subtype being the dominant strain at the time of isolation. A review by Orsi *et al.* (2011) demonstrated the urgency and importance of improvement and maintenance of an effective *Listeria* management plan within the factory. Failure to do so could result in product recalls with serious repercussions to consumer health, regardless of lineage.

In order to establish and possibly improve current sanitation and control protocols, the contamination mechanisms driving the spread and cross contamination of *L. monocytogenes* within the factory needs to be identified. The large amount of similar subtypes found on the floor in the factory in various geographical locations was indicative of crates and/or trolleys being a contamination mechanism driving the spread of the *Listeria* throughout the factory. DuPont ID 1041 was found in various produce chillers (cold rooms) as well as in the office floor area within the high-risk processing area. This was indicative of work boots of personnel being the contamination mechanism. Isolate 102 and 135 were both assigned DuPont ID 1042, with isolate 102 being found on the scullery floor and isolate 135 was found in a nearby drain. This was an indication of drains as good hygiene indicators of the microbial community within a factory, since an isolate contaminating the floor was likely to move or accumulate to the nearest drain (Dzieciol *et al.*, 2016).

Isolations of strains from both wet and dry areas as well as ambient and cold temperatures were indicative of the adaptability and high tolerance of *L. monocytogenes* to various environmental conditions (Figure 4.2). Clustering further provides a good snapshot of the *L. monocytogenes* flora at different time periods since isolates from each year showed a tendency to cluster together. It demonstrates how the environment and sanitation practises can influence the current microbial subtypes being detected. Moreover, a persistent strain could not be identified, since no isolates were repeatedly isolated between 2016 and 2017. This could be supportive of the hypothesis that the factory was housing various biofilms. Certain strains could be in a sessile state and thus not be isolated during the *Listeria* management protocols. A change in sanitiser or cleaning methods could dislodge the biofilm and increase the presence of previously “dormant” strains as seen by the difference in dominant DuPont ID isolates between 2016 and 2017.

The factory's in-house laboratory positive *L. monocytogenes* control, was shown to be a true *L. monocytogenes* positive strain with an assigned DuPont ID 1042. This was a suitable positive control as it is this DuPont ID that was most frequently isolated within the RTE factory. It is evident that although they share similar DuPont ID's, the isolates and positive control do not share an isolation location, since only an 84% correlation was seen between them on the dendrogram clusters in Figure 4.2.

Correlation was seen between the results obtained regarding DuPont ID and isolation location type in a study by Kabuki *et al.* (2004) in a cheese processing plant. DuPont ID 1062 and 1042 were also isolated from drains within the processing areas. However, Kabuki *et al.* (2004) reported that the majority of the strains isolated from the study were represented by DuPont ID 1044. This specific strain was not isolated once within this current study. Together with DuPont ID 1039 being exclusively isolated from a different factory during the current study it is indicative of how the in-house flora within different factories can vary. It can be atribbuted to the difference in original and initial contamination sources. A contamination scenario described by Bolocan *et al.* (2016) describes how a specific strain of *L. monocytogenes* can establish itself within the processing environment by simply entering on contaminated raw products. The variety of subtypes found within this environment is indicative of the good possibility of the large number of raw products that enter the facility, as expected from a RTE food factory.

Of the 29 isolates ribotyped, nine different DuPont ID's were assigned (Table 4.1), thus for comparison purposes, one could say three samples per ribotype. This is in alignment with another RTE processing environment study, where 46 ribotyped isolated yielded 17 DuPont ID's (Meloni *et al.*, 2009), also approximately three samples per ribotype. In contradiction, a single product factory such as a smoked salmon plant, only 16 DuPont ID's were assigned to 226 subtyped isolates (Klaeboe *et al.*, 2010), thus 14 samples per ribotype. A small subset of samples representing a ribotype is indicative of the diversity of the strains found within a RTE food processing environment when compared to a factory that only produces a single product. This diversity can be attributed to the large variety of raw products entering the production area (Bolocan *et al.*, 2016).

**Table 4.1** Distribution of 9 *L. monocytogenes* ribotypes in RTE food processing environment

| DuPont ID | Total isolates | Drain | Chiller | FCS* | Food | Cleaning equipment | Floor |
|---|---|---|---|---|---|---|---|
| 1027 | 2 | 1 | | | | | 1 |
| 1038 | 4 | | 3 | | 1 | | |
| 1041 | 6 | | 5 | | | | 4 |
| 1042 | 9 | 4 | | | | | 4 |
| 1045 | 1 | | 1 | | | | 1 |
| 1062 | 1 | 1 | | | | | |
| 18596 | 4 | | 1 | | | 2 | 1 |
| 19186 | 1 | | | | | | 1 |
| 20243 | 1 | | | 1 | | | |
| | 29 | 6 | 10 | 1 | 1 | 2 | 12 |

*Food contact surfaces

Comparison of the isolation locations of ribotypes (DuPont ID) from RTE food factory with isolation locations of similar ribotypes logged on Food Microbe tracker database (Table 4.2), showed conformation of results with global trends. Correlation with these trends is make since the locations of isolates logged on the database were similar to what was found in this study.

**Table 4.2** DuPont ID isolates logged on Food Microbe Tracker *(*as of 14/09/2017)

| **DuPont ID** | **Number of isolates** | **Sources** |
|---|---|---|
| 1027 | 46 | Sporadic human isolates, drains and raw fish |
| 1038 | 316 | Sporadic human, environment (nfcs*), RTE products |
| 1041 | 25 | Environmental (nfcs*) and RTE meat products |
| 1042 | 872 | Sporadic human isolates, RTE food products, animal (bovine and avian) |
| 1045 | 316 | Environmental (nfcs*), human and animal clinical sources |
| 1062 | 589 | In food but mainly from environmental sources like drains and display cases |
| 18596 (1062D) | 2 | Smoked Scottish salmon |
| 19186 (1062C) | 12 | Diary, drain cold storage floor |
| 20243 | - | - |

*non-food contact surfaces

Samples 184, 188, 189, 190 and 187 were isolated from the RTE food factory's Johannesburg branch, however the isolate locations remain non-disclosed. They were received in the form of presumptive positive *Brilliance Listeria* agar, instead of Rapid'*L.mono agar*. The samples were processed and confirmed as positive *L. monocytogenes* by PCR in a similar manner as samples from Western Cape samples. However, automated ribotyping identified the samples, with unknown locations, as *Staphylococcus sciuri.* The positive identification of the samples by PCR as *L. monocytogenes* is due to the crude DNA extraction methods that were used. It is well known that *L. monocytogenes* and *S. sciuri* can be found together in the environment, competing for the same nutrient sources (Leriche & Carpentier, 2000). This may be why *S. sciuri* can be seen growing on Rapid'*L.mono* agar (Figure 4.3), which is supposed to inhibit the growth of any micro-organisms not included in the *Listeria* genus. This is evidence of the observations made by Stessl *et al.* (2009), who after a challenge study of chromogenic agars, recommended further investigation into the reliability of chromogenic agar methods, due to competing micro flora and interspecies enrichment bias.

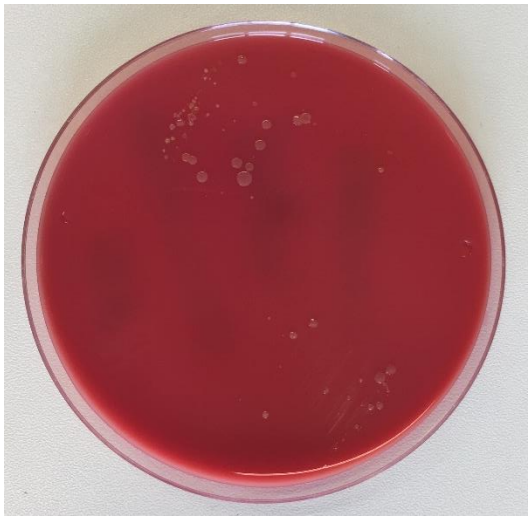

**Figure 4.3** Growth of *Staphylococcus sciuri* observed on Rapid'*L.mono* chromogenic, selective agar.

These two species, from different genera are natural competitors, with reports of *Staphylococcus sciuri* inhibiting biofilm growth of *L. monocytogenes* (Leriche & Carpentier, 2000; Overney *et al.*, 2016). This occurrence is also a possible explanation of why both species were isolated from the presumptive positive *L. monocytogenes* Rapid'*L.mono* plates. The isolation thereof from a factory environment is not unprecedented, since Marino *et al.* (2011) readily isolated this specific *Staphylococcus* specie from a food processing environment. What was of concern however, was the fact that *S. sciuri* survived the enrichment process of the method as well as the inhibiting agents present in the agar (lithium chloride, polymyxin B and nalidixic acid and amphotericin) (Anonymous, 2017).

## 4.5 Conclusion

This study demonstrated the usefulness of automated ribotyping in efforts to establish possible contamination sources within a food processing environment. The standardisation ability of this method allowed for reliable inter-laboratory comparison, to increase the information regarding isolates as well as gain better understanding of how this RTE food factory compares to current global trends. Inter-laboratory comparison is done by comparing results from this study with results from previous studies in order to broaden the information pool regarding the strains. This includes lineage assignments, that would not have been possible due to the scope and limitations of this study.

The variety of strains isolated within the duration of this study was indicative of a diverse microflora present within the processing environment. The movement of isolates, between different isolation locations, were indicative of definite mechanism that drive cross-contamination within the factory environment. Work boots, trolleys and crates were carriers of *L. monocytogenes* and the drains were distinct harbourage sites of both lineage I and II isolates. After establishing contamination sources, it was recommended that these mechanisms be targeted within the RTE food factory's *Listeria* management program. By gaining perspective of the omnipresence of a diverse *L. monocytogenes* microflora within the facility, awareness of its adaptability as well as the importance to maintain control thereof was reiterated. Furthermore, the simultaneous isolation of *L. monocytogenes* and *S. sciuri* prompts the recommendation for further investigation into the interaction and proliferation of these two species within the food chain.

After comparison of the results and subsequent trends from this study with similar studies and global trends, it was seen that the RTE food factory is not unique in regard to its diverse and ever-present *L. monocytogenes* microflora.

## 4.6 Acknowledgements

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# CHAPTER 5

# RESPONSE OF *LISTERIA MONOCYTOGENES* BIOFILMS TO SANITISERS USED IN READY-TO-EAT PROCESSING ENVIRONMENT

## 5.1 Abstract

Biofilms are widespread in the food processing environment and grow and proliferate under various conditions. Dissimilar growth conditions result in diverse biofilm structures and communities that respond differently to sanitation efforts. In this study, the response of *L. monocytogenes* monospecie biofilms, to sanitation chemicals was evaluated using a $CO_2$ evolution measurement system (CEMS). The CEMS is an effective method with which to study biofilms under flow conditions, since it accurately simulates conditions in water drain environment. Protocol development was conducted since *L. monocytogenes* biofilms have not been studied using this system. Four sanitisers representing quaternary ammonium compounds, peracetic acid and alternative chemicals were evaluated using their manufacturer prescribed minimum concentration and contact time. Responses were classified as the biofilm displaying development of resistance over time or otherwise being eradicated. Peracetic acid sanitiser and the proprietary QAC chemical showed no bactericidal effect. A general use QAC and proprietary QAC-free chemical yielded satisfactory results.

## 5.2 Introduction

The food industry has become more aware of the presence and effect of biofilms in the processing environment. It is important when considering that the main mechanism of *Listeria monocytogenes* contamination is said to occur post production (Kerouanton *et al.*, 2010; Vongkamjan *et al.*, 2013; Nyarko & Donnelly, 2015). Post production contamination of RTE foods is of concern; critical control points (CCP) during production ensures successful microbial reduction in food products but these foods can be re-contaminated during handling and storage. These contamination mechanisms include contaminated food contact surfaces (FCS) as well as other non-food contact surfaces (NFCS) such as machinery and processing equipment (Hansen & Fonnesbech, 2011; Fouladynezhad *et al.*, 2013). Sanitation and disinfection protocols form part of *Listeria* control plans, however *L. monocytogenes* in a sessile state shows more resistance to disinfection mechanisms than *L monocytogenes* in a planktonic state (Klaeboe *et al.*, 2010).

It is known that *L. monocytogenes* biofilms develop networks of cocci-like microcolonies surrounded by “knitted”-chains (elongated cells) under flow conditions which is in contrast to the

heterogenous layers of microcolonies and rod cells that form under static conditions (Rieu *et al.*, 2008; Da Silva & De Martinis, 2013). Considering that the response and recovery ability of bacteria within a biofilm can fluctuate due to the dependence on its relationship and position in the microbial community (Orgaz *et al.*, 2013), the response of a drain biofilm to sanitation efforts would differ from a biofilm found on machinery or FCS. In addition, quorum sensing is a major contributor to the construction the biofilm (Cappitelli *et al.*, 2014). Increased resistance to sanitation is found in matured biofilms (Ibusquiza *et al.*, 2011), with a response that counteracts sanitation efforts.

In this study resistance is defined as the ability of a biofilm to recover after a biocidal treatment and continue growing and proliferating as in its pre-treatment state. Biofilms mature when they are not subjected to either mechanical removal or chemical bactericidal treatment. Due to lower sanitation frequencies, drains are excellent harbourage sites for matured biofilms. A large part of successfully controlling *L. monocytogenes* within a RTE food processing environment is identifying and eradicating persistent strains. Many studies have attempted to define the factors that contribute to the persistence ability of *L. monocytogenes* and although definite parameters have not yet been identified, harbourage sites have been identified as a major driving force of persistence (Carpentier & Cerf, 2011; Orgaz *et al.*, 2013). This adds to the need to study the response of biofilms that have matured under flow conditions mimicking harbourage sites that facilitate persistence.

The $CO_2$ Evolution measurement system (CEMS) is a non-destructive, non-invasive method of studying mature biofilm response under flow conditions. It is a once-through flow system that in essence, evaluates biofilm metabolism by measuring $CO_2$ produced by the bacteria during respiration (Loots, 2016). The systems set-up and parameters were described in great detail by Kroukamp and Wolfaardt (2009). The response of a biofilm is measured as a decrease or increase in $CO_2$ production ($\mu mol.h^{-1}$) and resistance would be defined as an increase in $CO_2$ production ($\mu mol.h^{-1}$) after treatment. This would be indicative of a recovery effort and resuscitation of injured cells. Sanitiser treatment and the subsequent response of the biofilm in CEMS system is an excellent demonstration of how a single biofilm builds up resistance against a sanitiser in a matter of hours.

The biofilm eradication concentration (BEC) is the concentration of sanitation treatment where the biofilm is unable to resuscitate and microbial growth is stopped (Poimenidou *et al.*, 2016) and is generally prescribed by the manufacturer of the specific sanitation chemicals. ISO EN 13697 states that a disinfectant should provide a four log reduction (99.99%) of microbial count on a clean and soiled surface (Gram *et al.*, 2007). It does not, however state the requirements for destruction of the cells themselves.

Sanitation chemicals used by the food industry mainly entail acetic acid, lactic acid, quaternary ammonium containing chemicals (QAC), sodium hypochlorite (Da Silva & De Martinis, 2013) and other proprietary, novel and alternative methods. Microbial resistance to a majority of

chemicals used have started to emerge (Klaeboe *et al.*, 2010) and currently combination treatments of biofilms to avoid increase in biofilm resistance have been recommended (Pricope *et al.*, 2013).

Sanitation protocols are dual processes that entail cleaning and disinfection steps (Walton *et al.*, 2008). The aim of cleaning is to remove, not kill, approximately 90% of bacteria adhering to surfaces and to disintegrate the extracellular polymeric substance (EPS) of biofilms (Srey *et al.*, 2013). Removal of organic material such as protein, fat and carbohydrates, increases the efficacy of disinfectants. Disinfectants are aimed at killing bacterial cells exposed, through cleaning and subsequently reducing the viable population of pathogens and spoilage organisms (Srey *et al.*, 2013). In this study, sanitisers refer to chemicals used for both cleaning and disinfection.

The majority of *L. monocytogenes* isolates in the previous chapters were from drains in the high and low risk areas of the RTE factory. This was indicative of the predominant presence of *L. monocytogenes* strains within the drains. The aim of this study was therefore to examine the response of *L. monocytogenes* biofilms cultivated under conditions found in a drain, to prescribed sanitisers using the CEMS. These sanitisers include Byotrol, Byotrol QFC, Perasan and Divosan QC. This study was conducted to lay the ground work for future studies into treatments that could potentially contribute to the global food industry efforts of controlling *L. monocytogenes* in the RTE food processing environments.

## 5.3 Materials and methods

### 5.3.1 Flow system set-up and preparation

The CEMS system was assembled as described by Kroukamp and Wolfaardt (2009) and system was disinfected according to Loots (2016). System parameters and set-up was done as illustrated in Figure 5.1. On the inflow connection of each CEMS line, a tube connecting the growth medium and $CO_2$-free gas is attached. On the outflow connection of the CEMS line, was a tube carrying the gas containing the $CO_2$ from the growing biofilms to the $CO_2$ analyser, as well tubes allowing the outflow growth medium to be collected in flask containing industrial bleach. The four CEMS lines are placed in the water bath as a means of controlling the temperature (Figure 5.1).

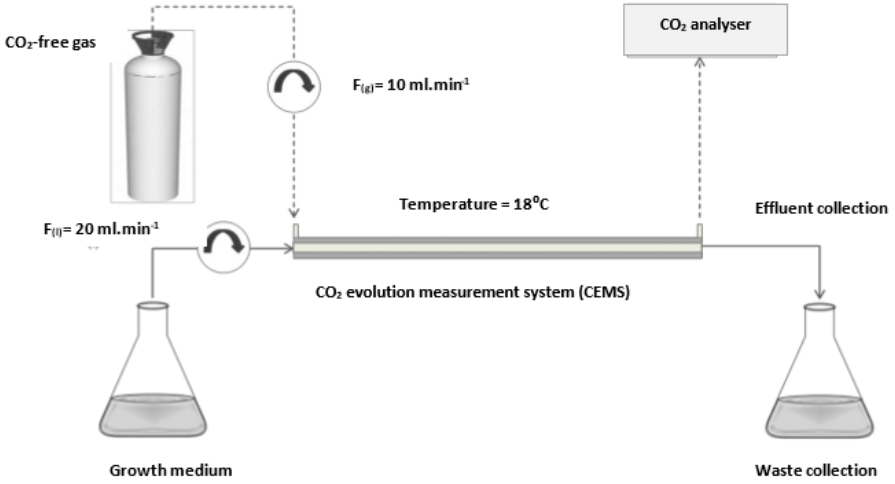


**Figure 5.1** CEMS system set-up (adapted from Loots (2016)).

### 5.3.2 Monoculture inoculum preparation

For preliminary testing to determine the experimental parameters of growing *L. monocytogenes* in the CEMS system, two Du Pont ID strains/ribotypes were selected. Experimental parameters included: time (h) required for the maturation of the biofilm and growth trends of *L. monocytogenes* under these conditions.

Sample 135 (Du Pont ID: 1042) was selected since it represents the most prominent ribotype isolated from the factory environment and it was also a strain isolated from a drain (Figure 4.2). Sample 51 (Du Pont ID: 1062) was selected based on it being reported as a persistent strain in a study by Klaeboe *et al.* (2010) which examined Norwegian salmon processing plants. Sample 51 was also isolated from a drain (Figure 4.2).

Stock cultures of selected samples were resuscitated by inoculation of Tryptic Soy Broth (TSB) with loopful of culture and incubation at 37ºC for 15 - 18 h. The broth cultures were then standardised to 0.1 OD at 600 nm (approximately $10^{-5}$ CFU.ml$^{-1}$) using sterile saline solution (0.9% w.v$^{-1}$ NaCl). This inoculum preparation procedure was applied to all test done within this study.

### 5.3.3 System inoculation

The flow of sterile TSB was paused as each of the four CEMS were aseptically inoculated with 1 mL standardised overnight cultures using a sterile needle and syringe. The cultures were allowed 1 h to adhere to the silicon tube before the peristaltic pump resumed the flow of TSB at 20 ml.h$^{-1}$.

### 5.3.4 Enumeration of free cells in CEMS outflow

Outflow from each line was collected by disconnecting the tube attached to the outflow tubes and inserting a 1.5 mL Eppendorf tube at the end (Figure 5.2).


**Figure 5.2** Effluent collection from CEMS.

From the outflow samples, a dilution series was plated onto Nutrient Agar (NA) plates and incubated at 37°C for 24 h, after which it was examined for any signs of contamination i.e. growth of other micro-organisms that do not have the characteristic cream, pin sized round *Listeria spp*. colony. A standard method for the enumeration of *L. monocytogenes* on Rapid'*L.mono* agar is available (Anonymous, 2017b). However, enumeration on Nutrient Agar was sufficient because the biofilm grew and consequently the outflow is a single strain and therefore no contamination or competition will be in effect. Nutrient agar is a non-selective media and is used to check for contamination as it will not inhibit the growth of another micro-organism as the chromogenic agar would.

### 5.3.5 Testing the effect of various industry based sanitisers

The four selected sanitisers, used by the RTE food factory (Table 5.1), were prepared to concentration and conditions as per the manufacturer specifications for food preparation surfaces and maintenance (Table 5.2). Stronger concentrations and extended contact times were excluded as such protocols were excluded from the scope of this study.

**Table 5.1** Description and industry recommended application of sanitisers used to study biofilm response

| Sanitiser | Additional description | Approved standard | Active ingredients<br>Chemical name | <br>% |
|---|---|---|---|---|
| Perasan[1] | Acidic disinfectant /descaler | SABS 1853 | Hydrogen peroxide<br>Acetic acid<br>Peracetic acid | 5-15<br>15-30<br>5-15 |
| Divosan[1] | Terminal disinfectant and cleaner | SABS 1853 | Dicecyldimethylammonium chloride (QAC) | 1-10 |
| Byotrol[2] | Antimicrobial surface sanitiser | SABS 1853 | Dicecyldimethylammonium chloride (QAC)<br>Benzylkonium chloride<br>Polyhexamethylenebiguanide | 2.5-10<br>2.5-10<br><2.5 |
| Byotrol (QFC)[2] | Antimicrobial surface sanitiser | SABS 1853 | Dodecyl dipropylenetriamine<br>Lactic acid<br>Polyhexamethylene biguanide hydrochloride | 5-10<br>5-10<br><1 |

[1](Jaftha, I. 2017, Customer Service agent, Sealedair, Cape Town, South Africa, personal communication, 12 September) [2] (Anonymous, 2017a)

**Table 5.2** Sanitisers and contact parameters for treatment of *L. monocytogenes* biofilms cultured in CEMS

| Test | Line | Sanitiser | Application (% v/v) | Contact time (min) |
|---|---|---|---|---|
| 1 | B | Byotrol | 1.0 | 5 |
| | Q | Byotrol (QFC) | 1.0 | 5 |
| 2 | P | Perasan | 0.5 | 5 |
| | D | Divosan QC | 3.0 | 10 |
| 3 | B | Byotrol | 1.0 | 5 |
| | Q | Byotrol (QFC) | 1.0 | 5 |

The first treatment was applied once it was evident that the biofilms had stabilised. Stabilisation was characterised by a non-fluctuating stationary phase of the log curve of the $CO_2$ production graph. The peristaltic pump was stopped as the inflow tubes were connected aseptically to outflow tubes of sanitiser reservoirs. After the connection was completed and air bubbles were minimised the pump was restarted at 1.5 rpm to resume flow for the set treatment time. After treatment time was reached the peristaltic pump was stopped and inflow tubes were reconnected to the flow medium reservoir and the flow was resumed at pre-treatment parameters (Figure 5.3).

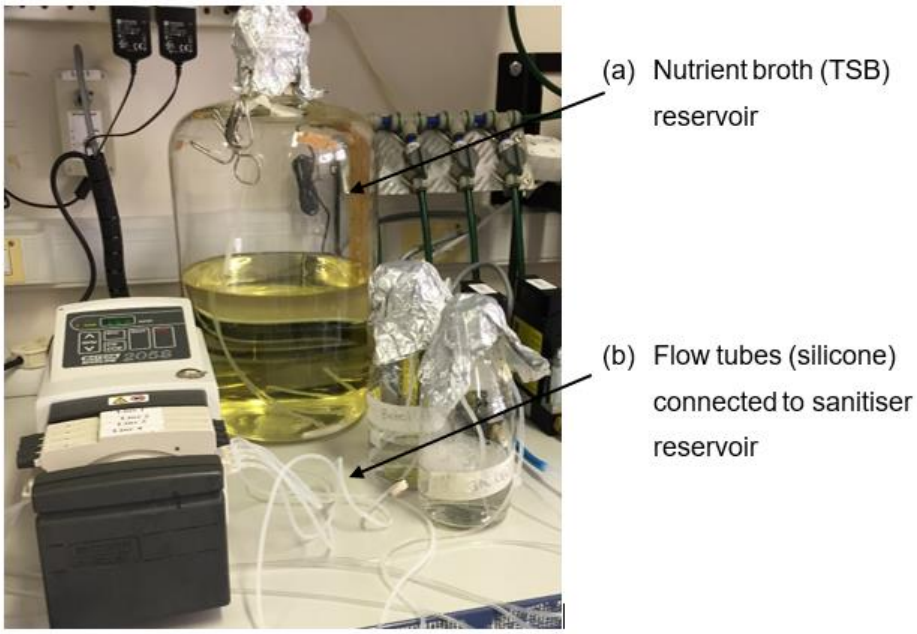


**Figure 5.3** Treatment set up for CEMS (a) Nutrient broth (TSB) reservoir disconnected during treatment; (b) sanitiser reservoir directly fed into CEMS system aided by peristaltic pump.

## 5.4 Results and discussion

It is very important to note that there are various methods and parameters in which the CEMS can be used to test and observe sanitation actions of various industry based sanitisers. The results seen should be used as a way to learn and understand how biofilms react to sanitisers. It is not the aim of this study to critique the company or the product itself, but rather to demonstrate the effect that inadequate contact times and misuse of these chemicals contribute to increased resistance of biofilms in food processing environments. Thus, remarks, conclusions and recommendations made in this study were based on observations made under the said parameters and conditions.

### 5.4.1 Protocol development for biofilm cultivation in CEMS

The aim of this test was to select a *L. monocytogenes* strain (previously isolated in this study) that could form a biofilm as well as represent isolates from the factory environment. This selection is due

to the strain's previous exposures to chemicals used within the RTE food processing environment. The growth trends of both strains were examined in CEMS (Figure 5.4) to aid in the selection of a strain for sanitiser tests.

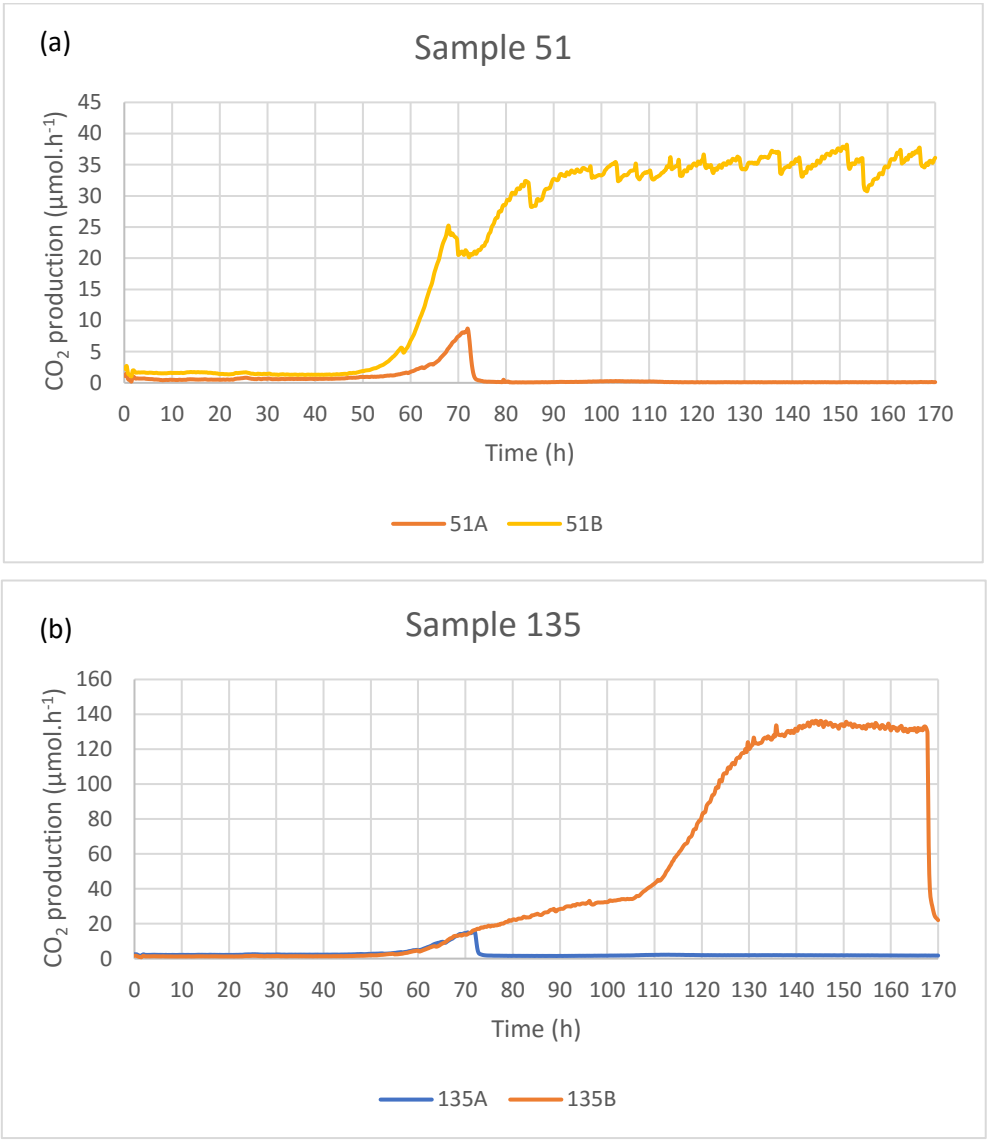


**Figure 5.4** Comparison of $CO_2$ production (µmol.h$^{-1}$) for selection of strain to be used for subsequent tests (a) sample 51, (b) sample 135.

There are distinct differences in growth behaviour pertaining to the *L. monocytogenes* subtypes evaluated in this test. In regard to reaching the exponential phase of the curve (establishment of biofilm), sample 51 started 56 h prior to sample 135 and reached stationary phase at approximately 4 days as opposed to 5 days for sample 135 (Figure 5.4). Nevertheless, Sample 135 displayed more stable behaviour, identified by less fluctuation of $CO_2$ production (µmol.h$^{-1}$) at stationary phase of curve (Figure 5.4). Due to this and its maturation period of 5 days, sample 135 was selected as the strain that would be used to the study the response of *L. monocytogenes* to sanitisers. The selection

of sample 135 was further confirmed by Henriques and Fraqueza (2017), who found their isolates to also be strong biofilm formers after a 5 day growth period.

The sudden drop in $CO_2$ production (µmol.h$^{-1}$) by line 51A and 135A at 72.25 h is due to these CEMS lines being disconnected to ensure that enough nutrient flow would be available for the other lines to complete the growth curve and the biofilm to stabilize. Since this test was done as a protocol development step the growth time and behaviour of *L. monocytogenes* in this system, under these conditions, were unknown. Thus, the amount of Tryptic Soy Broth (TSB) for all lines could not be anticipated. A sufficient amount of TSB to support flow of four CEMS lines was not prepared, hence disconnection of replicate lines and consequently the sudden drop in $CO_2$ production. The test was discontinued at 170 h as the sample selection could be made with data obtained.

### 5.4.2 Test 1: Protocol development for sanitiser treatment of *L. monocytogenes* biofilms in CEMS

Due to the novelty of using CEMS to study *L. monocytogenes* biofilms, protocol development was undertaken by treatment of biofilms to establish what was to be expected upon treatment and how recovery trends would present themselves. The aim of this test was to establish parameters for testing biofilm response to industry based sanitisers using CEMS and also to ascertain trends that could be anticipated. Each set of treatments for both Byotrol and Byotrol QFC were done in duplicate (Figure 5.5), which confirmed the growth behaviour and biofilm response of each treatment. The response trends were used as a guideline for tests to follow.

First treatment was done on day 5 (121 h) for both sanitisers (Table 5.2). Both B1 and B2 showed recovery behaviour after merely 3 h, which is indicative of resistance by the biofilm (Figure 5.5). Both Q1 and Q2 showed no recovery trends after one treatment, meaning the biofilm was completely eradicated from the silicon surface inside the CEMS.

However, the free cells collected from all CEMS were still culturable which suggests that the drop in $CO_2$ production (µmol.h$^{-1}$) could be attributed to the loosening or sloughing of the biomass. Therefore, neither Byotrol nor Byotrol QFC caused a bactericidal effect. It should be noted that collection of effluent was also done as a means to monitor possible contamination, since only *L. monocytogenes* should be seen during culturing. Upon final effluent collection after 170 h, the presence of presumptive *Pseudomonas* spp. was detected. It was decided that the results obtained could still be used since the aim of this test was only to establish the protocol and parameters for sanitiser treatments as well as to anticipate trends for tests to follow. The test was discontinued after 195 h since satisfactory conclusions could be made regarding responses and recovery trends.

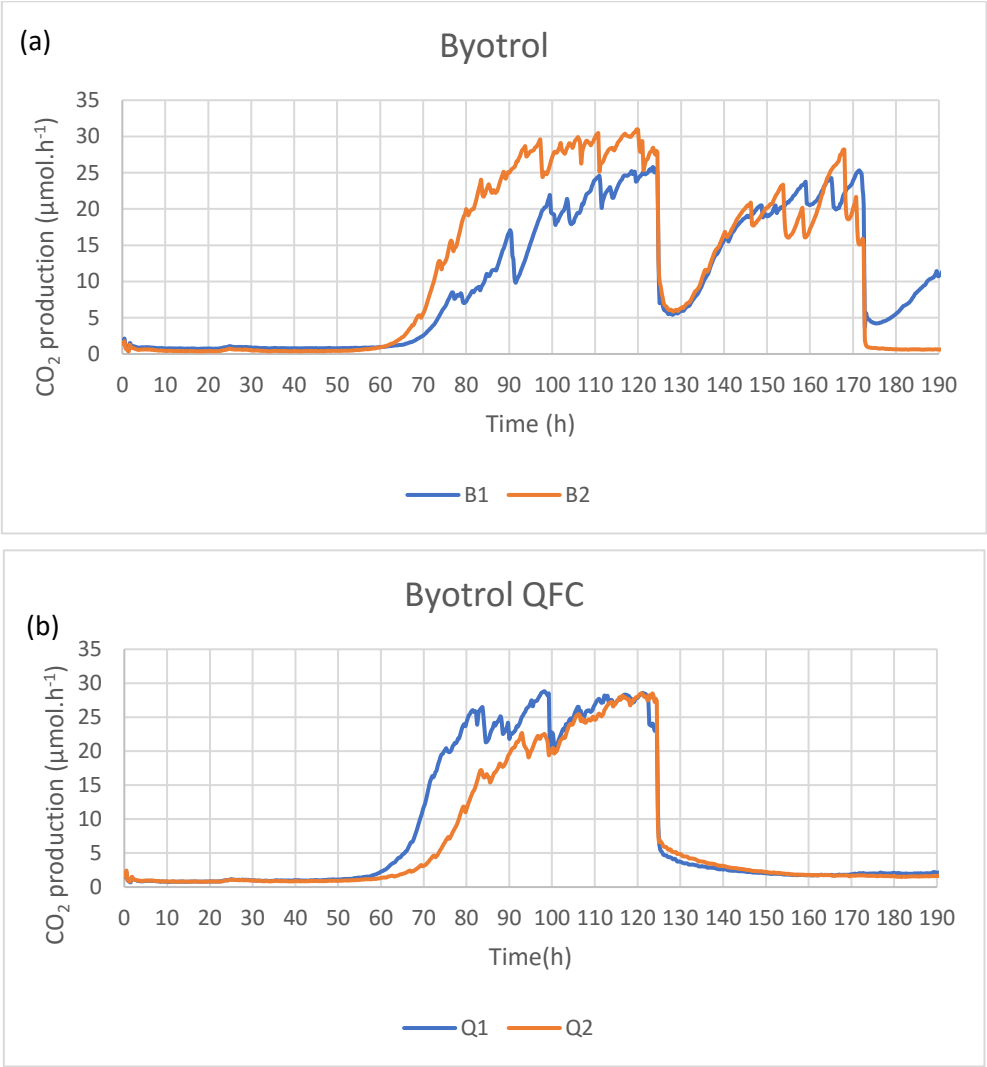


**Figure 5.5** Establishing trends for subsequent tests of $CO_2$ production (µmol.hr$^{-1}$) of biofilm in response to sanitisers (a) Byotrol, (b) Byotrol QFC

### 5.4.3 Test 2: Response of *L. monocytogenes* biofilms to QAC based and Peracetic acid based sanitisers

During the conduction of this test a momentary power failure occurred at approximately 97 h, clearly visible in Figure 5.6. This accounts for the irregular spikes seen on Figure 5.6 and Figure 5.7. This in no way effected the formation of the biofilm as the curve trend is still intact. The $CO_2$ analysers were rebooted and allowed to stabilize as seen at 106 h.

Divosan QC is a terminal disinfectant containing Dicecyldimethylammonium chloride (Table 5.1), a known Quaternary Ammonium Chloride compound. It is referred to as terminal since it can be used to clean whole rooms (floor, walls and ceiling). It has a recommended contact time of 10 min at a concentration of 0.5% (v.v$^{-1}$) (Table 5.2).

First treatment was performed on day 5 (121 h) (Figure 5.6). A decrease of 29.08 µmol $CO_2.hr^{-1}$ was observed. The biofilm may seem to be eradicated in both D1 and D2, however, resuscitation can be seen after 50 h for line D1. Although D2 took approximately 53 h longer to resuscitate (at 245 h), it still reached the same $CO_2$ production (µmol.$h^{-1}$) as D1 at 275 h. The recovery trend observed within the CEMS after 56 h and 103 h in D1 and D2 respectively, is evidence that although the majority of the biomass was eradicated, Divosan QC did not have a sufficient bactericidal effect. In addition, outflowing free cells were still culturable, further evidence of the lack of bactericidal effect and subsequently a possible contamination mechanism.

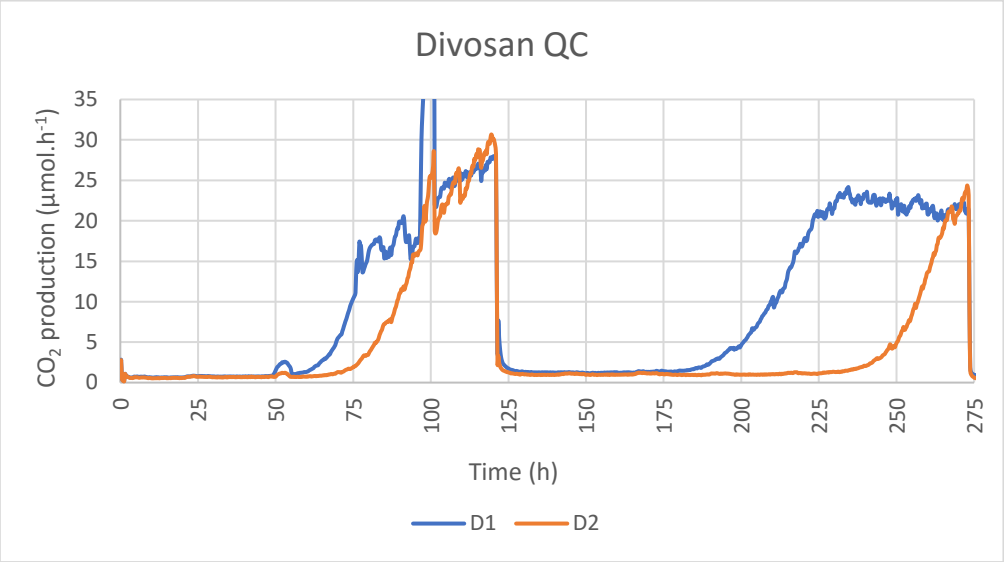


**Figure 5.6** Response of *L. monocytogenes* monoculture biofilm to QAC based sanitiser (Divosan QC).

Perasan is a peracetic acid based sanitiser, with a recommended contact time of 5 min at concentration of 0.5 % (v.$v^{-1}$) (Table 5.2). The first treatment on day 5 (121 h) (Figure 5.7) resulted in a 24.97 µmol $CO_2.h^{-1}$ drop after which resuscitation occurred rapidly, reaching full recovery after only another 47 h. The biofilm stabilised at a $CO_2$ production level of only 2.4 (µmol.$h^{-1}$) lower than before the treatment. It is at this point that the biofilm can be said to start showing resistance to the treatment parameters, since it recovers to its original metabolic state in only two days.

The treatment was repeated for both P1 and P2, at 168.25 h which saw an average 21.11 $CO_2$ production (µmol.$h^{-1}$) drop, which is similar although slightly less than the previous treatment. The recovery of the biofilm took 11 h longer than the previous recovery episode, however upon stabilisation it once again showed the same $CO_2$ production (µmol $CO_2.h^{-1}$) as before the second treatment (Figure 5.7)

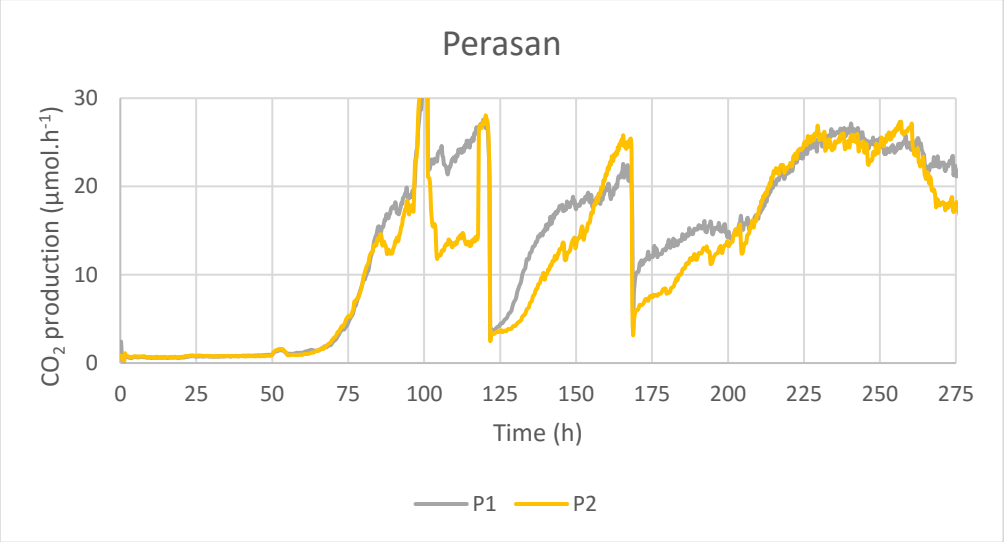


**Figure 5.7** Response of *L. monocytogenes* monoculture biofilm to peracetic acid based sanitiser (Perasan).

This resistance patterns/trends shows that the recommended treatment parameters of Perasan are inadequate to completely eradicate the biofilm from CEMS. In addition, the free cells in the effluent were still culturable. It is thus suggested to increase the recommended minimum contact time and to ensure that this sanitation protocol is followed once it is adjusted, since deviation or lowering of contact time would be deemed inadequate

### 5.4.4 Test 3: Observing the response of *L. monocytogenes* monoculture biofilms to treatment with QAC-sanitiser (Byotrol) and QFC-sanitiser (Byotrol QFC).

Byotrol is a QAC sanitiser containing Dicecyldimethylammonium chloride, Benzylkonium chloride and Polyhexamethylenebiguanide. It delivers the chemicals to the environment using Amphicelles technology (Anonymous, 2017a).

Considering the trends observed in both B1 and B2 in comparison with treatments in Test 2 (Figure 5.6 and 5.7), the *L. monocytogenes* monoculture biofilms shows significantly more resistance to Byotrol. The first treatment was performed on day 5 (125 h) and saw resuscitation of both B1 and B2 commence almost immediately after the average 14 µmol $CO_2.h^{-1}$ reduction. The second treatment was performed on day 7 (174 h) and a similar trend was observed (Figure 5.8). An average 12.8 µmol $CO_2.h^{-1}$ decrease was observed, which is similar to the first treatment. Also, comparable to the first treatment, is the almost immediate resuscitation of the biofilm with stabilisation occurring approximately 14 h later.

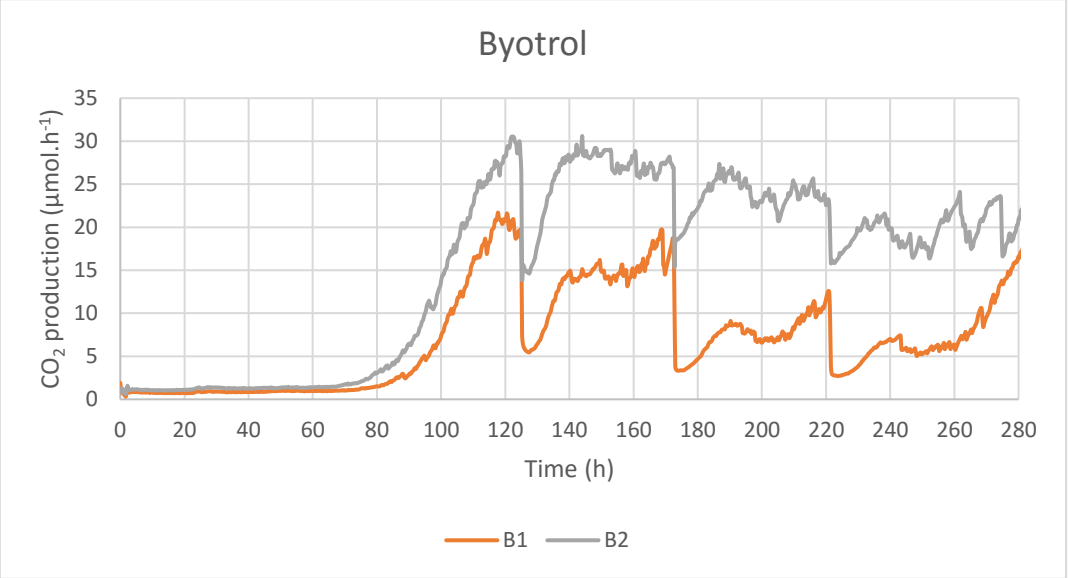


**Figure 5.8** Response of *L. monocytogenes* monoculture biofilm to QAC based sanitiser (Byotrol).

A third and final treatment was performed on day 9 (224 h) and saw an average reduction of 7.4 µmol $CO_2.h^{-1}$, which is 5.4 µmol $CO_2.h^{-1}$ less than the previous treatment (Figure 5.8). This is indicative of the increase in resistance that the biofilm built up over the course of the three treatments. The biofilm continued to recover and increase in biomass until the test was terminated at 280 h.

Byotrol QFC is claimed to be the exactly the same as the original Byotrol product, however it does not contain any QAC. It is comprised of Dodecyl dipropylenetriamine, Lactic acid, Polyhexamethylene biguanide hydrochloride (Anonymous, 2017a).

In an overview of the biofilm response to the treatment, the biofilm shows less resistance to the QFC (Figure 5.9) than the original Byotrol product (Figure 5.8). This could be the first evidence that the *L. monocytogenes* strain, isolated from the processing environment, has developed a resistance to QAC. Further molecular testing will be required to confirm this observation.

Similar to the Byotrol test (Figure 5.5), the first treatment was performed on day 5 (125 h), with an initial average 11.9 µmol $CO_2.h^{-1}$ reduction. However, the $CO_2$ production (µmol.$h^{-1}$) continued to reduce over the course of the following 25 h by a further 7.29 µmol $CO_2.h^{-1}$. Only 26 h after treatment, did the biofilm show any sign of recovery behavior, signified by an increase of $CO_2$ production (µmol.$h^{-1}$). The second treatment was performed on day 9 (219 h) after which the biofilms showed no sign of recovery as $CO_2$ production (µmol.$h^{-1}$) plateaued off reaching the baseline $CO_2$ until the test was terminated at 280 h. The lack of resuscitation of the biofilm after two treatments, was indicative of the effectiveness of Byotrol QFC in removing the biomass from the system as it would in the drainage area of a factory environment.

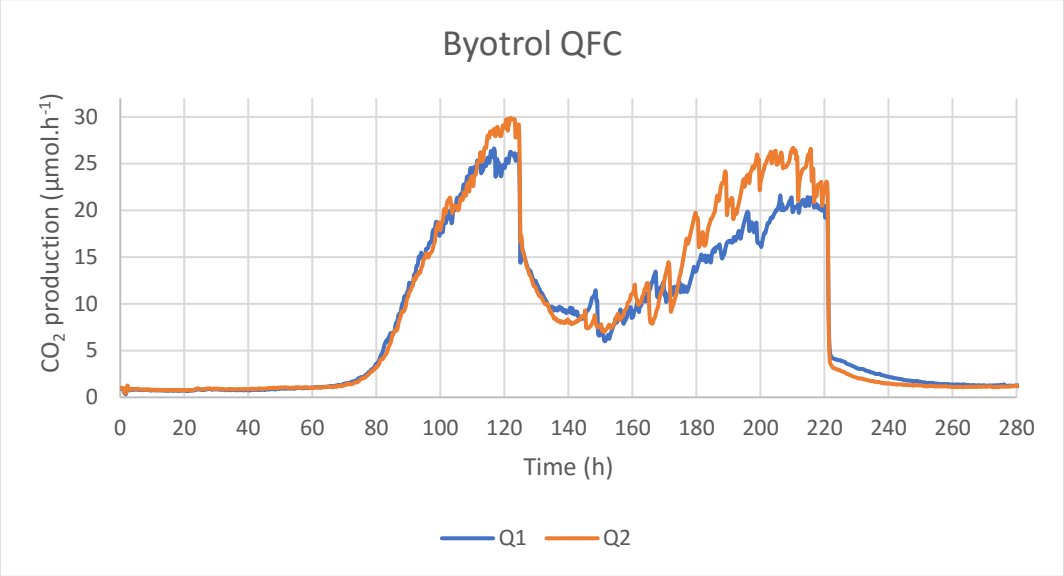


**Figure 5.9** Response of *L. monocytogenes* monoculture biofilm to QAC-free sanitiser (Byotrol QFC).

## 5.5 Conclusion

This study demonstrated the value of using CEMS as a tool to observe and evaluate the effectiveness of sanitisers in management and possible eradication of *L. monocytogenes* biofilms. Parameters and procedures to do these evaluations were optimised and resulted in a method in which to study biofilms in a novel, non-static way that more accurately reflected environmental conditions found in food processing environments. The reliability of the system was reflected in the excellent replication of response curves, despite the inherent variability of biofilms.

Peracetic acid was confirmed to have no bactericidal effect on *L. monocytogenes* biofilms, with resuscitation occurring even after two treatments where applied. It is evidence of PAA's inability to oxidise the biofilm structure. It is therefore not suitable as a sanitation product within this context, and further investigation into its disinfection capabilities and recommended treatments protocols are advised.

Sanitisers tested were representative of the common chemicals used in food factories, but more specifically sanitisers used in the RTE food factory's sanitation program. Of the QAC containing chemicals, Divosan QC was more effective than Byotrol, since immediate resuscitation of the biofilm did not occur after treatment. Resuscitation was only observed much later. This might be attributed to its recommended application that is more effective, however the inability of both the QAC chemicals to completely eradicate the *L. monocytogenes* biofilm, prompts the possibility of QAC resistance strains. Further investigation into QAC resistant genes among the RTE food factory's isolates is recommended.

The possibility of QAC resistance among the isolates is further supported by the response of *L. monocytogenes* biofilms to the QAC-free (QFS) version of Byotrol. No resuscitation of the biofilm was observed after only two treatments. This is evidence of its effectiveness in the eradication of *L. monocytogenes* biofilms found within the drains of the RTE food factory environment.

The aim of this study was to observe the response of *L. monocytogenes* biofilms to sanitation chemicals used by the RTE factory in order to make recommendations to aid in improving current practises and management protocols. Byotrol QFC was thus recommended, based on the observations of the study, under the conditions of the disclaimer previously discussed.

Finally, it was also the aim of this study to lay the ground work for future studies into foodborne pathogen biofilms and their response to environmental conditions and control protocols. Considering the outcome of this study and the conclusions that could be made. For future studies, it is recommended that combination treatments, alternative environmental conditions, multispecies biofilms, as well as the correlation between $CO_2$ production and biofilm CFUs be explored. Finally, it is recommended that a more in-depth data analysis (principle component analysis) be done taking into account all the data obtained from the $CO_2$ analysers as well with other factors such as temperature and pH.

## 5.6 Acknowledgments

A most sincere thank you to Dr Elana Bester, Department of Microbiology, Stellenbosch University. Her knowledge, time and assistance proved invaluable to this study

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# CHAPTER 6

# GENERAL DISCUSSION AND CONCLUSIONS

The main aim of this study was to examine the survival and proliferation of *L. monocytogenes* within a specific RTE food factory situated in the Western Cape, South Africa. The first objective was to isolate and positively identify *L. monocytogenes* isolates from the factory environment. Presumptive positive agar samples collected from the factory's *Listeria* control and management plan were subjected to molecular identification using PCR.

This study attempted to optimise a multiplex PCR protocol using the most cost effective and simple components to maintain its applicability to routine analysis within the food industry. These components include crude DNA extraction and One*Taq* endonuclease. A multiplex PCR protocol using One*Taq* endonuclease designed for the amplification of *iap* (*Listeria* spp.) and *lmo2234* (*L. monocytogenes*) (Chen and Knabel, 2007), was unsuccessful. Singleplex PCR protocols for each target gene were then optimised using One*Taq*. It is recommended that alternative endonuclease be used for a multiplex protocol that targets the specified genes simultaneously.

The Rapid'*L.mono* method was also evaluated for its ability to detect *L. monocytogenes* in the presence of *L. innocua.* A protocol to evaluate enrichment bias in half Fraser broth was developed that entailed the simultaneous inoculation and incubation with the same CFU of both species. It was found that *L. monocytogenes* was still detectable in the presence of *L. innocua* when streaked onto Rapid'*L.mono*. This includes streaking as prescribed by the manufacturer (Anonymous, 2014) or by standard streaking methods. For a more in-depth evaluation of enrichment bias as it occurs in naturally contaminated food environments and products (Zitz *et al.*, 2011), inoculation with a lower *L. monocytogenes* CFU count than *L. innouca* is recommended. This can be achieved by using a lower concentration of *L. monocytogenes* starting inoculum.

*L. monocytogenes* isolates from the RTE factory were subjected to subtyping through automated ribotyping using the DuPont RiboPrinter®. A total of 29 samples underwent riboprinting and were assigned DuPont ID's. The assigned ID's included, amongst others, DuPont ID 1038, 1041, 1042 and 18596. Through comparison of these IDs with similar studies as well as using the Food Microbe Tracker database (Anonymous, 2013), it was found that these isolates have been implicated in various human listeriosis cases and product recalls (Meloni *et al.*, 2009; Rosef *et al.*, 2012). Although previously isolated by Gambarin *et al.* (2012) in a salmon product, DuPont ID 20243, isolated from the produce area in this study, has not yet been logged on Food Microbe Tracker. Lineage assignments could also be made, using existing literature, to identify the virulence potential of strains found in the environment. The ribotype data was also subjected to cluster analysis using

a Pearson correlation dendrogram. This aided in creating a better understanding of the geographical distribution of the isolates in terms of their strain similarity. It was found that the drains and floor harboured the majority of the isolated strains. The distribution of similar strains was indicative of cross contamination mechanisms that included work boots, trolleys and crates. It was recommended to the factory to increase sanitation and monitoring of drains, as the majority of the strains were classified as lineage I, meaning an increased virulence potential (Orsi *et al.*, 2011; Vongkamjan *et al.*, 2016). The distribution of clusters across 2016 and 2017 was also indicative of the presence of biofilms, were some DuPont IDs were more prevalent in 2016 than in 2017.

A further aim was to observe the response of biofilms to the current sanitisers used within the RTE food factory's environment. Isolates obtained from the factory environment was selected to cultivate the biofilms, since these strains have been previously exposed to the specific RTE factory environment and would best represent the in-house microflora. The biofilms were studied under flow conditions to best mimic conditions found in drains, and subsequently to gain a better understanding of the effect of sanitiser on biofilms found in drains.

The $CO_2$ evolution measurement system (CEMS) was used for this study. *L. monocytogenes* biofilms have not yet been studied under these conditions in this system, adding to the novelty of the study. CEMS is a once-through, non-destructive method to study the response of biofilms (Loots, 2016) to sanitiser using $CO_2$ as an indicator of biofilm metabolism. Four sanitisers used by the food factory were tested and the QAC-free chemical resulted in the best eradication of the biofilm. Peracetic acid based product had no effect on the biofilm as increased recovery was seen throughout the test period, which averaged nine days. The results obtained from the biofilm study showed that using these chemicals at their minimum contact time and concentration has no bactericidal affect, but only resulted in the sloughing and loosening of the biofilm. This occurrence was of concern, since this type of sanitation effect will only aid in the increased spread of the pathogen within the factory. Nevertheless, with the current sanitisers at the disposal to the RTE food factory, it was recommended that Byotrol QFC (QAC free) chemicals be used for the management of *L. monocytogenes* biofilms.

The developed protocol and results from this study can now be used as a guideline to further study *L. monocytogenes* biofilms within food processing environments. It is recommended that the response of multispecies biofilms be compared to the findings of this study. Furthermore, the effect of different environmental conditions, such as temperature fluctuations, change in pH and fluctuating nutrient availability, be studied. It is also recommended to evaluate genetic stress responses of *L. monocytogenes* when treated with various sanitation chemicals.

Apart from achieving the set objectives, this study created awareness within the RTE food factory and associated stakeholders regarding the complex nature of *L. monocytogenes*. This pathogen should no longer be seen as a by-the-way organism, but should be considered a high

priority when it comes to ensuring food safety, especially in South Africa. This study hopes to set in motion discussion within the food industry, to increase efforts into understanding *Listeria monocytogenes*, in order to effectively manage it within the South African RTE food industry.

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