The prevalence of sanitizer resistant *Listeria monocytogenes* in South African Food Processing Environments

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Listeria monocytogenes is a ubiquitous, Gram-positive bacteria, that can survive and proliferate within food processing environments (FPEs), form biofilms on a variety of surfaces, survive the application of the quaternary ammonium compound (QAC)-class of sanitizer and cause the human disease listeriosis. Cleaning of FPEs follows a multistep process which include the application of a detergent and mechanical action (scrubbing), facilitating the removal of soil and debris, which may help shield bacteria from the effects of the cleaning chemicals. This multistep cleaning process culminates in the application of a terminal disinfectant (sanitizer) which helps remove any surviving, free cells. If the prior steps of cleaning are not adequately carried out, sublethal exposure of the sanitizer in use may occur which may lead to a rise in resistance towards the sanitizer applied. Citizens of the Republic of South Africa (RSA) were subjected to the world's largest outbreak of listeriosis over the 2017-2018 period. Based on previous global outbreaks of listeriosis and an association between the presence of resistance genes, conferring resistance to QAC-class of sanitizer, an investigation into the prevalence of sanitizer resistant *L. monocytogenes* from six (6) South African FPEs from across RSA was carried out with a sample size of 50 (N=50). The 50 isolates were obtained from presumptive positive RapidL'mono (Biorad, FR) selective media plates through visual confirmation and polymerase chain reaction (PCR) confirmation by screening for the *hly* gene. The isolates were then categorized into lineage types with 14 being assigned to lineage I and 36 being assigned to lineage II. The origin of the isolates were either from drains, the food processing environment or food contact surfaces. All 50 isolates were confirmed to be *L. monocytogenes*. Due to their presence in clinical outbreaks of listeriosis and their high prevalence of reporting in international literature, four QAC resistance genes: *bcr*ABC, *emr*C, *emr*E and *qac*H were screened for in the samples using conventional PCR. Contrary to international literature, the *emr*E and *qac*H genes were not found in any of the isolates, however, a high prevalence of the *bcr*ABC gene (68%) and *emr*C gene (62%) were found in the isolates which indicates a high prevalence of QAC resistance amongst the isolates. Phenotypic testing was also carried out using a modified disk diffusion method. A high prevalence of phenotypic resistance towards different generations of QACs and QAC cocktails was found as well. Although, there were differences in sanitizer susceptibilities between isolation source, benzalkonium chloride (a first generation QAC) (BAC) was found to be the least effective while a QAC-free sanitizer from Byotrol was found to be the most effective. The phenotypic tests were carried out to represent a worst-case scenario of contamination. Finally, using a novel technique to measure biofilm growth in real time, known as the CO_2 Evolution Measurement System (CEMS), one isolate was used to measure the efficacies of BAC, peracetic acid (PAA) and the QAC free sanitizer from Byotrol against a *L. monocytogenes* biofilm's CO_2 output in real time. The isolate chosen was a lineage II and held both the *bcr*ABC and *emr*C resistance genes. Resistance towards BAC was encountered 15 hours after initial treatment and no resistance was encountered when the isolate was treated with PAA and the QAC free sanitizer from Byotrol. To conclude, a high prevalence of resistance towards QAC-based sanitizers was found amongst the isolates from

the six South African FPEs. Industry should therefore carefully consider their choice of sanitizers going forward and select a sanitizer that is effective against their FPE's unique sanitizer resistance profiles.

Opsomming

Listeria monocytogenes is ‘n alomteenwoordige Gram-positiewe bakterie wat kan oorleef en versprei binne voedselverwerkingsomgewings (FPEs), biofilms kan vorm op ‘n verskeidenheid van oppervlaktes, die toepassing van kwaternêre ammoniumverbindings (QAC)-klas ontsmettingsmiddels kan oorleef en die menslike siektetoestand, listeriose, kan veroorsaak. Die skoonmaak van FPEs volg ‘n multistap-proses wat verskeie stappe bevat, insluitend die toepassing van ‘n skoonmaakmiddel en meganiese aksie (skrop) wat help om grond en ander stowwe, wat kan help om die bakterieë te beskerm teen die effek van skoonmaak middels, te verwyder. Hierdie multistap skoonmaakproses kulmineer in die toepassing van ‘n terminale ontsmettingsmiddel wat help om enige oorlewende, vry selle te verwyder, en indien die voorafgaande skoonmaakstappe nie voldoende uitgevoer is nie, kan subletale blootstelling aan die ontsmettingsmiddel wat gebruik word, voorkom, wat kan lei tot ‘n toename in weerstandbiedendheid teen die ontsmettingsmiddel. Burgers van die Republiek van Suid-Afrika (RSA) is gedurende die periode 2017-2018 onderwerp aan die wêreld se grootste uitbreek van listeriose. Op grond van vorige globale uitbreke van listeriose en ‘n verband tussen die voorkoms van weerstandsgene wat weerstandbiedendheid teen QAC-klas ontsmettingsmiddels kan oordra, is ‘n ondersoek geloods na die voorkoms van ontsmettingsmiddel weerstandbiedende *L. monocytogenes* vanuit ses (6) Suid-Afrikaanse FPEs regoor die RSA met ‘n steekproefgrootte van 50 (N=50). Die 50 isolate is verkry uit vermoedelik-positiewe RapidL’mono (Biorad, FR) selektiewe media plate deur visuele bevestiging en polimerase kettingreaksie (PCR) bevestiging deur die *hly* geen te gebruik. Die isolate is dan in geslagslyne gekategoriseer, met 14 wat toegeken is aan geslagslyn I en 36 aan geslagslyn II. Die isolate het vanuit dreine, die voedselprosesseringsomgewing of voedselkontakoppervlaktes gekom. Daar is gevind dat al 50 isolate *L. monocytogenes* is. Daar is vir vier QAC weerstandsgene: *bcr*ABC; *emr*C; *emr*E; en *qac*H; gekontroleer in die monsters met behulp van konvensionele PCR vanweë hul teenwoordigheid in kliniese uitbrake van listeriose en hul hoë voorkoms van rapportering in internasionale literatuur. In teenstelling met internasionale literatuur is die *emr*E en *qac*H gene nie in enige van die isolate opgespoor nie, maar ‘n hoë voorkoms van die *bcr*ABC geen (68%) van isolate en *emr*C (62%) is gevind, wat ‘n hoë voorkoms van QAC-weerstandbiedendheid onder isolate aandui. Fenotipiese toetsing is ook uitgevoer deur middel van ‘n gewysigde skyfdiffusie metode. ‘n Hoë voorkoms van fenotipiese weerstand tot verskillende generasies van QACs en QAC-mengsels is ook gevind. Daar was verskille in vatbaarheid tussen isolasiebronne, maar in die algemeen is gevind dat bensalkoniumchloried (‘n eerste generasie QAC) (BAC) die minste effektief was en ‘n QAC-vrye ontsmettingsmiddel van Byotrol, die mees effektiewe ontsmettingsmiddel was. Die fenotipiese toetse is uitgevoer om die ergste geval van kontaminasie voor te stel. Uiteindelik, met behulp van ‘n nuwe masjien om biofilmgroei in reële tyd te meet, bekend as die CO2 Evolution Measurement System (CEMS), is een isolaat gebruik om die effektiwiteit van BAC, perasynsuur (PAA) en die QAC-vrye ontsmettingsmiddel van Byotrol te meet teenoor ‘n *L. monocytogenes* biofilm se CO_2-produksie, in reële tyd. Die isolaat wat gekies is, was ‘n geslagslyn II en het beide die *bcr*ABC en *emr*C weerstandsgene bevat. Weerstand teen BAC is 15 uur na die aanvanklike behandeling ondervind en geen weerstand is ondervind

wanneer die isolaat met PAA en die QAC-vrye ontsmettingsmiddel van Byotrol behandel is nie. Ten slotte, ‘n hoë voorkoms van weerstandbiedendheid teen QAC-gebaseerde ontsmettingsmiddels is gevind in die isolate van die ses Suid-Afrikaanse FPEs. Die industrie moet dus in die toekoms hul keuse van ontsmettingsmiddels noukeuring oorweeg en ‘n ontsmettingsmiddel kies wat effektief is teen hul FPE se unieke ontsmettingsmiddel-weerstandige profiele.

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Research contributions

Abstract for the 2021 International Association for Food Protection (IAFP) in Phoenix, Arizona, United States of America. Approved for poster presentation. Poster title: The prevalence of Quaternary Ammonium Compound (QAC) Resistance in *Listeria monocytogenes* isolated from South African Food Factories.

Table of contents

Abbreviations

ALOA- Agar Listeria Ottaviani and Agosti

BAC- Benzalkonium Chloride

CCP- Critical Control Point

Cfu / g- Colony forming units per gram

DOH- Department of Health

ECDC- European Centre for disease control

EFSA- European Food Safety Authority

*emr*E- multidrug transporter

FCS- Food contact surface

FPE- Food Processing Environment

HACCP- Hazard Analysis Critical Control Point

HIV- Human immunodeficiency virus

hly- Listeriolysin O gene

iap- Invasive associated protein gene

*inl*A- Internalin A gene

LA- Lactic Acid

LLO- Listeriolysin O gene

MATE- Multidrug and toxic compound extrusion

*mdr*L- Multidrug resistance L gene

MFS- Major facilitator Superfamily

MIC- Minimum inhibitory concentration

mpl- thrombopoietin receptor metalloprotease gene

NFCS- Non- food contact surface

NICD- National Institute for Communicable Diseases

PAA- Peracetic Acid

PFGE- Pulse field gel electrophoresis

*plc*A- Phospholipase A gene

*plc*B- Phospholipase B gene

*plc*C- Phospholipase C gene

*prf*A- Positive regulation factor A gene

PRP- Pre-requisite program

PVC- Polyvinyl Chloride

QAC- Quaternary ammonium compound

*qac*H- Quaternary ammonium compound-resistance protein H

RNA- Ribose nucleic acid

RND- Resistant nodulation division

RSA- Republic of South Africa

RTE- Ready to Eat

SigB- Sigma B stress factor

SMR- Small multidrug resistance family

TetR- Tetracycline resistance family

USA- United States of America

UTR- 5'- untranslated regions

WGS- Whole genome sequencing

σ^{B}- Sigma B stress factor

List of tables

List of figures

Chapter 1

Introduction

1.1 Introduction

Foodborne pathogens are a threat to human life and economic productivity because they can affect a variety of food products and their effects on human health can range from mild or moderate illness to life threatening infections or intoxications. Numerous bacterial species are responsible for foodborne infections in humans such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella spp.*, *Campylobacter spp.*, *Yersinia spp.*, *Shigella spp.*, *Clostridium spp.* and *Staphylococcus aureus* (Scallan *et al.*, 2011). With an increasing demand for ready to eat (RTE) or convenience foods that require no subsequent cooking steps, instances of listeriosis, a foodborne disease caused by *L. monocytogenes*, have been reported to be on the rise globally (Wang *et al.*, 2016).

With an increase in pathogen surveillance and the increasing prevalence of foodborne pathogens becoming notifiable diseases: the accountability that food producers are being held to is increasing as well. Food producers can mitigate the risk of foodborne pathogen contamination by ensuring that their food safety systems such as Hazard Analysis Critical Control Points (HACCP) and Good Manufacturing Processes (GMPs) are in place and are being correctly implemented (Wallace *et al.*, 2012).

Despite these systems and standards to which they are audited against: outbreaks still take place. Thus, food producers constantly need to re-evaluate and challenge conventional food safety systems as although they are in place, slip-ups are taking place, costing lives. An example of this need to re-evaluate and challenge existing protocols, comes in the form of factory hygiene and sanitation: an example of a GMP.

Factory hygiene and sanitation is a pivotal component in the production of safe food, and cleaning a factory regularly and correctly is a key component of the concept. Debris from food processing such as soil like skin, feathers, fat, plastic, meat, and fruit pulp need to be removed and surfaces that they were in contact with need to be cleaned to prevent bacterial growth and proliferation.

Between 2017 and 2018, citizens of the Republic of South Africa were subjected to the world's largest outbreak of listeriosis, an infection caused by the bacteria *Listeria monocytogenes*, chiefly associated with ready to eat (RTE) meat products, known locally as "polony" which closely resembles mortadella from Italy (Olanya *et al.*, 2019). Moreover, clinical *L. monocytogenes* isolates from listeriosis outbreaks associated with food products from various countries, have been found to be carrying genes which confer resistance to a class of compounds known as a Quaternary Ammonium Compounds (QACs). These QACs are routinely used

as terminal disinfectants or sanitizers in Food Processing Environments (FPEs) and resistance towards them has been detected internationally.

The causative agent in the South African outbreak was identified to be *L. monocytogenes*, lineage type I, serotype 4b, ST-6 (Olanya *et al.*, 2019) which has been shown, in the Netherlands, to harbour the *emr*C QAC resistance gene (Koopmans *et al.*, 2017). This suggests, as seen in the United States of America (USA) and Canada, the *L. monocytogenes* isolate responsible for the outbreak could be carrying a gene encoding for QAC resistance (Kovacevic *et al.*, 2016). The QAC resistance genes responsible for the USA and the Canadian outbreaks were the *bcr*ABC and *emr*C resistance genes which were respectively isolated from *L. monocytogenes* in infected persons (Kovacevic *et al.*, 2016; Müller *et al.*, 2014).

Koopmans *et al.* (2017) suggested that the *emr*C gene encodes resistance to a QAC known as benzalkonium chloride (BAC), which is often referred to as a first generation QAC that is commonly used as a terminal disinfectant in many FPEs. QACs have undergone many generational changes since BAC was first introduced as a sanitizer (Gerba, 2015). Many FPEs are now beginning to move away from BAC in favour of later generations of QACs, or cocktails of various generations of QACs or QAC-free sanitizers such as peracetic acid (PAA), or other alternatives from companies with novel technologies such as Byotrol QFS (Byotrol, GBR). Globally, there have been numerous studies focusing on BAC and resistance of pathogens (particularly *L. monocytogenes*) towards it, however very few discuss the alternative types of sanitizers available, nor investigate resistance towards them. This study aims to fill in knowledge gaps regarding QAC-resistance in FPEs within RSA, as well as try to identify any resistance towards QAC cocktails and QAC-free sanitizers.

Resistance of pathogenic bacteria towards antimicrobials is not a new concept and extensive studies have been carried out on the risk of antibiotic resistance on human health. Sanitizers, themselves, are antimicrobial agents and they have not been treated, historically, with the same respect or controls that antibiotics have been. As seen in cases from the USA and the Netherlands, QAC resistance genes have been detected in clinical isolates which suggests that those isolates can survive the application of a terminal disinfectant, contaminate a food product and subsequently cause listeriosis. Resistance towards sanitizers in South Africa has been investigated (Ackerman, 2017), however a study on the presence of certain QAC resistance genes and an association towards resistance to different commercial sanitizers: both in the planktonic and sessile state has not yet been conducted. This would prove advantageous to industry.

Understanding *L. monocytogenes* sanitizer resistance profiles in a FPE can be advantageous for the producer as it will allow for a targeted approach to the eradication all *L. monocytogenes* cells within an FPE. This would furthermore help prevent cases of product contamination and ultimately listeriosis which in the long run would save lives and also result in a good fiscal outcome for the producer.

L. monocytogenes can form biofilms on a variety of surfaces within an FPE such as stainless steel, and cells in the biofilm state can be up to 1000 times more resistant to antimicrobials than cells in the planktonic state. This shows that the control of *L. monocytogenes* within an FPE should be carried out against planktonic cells to help ensure that biofilms are not formed, due to their hard to eradicate nature (Mah, 2012).

The focus of this study is therefore to identify and categorize *L. monocytogenes* isolates from six FPEs across the Republic of South Africa and categorize them in terms of lineage type and isolation location as well as to identify the prevalence of three resistance genes and one resistance cassette and explore responses of the isolates towards commercial sanitizers in both the planktonic and sessile state.

The first objective of this study was to identify the presence of four resistance genes in 50 *L. monocytogenes* isolates from six FPEs across RSA, the genes were: *bcr*ABC, *emr*C, *emr*E and *qac*H. Thereafter, identify a correlation between lineage type and resistance gene prevalence. Two reference strains were used *L. monocytogenes* (ATCC 7644) and *L. innocua* (ATCC 33090).

The second objective of this study was to test various commercial sanitizers against the 50 *L. monocytogenes* isolates using a diffusion disk method to ascertain the phenotypic expression of resistance of the isolates.

The third research objective was to determine the response of a biofilm culture from one of the 50 isolates to three commercial sanitizers, which was measured in real time using the CO_2 evolution measurement system (CEMS). This was done to determine whether the diffusion disk results aligned with the real-time measurement results.

1.2 References

Ackerman, E. (2017). Survival and proliferation of *Listeria monocytogenes* in a South African ready-to-eat factory. MSc Thesis. Stellenbosch University, South Africa.

Gerba, C.P. (2015). Quaternary Ammonium Biocides: Efficacy in Application. *Applied and Environmental Microbiology*. 81(**2**):464-469.

Koopmans, M.M., Bijlsma, M.W., Brouwer, M.C., van de Beek, D., van der Ende, A. (2017). *Listeria monocytogenes* meningitis in the Netherlands,1985-2014: a nationwide surveillance study. *Journal of Infection*. **75**. 12-19.

Kovacevic, J., Ziegler, J. Walecka-Zacharska, E., Reimer, A., Kitts, D.D. Gilmour, M.W. (2016). Tolerance of *Listeria monocytogenes* to Quaternary Ammonium Sanitizers is mediated by a novel efflux pump encoded by emrE. *Applied and Environmental Microbiology*. 82(**3**): 939-953.

Mah T.F. (2012). Biofilm-specific antibiotic resistance. *Future Microbiology.***7**.1061–72

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stressl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S. (2014). The *Listeria monocytogenes* tansponson Tn6188 provides increased tolderance to various quaternary ammonium compounds and ethidium bromide. *FEMS Microbiology Research Letters*. **361**(2). 166-173.

Olanya, O.M., Hoshide, A.K., Ijabadeniyi Ukuku, D.O., Mukhopadhyay, S., Niemira, B.A., Ayeni, O.M. (2019). Cost estimation of listeriosis (*Listeria monocytogenes*) occurrence in South Africa in 2017 and its food safety implication. *Food Control*. **102**. 231-239.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases*. **17**. 7–15.

Wallace, C.A., Holyoak, L., Powell, S.C., Dykes, F.C. (2012). Rethinking the HACCP team: An investigation into HACCP team knowledge and decision-making for successful HACCP development. *Food Research International*. **47**. 236-245.

Wang, S., Weller, D., Falardeau, J., Strawn, L. K., Mardones, F. O., Adell, A. D., Moreno Switt, A.I. (2016). Food safety trends: From globalization of whole genome sequencing to application of new tools to prevent foodborne diseases. *Trends in Food Science & Technology*. **57**(Part A). 188–198.

Chapter 2

The control of *Listeria monocytogenes* within food processing sites requires more than an understanding of resistance to sanitizers: a review

2.1 Introduction

Sanitizer resistance amongst *Listeria monocytogenes* is of growing concern across all sectors of food production, and this prevalence continues to rise globally. This growing resistance amongst *L. monocytogenes* is especially prevalent against the quaternary ammonium compound (QAC) class of sanitizers and in particular, benzalkonium chloride (BAC) or first generation QAC. This has been found across the globe and across food processing industries and also in other species of *Listeria* such as *L. innocua*, *L. ivanovii* and *L. welshmeri* (Dutta *et al.,* 2013; Elhanafi, *et al.,* 2010; Jiang, *et al.,* 2016; Korsak & Szuplewska, 2016; Mereghetti *et al.,* 2000; Møretrø *et al.,* 2017; Mullapudi *et al.,* 2008; Ratani *et al.,* 2012; Xu *et al.,* 2016).

These resistant strains of *L. monocytogenes* are thus able to survive the final step of the cleaning process, should the incorrect sanitizer be applied, which gives them the ability to persist in factory floors and drains (Berrang, & Frank, 2010; Tompkin, 2002). The biofilm forming ability of *L. monocytogenes* is another great cause for concern as it allows *L. monocytogenes* to adhere to parts of the processing equipment within factories such as bowl choppers and conveyor belts, which thus increases the contamination risk further (Alonso, *et al.,* 2014; Beresford, Andrew & Sharma, 2001; Gamble & Muriana, 2007; Wang, *et al.,* 2012).

Combined, the ability of *L. monocytogenes* to colonise a varied array of food factory areas with inadequate sanitation protocols within factories (not following a cleaning regime), and inadequate sanitizer application (such as lowered concentration or insufficient contact time) results in the selection of resistant strains of *L. monocytogenes* or other bacteria species such as *Pseudomonas spp.* This can further facilitate the spread of sanitizer resistant determinants through mixed species biofilms or even plasmid mediated transfer (Kim *et al*., 2013; Simões *et al.,* 2010). These resident strains are then able to further contaminate various parts of the factory due to human and product movement and if finished product (especially ready to eat (RTE)) is contaminated then the effects can be incredibly detrimental as seen in the listeriosis outbreak of 2017/ 2018 in the Republic of South Africa (RSA) (Thomas *et al.,* 2020).

The correlations between outbreaks of listeriosis and the presence of genes conferring resistance to sanitizers (Dutta *et al.,* 2013; Kovacevic *et al.,* 2016), undeniably highlights the need to improve the understanding and implementation of cleaning practices within food producing environments. Further, an understanding of where in a food factory, *L. monocytogenes,* can form biofilms (in niche locations such as cracks in the floor and processing table-legs) would allow for a targeted approach to monitoring and ultimately controlling the spread of *L. monocytogenes*.

2.2 *Listeria monocytogenes*

2.2.1 Background on *L. monocytogenes*

Citizens of the RSA recently experienced the world's biggest outbreak of listeriosis, with panic and distrust surrounding processed meats ensuing. The Department of Health (DOH) and the National Institute for Communicable Diseases (NICD) determined that *L. monocytogenes* sequence type 6 (ST-6) associated with a processed, value added-deli meat locally called "polony" (Department of Health National, *Listeria* Incident Management Team, 2018) was the cause of the outbreak. The outbreak was investigated by the DOH and NICD for a total of 60 weeks, which saw a total of 204 individuals succumb to the illness (mortality rate of nearly 20%) with a total of 1034 confirmed infected persons (Boatemaa *et al.,* 2019; Lepe, 2020; Olanya, *et al.,* 2019). The 2017/2018 outbreak of listeriosis in the RSA further highlighted the need for better *L. monocytogenes* control measures across the food processing chain.

L. monocytogenes belongs to the phylum Firmicutes and resides within the family *Listeriaceae* which is comprised of the non-pathogenic *Brochothrix* and *Listeria* (Huillet *et al.*, 2005). Currently there are twenty known species of *Listeria* with *Listeria thailandensis* being the latest addition (Leclerq *et al.,* 2019). *L. monocytogenes* is a ubiquitous, Gram-positive, non-spore forming, motile bacterium that can live and propagate at refrigerated temperatures (Forsythe, 2010). *L. monocytogenes* can adhere to stainless steel, polystyrene, and form biofilms (Poimenidou *et al*., 2016). The cells of *L. monocytogenes* in the sessile state have a much greater resistance to external stresses such as sanitizers, desiccation, pH, and temperature than those cells in the planktonic state (Costa *et al.,* 2018).

The genus *Listeria* contains multiple species which are predominantly non-pathogenic such as *Listeria innocua*. *L. monocytogenes* is the species within the genus of most concern due to its pathogenic nature, however pathogenic strains of *L. innocua* have been isolated (Osri & Wiedmann, 2011; Johnson *et al*., 2004; Perrin *et al*., 2003; Favaro *et al*., 2014). Listeriosis is the human disease associated with infection by *L. monocytogenes* with a lethal dose of 10^6 cells of the organism (Pouillot *et al.,* 2016). Symptoms of listeriosis include meningitis, encephalitis, septicaemia or even miscarriages in pregnant women. Immunocompromised individuals such as those suffering from cancer, or otherwise immunocompromised are at much higher risk of succumbing to listeriosis (Colagiorgi *et al.,* 2017).

Various genes contribute to the virulence of *L. monocytogenes*. The phospholipase A (*plc*A), phospholipase B (*plc*B), encoded hemolysin (*hly*), positive regulation factor A (*prf*A), invasive associated protein (*iap*) and thrombopoietin receptor metalloprotease (*mpl)* genes are all associated with the virulence of the organism (Shi *et al.,* 2013; Vines *et al*., 1992). There were initially three lineage types that *L. monocytogenes* was classified according to: Lineage type I, Lineage type II and Lineage type III (Piffaretti *et al.,* 1989; Rasmussen *et al.,* 1995; Wiedmann *et al.,* 1997). However, work carried out by Ward *et al.,* (2008)

and Osri *et al.,* (2011) has determined that there are four lineage types of *L. monocytogenes*, with lineage type III being split into two distinctive lineage types. Their distribution within food factories is rather disproportionate with lineage II being the dominate lineage type found within food factories, followed by lineage I.

Lineage type I contains serotypes 1/2 b, 3b, 4b, 4d and 4e (Cheng *et al.*, 2008; Osri *et al.,* 2011). Lineage types I and II dominate the food processing environment with high prevalence of 1/2 a (Lineage type I) and 4b serotypes (Swaminithan & Gerner-Smidt, 2007; Skowron *et al.,* 2018). Serotypes 4d and 4e are rare amongst food and clinical isolates, they are however understood to be very similar to 4b (Cheng *et al.,* 2008) and are therefore designated the classification of "serotype 4b complex" (Eifert *et al.,* 2005).

Lineage type II is comprised of serotypes 1/2 c, 1/2 a, 3a and 3c (Borucki et al. 2003; Cheng *et al.,* 2008; Kathariou, 2002). Studies have shown that lineage type III (serotypes 4a, 4c and an atypical 4b) is comprised of three distinct groups; Lineage IIIA, lineage IIIB and lineage IIIC (Liu *et al.,* 2006). Through further studies on lineage type III, it was determined that lineage type IIIB should be reclassified as lineage type IV due to it being a distinct group with remarkable phylogenetic differences between other lineages (Osri *et al.,* 2011; Ward *et al.,* 2008). Lineage type III and lineage type IV were the most recently classified, being classified in 1995 and 2008 respectively (Rasmussen *et al.,* 1995; Ward *et al.,* 2008). Table 2.1 shows the serotypes most commonly associated with human listeriosis from lineages I and II, as could be encountered in a FPE.

Strains of *L. monocytogenes* belonging to lineage type IV are said to be atypical and incredibly rare, their existence was often brushed by earlier articles however the existence of a fourth lineage group does not have major implications for the food industry as lineage type II dominates food processing environments, with lineage I being slightly less prevalent than lineage II (Melero *et al.,* 2019; Alia *et al.,* 2020; Baria *et al.,* 2020; Aalto-Areneda *et al.,* 2019). It is important to note that lineage type IV is atypical and has only been isolated from animals thus far (Kuenne *et al*., 2013).

Table 2.1 *L. monocytogenes* lineage type and serotypes associated with linage type I and II (Rip & Gouws, 2020)

Lineage Type	Serotypes associated
I	1/2 b; 3b; 4b; 4d; 4e; 7
II	1/2 a; 1/2 c; 3a; 3c

Three major serotypes are most often associated with the disease in humans and they are 1/2 a, 1/2 b and 4b with serotypes 4a and 4c (lineage III) being rarely associated with listeriosis (Wiedmann *et al.,*

1996; Jacquet *et al.,* 2002). Identified functions of the pathogenicity genes above have been identified and documented which help explain the lethality of listeriosis. The internalin A gene (*inl*A) is responsible for the entry of the organism into the cell, the *hly* and *plc*A genes are responsible for the lysis of the vacuole and intracellular division, the *act*A gene is responsible for actin polymerization and intracellular movement and finally *plc*B is responsible for cell-to-cell spread of the *L. monocytogenes* cells (Montville *et al.,* 2012).

Listeriosis can be differentiated as either a non-invasive and self-limiting gastrointestinal manifestation of the illness or as an invasive clinical condition which affects those with compromised immune systems, deemed as "at risk" individuals, example, those suffering from cancer, human immunodeficiency virus (HIV), the elderly, young or pregnant. Manifestations can be neonatal infections, maternofetal listeriosis, septicaemia or meningoencephalitis (Swaminathan & Gerner-Smidt, 2007; Low & Donachie, 1997). Cutaneous listeriosis is rare and is usually observed in those working with animals such as farmers and veterinarians (Godshall *et al*., 2013). The self-limiting, febrile gastroenteritis is often described as being non-specific and is often not diagnosed as listeriosis: it also has a much shorter incubation time and is self-limiting in nature (where no medication or hospital treatment is required to recover) and usually occurs in healthy adults who have had a ingested a high dosage of the pathogen (Swaminathan & Gerner-Smidt, 2007). Studies in Canada have described sporadic gastroenteritis due to *L. monocytogenes* as an uncommon illness through a two-year stool sample screening period (Schlech *et al.,* 2005). Later studies have shown that this form of listeriosis may actually be underrepresented with a self-reported gastroenteritis survey reporting that only 36.5% of infected people sought assistance with only 2.3 % of those submitting a stool sample, suggesting that the sample size may have not been representative (Scavia *et al.,* 2012).

The invasive form of listeriosis yields incredibly high mortality rates of between 20-30%, despite antibiotic treatment (Swaminathan & Gerner-Smidt, 2007). Even higher mortality rates have been observed in the hospital setting where outbreaks have been seen to occur (Gaul *et al.,* 2013). The infectious dosage of *L. monocytogenes* is generally considered to be between 10^4 to 10^6 cfu/g (colony forming unites per gram) (Vázquez-boland *et al.,* 2001; Swaminathan & Gerner-Schmidt, 2007; Lamont, *et al.*, 2011). However exact estimates and studies have been affected by the long incubation times (upwards of 60 days). Certain groups of individuals (such as those suffering from cancer, individuals undergoing dialysis or the elderly) presented with more serious manifestations of the disease than those under the age of 65 with no underlying medical conditions (Pouillot *et al.,* 2016). Underlying conditions such as lung and pancreatic cancer have been shown to increase mortality rates by invasive listeriosis infections (Goulet *et al.,* 2012; Pouillot *et al.,* 2016; Silk *et al.,* 2012).

A study looking at listeriosis between 2004 and 2006 found that the highest frequencies of *L. monocytogenes* were found in RTE food products and in meat and fish products (EFSA & ECDC, 2014). The

highest proportion of noncompliant food products in retailers were hard cheeses, fermented sausages, RTE fish products and soft and semi-soft cheeses (EFSA & ECDC, 2014). It is challenging to try and decipher trends and incidences of *L. monocytogenes* in RTE foods, since different sampling plans and methods hinder the comparability of different study results.

L. monocytogenes has many unique adaptions which are temperature dependent and convey a survival advantage upon the organism by either increasing its virulence potential or assisting in the formation of a biofilm (survival mechanism) (McGann *et al.,* 2007). Three distinct lifestyles of *L. monocytogenes* are described which are (i) as an intracellular pathogen utilising actin-based motility for cell to cell spread, (ii) as an extracellular, free living pathogen and (iii) as an extracellular bacterial member of a multicellular biofilm community (Lemon *et al.,* 2010). These lifestyles are triggered by various temperature changes/ zones of temperature which determine which genes are transcribed, thus aiding in the survival and success of this pathogen (McGann *et al.,* 2007). In the interests of remaining within a food safety scope, only the *prf*A and *sig*B (sigma B) transcription factors and their roles in pathogenicity and biofilm formation will be further discussed, however it is worth noting that nine different ribonucleic acid (RNA) and protein mediated virulence (or survival) gene transcription factors have been identified and described (Lebrenton & Cossart, 2017; Lemon *et al.,* 2010;) These include protein-mediated controls of virulence gene expression, *prf*A which can be described as the master controller of virulence genes in *L. monocytogenes*, sigma B (σ^B) stress response activator, *vir*R coordinator of surface components modification and antimicrobial resistance, *mog*R which represses motility genes, *cod*Y which regulates *Listeria* metabolism in host cells, 5'-Untranslated Regions (UTRs), Riboswitches, cis-regulation by antisense transcription and trans-acting functions of small RNAs (Lebreton & Cossart, 2017).

Through the presence of a thermoregulated transcription factor, known as *Prf*A, *L. monocytogenes* can regulate the expression of its virulence genes based on the temperature of its environment. *Prf*A was the first identified regulator of virulence genes in *L. monocytogenes* and was found to regulate the transcription of the *hly* housekeeping gene which codes for listeriolysin O (LLO) which forms pores in to-be invaded cells. The PrfA protein can be classified as belonging to the family of cyclic AMP receptor protein transcriptional regulators, which bind DNA as dimers on specific sites in gene promoters and thus activate transcription. Co-expression of *hly* with *prf*A was found with the *prf*A virulence cluster of genes (Lebreton & Cossart, 2017). The virulence genes have been shown to have maximum expression at 37 ^{O}C and virtually silent at 30 ^{O}C, this shows *L. monocytogenes* can control its gene expression, thus saving energy and further contributing to its pathogenicity inside a living host (Johansson *et al.,* 2002). The mechanism of the thermoregulation of PrfA can be attributed to an RNA-mediated control called a 5'-UTR which controls translation depending on temperature and a motif called a Shine-Dalgnaro (SD) sequence of PrfA. This SD sequence of PrfA is close to

a closed stem-loop structure which blocks the access of a small ribosomal subunit which therefore inhibits translation unless the organism is exposed to a specific temperature (Lebreton & Cossart, 2017).

The *sig*B (sigma B) stress factor plays a role in the activation of virulence genes within the intestine of a host as well as numerous genes associated with the survival of the organism, it also regulates PrfA expression at temperatures below that of a host (Nadon *et al.,* 2002; McGann *et al.,* 2007). SigB, furthermore, controls and prepares flagellum propelled *L. monocytogenes* cells for either host cell invasion or the formation of a biofilm on an abiotic surface (Lemon *et al.,* 2010). This highlights the fact that these two regulatory factors have the organism "primed" for survival with or without a suitable host, and truly set *L. monocytogenes* apart from other pathogens encountered in food production.

It is however important to note that *L. monocytogenes* is not the only foodborne pathogen of concern showing resistance to commercial sanitizers: *Salmonella Typhimurium* (Nguyen& Yuk, 2013), *Escherichia coli* O157:H7 (Wang *et al.,* 2020), *Staphylococcus aureus* (Da Silva Meira *et al.,* 2012) and *Clostridium perfringens* (Udompijitkul *et al.,* 2013) make up the most common causative agents of food poisoning globally (Maia *et al.,* 2020; Buzby, 2001).

The ability of *L. monocytogenes* to grow at temperatures as low as -0.4 ^{O}C and even survive a freezing process as low as -18 ^{O}C (Walker, *et al.,* 1990; Nowak, *et al.,* 2015) can be explained through bacterial membranes becoming more rigid and the metabolic rate of the organism decreasing, when exposed to lower temperatures. This therefore means that bacteria need to increase the expression of genes involved in cell membrane function and produce cold shock proteins as well as other metabolic strategies to maintain homeostasis (NicAogáin & O'bryne, 2016). A common cold adaptive strategy employed by *L. monocytogenes* is through the induction of osmolyte and peptide transporters which help maintain turgor pressure within the cell (Miladi *et al.,* 2017). Turgor pressure is maintained through the uptake of osmoprotectant molecules (osmolytes and short oligopeptides) which is facilitated through the cold adaption of *L. monocytogenes* (Wemekamp-Kamphuis *et al.,* 2004). Protein damage can also be experienced by bacteria in cold environments. Such damage can be counteracted via network of molecular chaperones which help assist in maintaining proteins in their various native states; by preventing protein aggregation (Kim *et al*., 2013). The responses of *L. monocytogenes* to the cold and other changing environmental conditions can be explained by the *sig*B stress factor which controls various metabolic pathways, transport associated proteins, stress proteins and other transcription factors (Chan *et al.,* 2007) especially the osmolyte carnitine. Other proteins associated with cold stress resistance are known as ferratin like proteins (Hebraud & Guzzo, 2000). A direct link between cryotolerance and *sig*B has not yet been obtained, however clear signs that this transcription factor has a role in *Listeria*'s adaption to cold temperatures is clear (Santos *et al*., 2019; NicAogáin & O'bryne, 2016).

2.3 Sanitizers

2.3.1 Sanitizer uses throughout food processing

A sanitizer is a chemical compound used at the end of a cleaning cycle to render any surviving bacterial cells as non-viable. They are commonly comprised of quaternary ammonium compounds (QACs), L-lactic acid, peracetic acid or hypochlorite. However, the selection of most appropriate sanitizer for a factory are adjusted based on the needs of the factory and its unique pathogenic residents and incoming raw materials (EcoWize, 2019). It is important to note that sanitizers are not a stand-alone solution when it comes to cleaning a factory, they are in fact the last step in a multistep cleaning process (Holah *et al*., 2004; Gibson *et al*., 1999).

2.3.2 Types of sanitizers

2.3.2.1 Quaternary ammonium compound (QAC)-based sanitizers

QAC based sanitizers are cationic detergents that reduce surface tension and form micelles in a liquid that makes for their easy application in a food-processing environment (Gerba, 2015). They were first noted for having antimicrobial activity over a century ago. However, QACs only came into widespread use after World War I due to their cost and effectiveness at eliminating microorganisms (Merianos, 2001). The mode of action of a QAC sanitizer involves the interaction of a cytoplasmic membrane of a bacteria and involves the following steps as described by McDonell (2020): the initial step is the adsorption of the QAC to the cell wall and its subsequent penetration, followed by a reaction with the cell's cytoplasmic membrane which causes membrane disorganization. Next the leakage of intracellular, lower-weighted materials occurs followed by the degradation of proteins and nucleic acids. Finally, cell wall lysis occurs, and this is facilitated by autolytic enzymes.

2.3.2.2 Peracetic Acid based sanitizers

Peracetic acid (PAA) is another commonly used sanitizer, particularly in the dairy industry due to its wide spectrum of effectiveness, particularly its effectivity at low temperatures (Lee *et al.,* 2017). PAA was patented for use in treating produce with the aim of reducing spoilage organisms in 1950 (Alvaro *et al.*, 2009; Greenspan & Margulies, 1950). PAA is a mixture of acetic acid and hydrogen peroxide in aqueous solution and is a very strong oxidizing agent and is also commonplace in factories as a surface disinfectant for food contact surfaces (Evans, 2000; Brinez *et al.,* 2006). PAA is effective against bacteria through its ability to oxidize the outer membrane of a bacterial cell. The transfer of electrons (the process of oxidation) is what destroys the cell, causing bacterial cell death. PAA is a stronger oxidizing agent than chlorine (Du *et al.,* 2018).

2.3.2.3 Chlorine based sanitizers

Chlorine as mentioned above is also a well-known oxidizing agent and has been used in many food industries for quite some time to control a variety of organisms including *L*. *monocytogenes* (Butterfield, *et*

al., 1943; Tonney *et al.,* 1928; Willet, 1980; El-Kest & Marth, 1988). Due to it being an oxidizing agent, its mode of action is identical to PAA; however, it is a weaker oxidizing agent than PAA and is therefore not as effective against Gram-positive or Gram-negative bacteria. Moreover, it has begun to fall out of use within many food industries due to the rise of awareness regarding the biofilm formation of *L. monocytogenes* and its enhanced resistance to antimicrobial application as well as environmental conditions. Based on historic misuse, much higher concentrations of chlorine are required on bacteria that are in the process of contaminating and colonising a FPE (De Luca *et al.,* 2008). *L. monocytogenes* has been shown to be more resistant to chlorine whilst in the sessile state when compared to planktonic cultures (Folsom & Frank, 2006). These revelations combined with the availability of alternative sanitizers has led to the decline in the use of chlorine-based sanitizers to control *L. monocytogenes*. Chlorates have subsequently had their use in FPE protection within the European Union (EU) phased out by industry. However, EC No. 396/2005 does still have a regulated maximum residue allowance (European Commission, 2019)

2.3.2.4 Lactic acid-based sanitizers

Lactic acid is the product of lactic acid fermentation and can be found in either L or D chiral forms with organisms such as *Lactococcus* and *Carnobacterium* producing L-Lactic acid and *Leuconostoc* producing D-Lactic acid (Liu *et al.*, 2003). An article by Gravesen *et al.,* (2004) showed that *L. monocytogenes* was more susceptible to D-Lactic acid than it was to L-Lactic acid. It is important to note that the penetration of lactic acid across the membrane of a cell is identical for both isomers but L-lactic acid had a greater overall antimicrobial effect than D-lactic acid against *Escherichia coli* O157 (McWilliam Leitch & Stewart, 2002). In addition to this non-stereospecific pH-related detrimental effect of lactic acid, there is an additional chiral-specific interaction taking place. Studies on *E. coli* and *Lactococcus delbrueckii* subspecies *bulgaricus,* both of which produce D-Lactic acid, showed that they are less susceptible to their produced chiral form of lactic acid when compared to L-Lactic acid, and this may be used to explain the decreased susceptibility of *L. monocytogenes* to L-Lactic acid (Benthin & Villadsen, 1995; Bunch *et al.,* 1997; Gravesen, *et al.,* 2004). This may be mediated through either the metabolism of intracellular lactate or a stereospecific efflux system (Benthin & Villadsen, 1995; Bunch *et al.,* 1997; Gravesen, *et al.,* 2004). Furthermore, Gravensen *et al.,* (2004) found that the sensitivities of tested *L. monocytogenes* strains to lactic acid corresponded to their sensitivities to hydrochloric acid. The varying levels of sensitivity between the tested strains of *L. monocytogenes* did not have a correlation to the intrinsic levels of L-Lactic acid produced. Gravesen *et al.,* (2004) found, that the strain and the variations in sensitivity to L-lactic acid were larger than the difference between the effects of L- and D- lactic acid and when compared to the work of McWilliam Leitch & Steward (2002).

L-Lactic acid has become the most common form of commercially available lactic acid, due to its ability to be scaled up by industrial fermentation and its application in a variety of other industries such as pharmaceuticals and cosmetics (Boomsma *et al.,* 2015).

2.3.2.5 Sanitizer selection

The choice of sanitizer used by a factory can be influenced by the following factors: the type of soil (contaminant such as leftover/ spilt food) that needs to be removed. In addition to the type of surface that needs to be cleaned; factors such as corrosion susceptibility as well as lamination need to be considered. The concentration required to be effective; economic factors; method of application and compatibility with water type and influence of hard water on chemical action need to also be considered. Finally, availability and cost and the amount of cleaning chemical required all play a role in the selection of a sanitizer (SurTech Deutschland GmbH, 2014).

2.4 Factory Cleaning operations

The standard process of cleaning a factory usually follows a 10-step process that is outlined as follows (Ecowize, 2019):

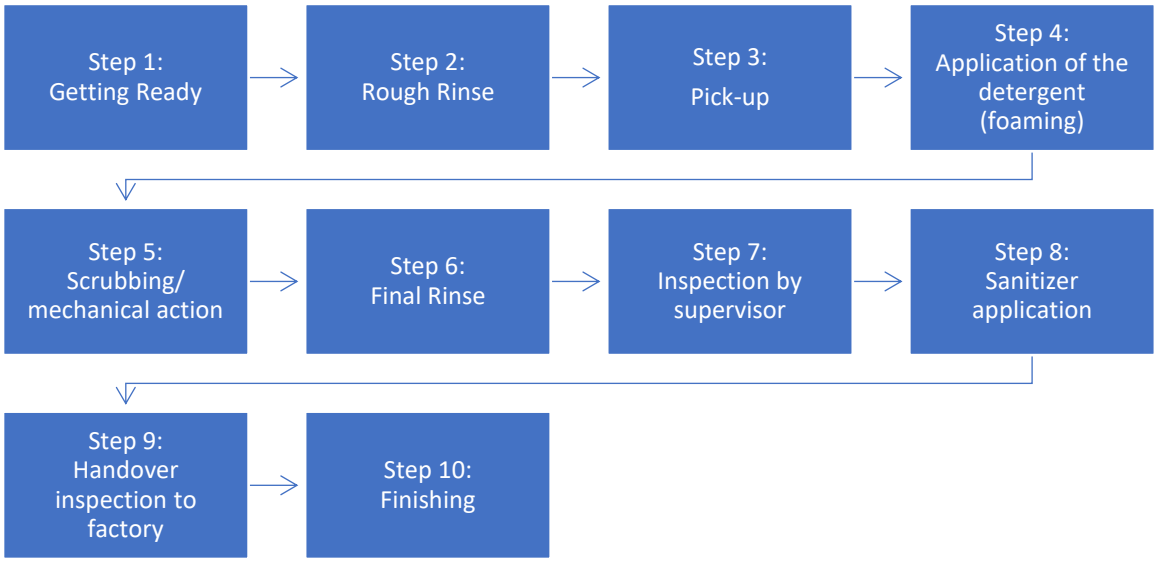


Figure 2.1 An illustration of the 10-steps of cleaning (adapted from Ecowize, 2019).

This 10-step process was development and formalized by EcoWize, an international cleaning company with its base in South Africa, to allow for cleaning to be audited to the ISO 9001 standard.

Step 1: the 'getting ready' step involves the following criteria: ensuring that the staff are wearing the company issued HACCP compliant uniform (trousers and covering long-sleeved pullover); ensuring that the staff are kitted in the correct personal protective equipment (PPE) (oilskin rain suits, goggles, earplugs, gloves and site specific PPE for example a hardhat). The issuing of the chemicals is done safely, and they are correctly labelled with familiarisation of first aid procedures taking place. The issuing of cleaning apparatus and consumables (brushes, scouring pads, ladders, hoses, hose nozzles, squeegees) is recorded. Ensuring lock out procedures are in place (especially when working with moving machinery). Ensuring that all machines such

as screens and scales are covered with Polyvinyl chloride (PVC) covers to prevent water damage. Any equipment that requires dismantling is stripped by qualified personnel. Packaging products, or raw materials must be moved to a separate and secure area as per customer requests. All loose debris prior, to rinsing, must be picked up and place in designated bins. Ensuring that all chemicals are tested with a kit to determine their concentration which are then recorded; thereafter the handover from customer to cleaning service is completed notifying the cleaning service of any changes from the normal procedure (EcoWize, 2019).

Step 2: the 'rough rinse' step involves the following: ensuring that all areas/ equipment that require a dry clean (scales, computers and control panels) are not wet or damaged during this step. Planning the sequence of rinsing to ensure that no cross contamination takes place. Ensuring that hoses are connected and that the correct nozzle attachment is selected and that the rinsing occurs from the ceiling to the floor. All gross debris, fat, proteins and allergens must be removed from the equipment after the rough rinse has been completed. It is important to ensure that no rinsing of any gross debris down the drain occurs and finally no rinsing should take place whilst sanitation is in progress (Ecowize, 2019; Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998).

Step 3: the 'pick-up step' involves: using the squeegee apparatus to move all debris to a point which is then picked up by hand and discarded in designated waste bins; removal of all large pieces of debris and its placement in designated waste bins and finally ensuring that no organic matter/ gross debris is rinsed, swept or soaked up with the sponge broom that can be trapped down the drain or in a drain's fat trap (EcoWize, 2019; ; Khalid *et al*., 2019; Meng *et al*., 1998).

Step 4: the 'foaming' or application of a detergent step involves: the application of the detergent via the detergent specific standard; planning the sequence of detergent application to avoid and prevent cross contamination; filling the correct coloured buckets with detergent/ foam for manual scrubbing application; verifying the mix concentration using specific tests; ensuring that any and all areas that require dry or waterless cleaning are not wet or damaged during the application of the detergent, and finally an acid wash step (if required/ specific to the site in question) (Ecowize, 2019; Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998).

Step 5: the scrubbing step involves the following: planning the sequence of scrubbing to ensure that no cross contamination occurs; scrubbing all areas of the food plant with scouring pads, disposable cloths, brooms to ensure removal of fat, protein, allergen or other organic matter; ensuring that no equipment is cleaned, scrubbed, rinsed directly on the factory floor- it is imperative that containers, bins, loose utensils and tools are cleaned on a table to prevent any further contamination from dirty boots and uncleaned areas of the factory (Ecowize, 2019; Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998).

Step 6: the 'final rinse' step involves ensuring that the required dry-cleaning areas are still protected, the sequence of events prevents cross contamination, and that the factory is rinsed from top to bottom (to prevent aerosol contamination). No visible chemical residue should persist within the factory. No visual fat, protein or allergen residue should be visible, ensuring that if an acid wash was used that it was rinsed correctly and finally all PPE in use including boots are cleaned (EcoWize, 2019).

Step 7: the 'inspection by supervisor' step involves: ensuring that the entire area is clean according to company standards and that any non-conformances are rectified; the inspection will make use of a torch and ladder to investigate difficult to see areas; any and all potential allergen issues must be rectified immediately and finally if findings are unsatisfactory steps four to seven are to be repeated (EcoWize, 2019).

Step 8: the 'sanitation step' involves: applying the sanitising agent to oilskins and PPE, with the chemical burn risk of the sanitizer taking top priority during the process; applying the agreed upon sanitizer (consultation with the service provider of the pre-requisite program for cleaning of the factory) to all food contact surfaces (FCS) and non-food contact surfaces (NFCS). Sanitizing to prevent cross contamination using a ceiling to the floor approach for sanitizer application. The sanitizer is to only be rinsed if specifically instructed to do so. Any sanitizer rotation that occurs is to be noted in documentation and finally all PPE is to be sanitized upon completion of sanitizer application step (Ecowize, 2019; Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998).

Step 9: the 'handover inspection to factory' is the responsibility of the cleaning supervisor and all issues that occurred during cleaning are to be reported to the factory. It is important to note that the factory's personnel must, wherever possible, work with the cleaning service provider to ensure that a minimum standard of cleanliness is maintained before cleaning occurs to facilitate the deep clean process. All non-conformances from the factory's side that were picked up during cleaning are to be raised at this point (EcoWize, 2019).

Step 10: the 'finishing step' involves: reassembling all equipment that were taken apart; rinsing of cleaning stations with clean water; ensuring that all hoses are drained and stored correctly along with other cleaning equipment and free from soil that may have been picked up during cleaning operations. Sanitization of all cleaning equipment; food contact and non-food contact equipment must be cleaned in separate areas. Storage of equipment with the head down (off the ground) on rails; buckets to be stored off the floor; all consumables such as scouring pads and cloths are to be returned as well as nozzles and cleaning PPE. PPE and boots must be cleaned and neatly stored, and finally an inspection by supervisor follows ensuring that

all equipment is ready and operational for the next cleaning session (Ecowize, 2019; Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998).

Cleaning should not occur whilst production is taking place as this will facilitate cross contamination, even with barrier sheets up as they have very little impact on preventing the spread of debris, soil and microorganisms that occurs when rough rinsing begins (Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998). Barrier sheets also do not help mitigate the risk of chemicals used in cleaning, contaminating either raw materials or final products, therefore this chemical hazard should be mitigated through the application of an effective HACCP program to ensure ultimate product safety (Ecowize, 2019; Khalid *et al*., 2019; Leu *et al*., 1998; Meng *et al*., 1998).

The type of surface influences the efficacy of the cleaning with smoother surfaces being cleaned more efficiently than rougher surfaces (Khalid *et al*., 2019).

2.4.1 Cleaning contact time and temperature of application

The contact time of a cleaning chemical agent is of great importance as it allows for greater interactions of the active compound against the bacterial cell (EcoWize, 2020). It was shown by Costa *et al.,* 2018 that increasing the contact time increased the efficacy of the cleaning protocol. Contact times are usually between 10 and 15 minutes for the cleaning of commercial plants due to time constraints because of production pressures put on cleaning service providers by factory management (Ecowize, 2019; Khalid *et al.,* 2019; Leu *et al.,* 1998; Meng *et al.,* 1998).

Tobin *et al.,* (2020) studied the effect of temperature of disinfection on six heat tolerant strains of *L. monocytogenes* and it was found that hot water (55 ^{O}C to 75 ^{O}C) played a significant role in decontamination efficacy by helping remove fat residue and other soil from the daily operations. Furthermore, South African meat producing regulations require hot water to be used during all cleaning procedures due to its ability to dislodge fat and other bodily soil which can shield bacterial cells such as *L. monocytogenes* from the active compounds in the cleaning chemical. Temperatures of between 40-60 ^{O}C are required in meat processing in RSA to assist in the cleaning process, particularly to dislodge fat that may have congealed (SANS:587).

2.4.2 Mechanical action

Mechanical action is an important part of the cleaning regime as it removes hardened soil on surfaces and breaks down bacterial biofilms. A scrubbing device with sturdy bristles has been shown to be more effective at removing fat-based soil (Khalid *et al.,* 2019). Cleaning without industrial brushes with sturdy bristles means that there is insufficient mechanical action which will leave behind soil resulting in further

contamination as well as hinder the efficacy of further cleaning steps such as sanitization. The importance of mechanical action is further illustrated in Figure 2.2.

Industrial brushes are however not the only way to achieve mechanical action sufficient to remove soil in the food factory setting, hoses with special nozzle attachments can be used to create a "pressure fan" which has a similar scrubbing motion to that of brushes (EcoWize, 2019). Furthermore, hoses with varying nozzle attachments for the different stages of cleaning have been shown in practice to reduce cleaning time and increase the quality of the clean (Ecowize, 2019; Khalid *et al.,* 2019; Leu *et al.,* 1998; Meng *et al.,* 1998). A similar phenomenon was described in literature stating that a nozzle distance closer to the surface being cleaned resulted in a higher fluid velocity, which resulted in a higher impact of the water jet, resulting in the effective removal of fouling deposits (Leu *et al.,* 1998; Meng *et al.,* 1998).

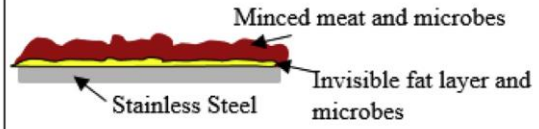
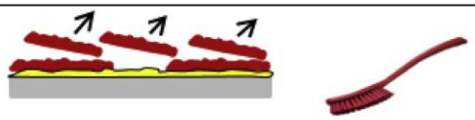
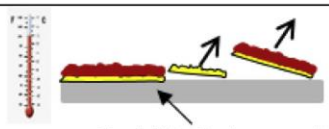
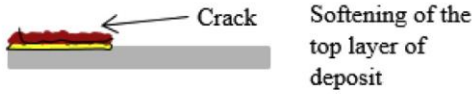
Cleaning action	Cleaning mechanism	Microbiological cleanliness	Physical cleanliness	
			Visual	Touch
Before cleaning t = 0 min		3.7 – 6.1 log CFU/ ml	X	X
Step 1 and 2 Pre-rinse and brush t=6 min		Not measured	X	X
Step 3 Hot water rinse (60°C): Using portable cleaning unit t=2 min		Not measured	X	X
Step 4 (a) Contact with alkaline wash t=3 min (total time for step 4 (a) and 4 (b))		Not measured	X	X
Step 4 (b) Mechanical action using brushes t=3 min (total time for step 4 (a) and 4 (b))		Not measured	X	X
Step 5 Rinsing with water t=2 min		Not measured	X	√
After cleaning Let dry		ND	√	√

Figure 2.2: Illustration of the importance of mechanical action whilst cleaning in a food factory (adapted from Khalid *et al.,* 2019). ND= not determined.

As seen in Figure 2.2. the detachment of debris (soil) (step 1 and 2) allows for easier colonisation by microorganism onto a surface within a factory. The inadequate removal of soil may also facilitate biofilm development (Gazula *et al.*, 2019), which may prove detrimental to product safety and quality: especially in the ready to eat sector. It is important to monitor areas such as those that are difficult to reach (such as the inside of equipment), as well as cracks such as those in the factory floor where water can pool for soil. Coupled with pooled water, both (deposit debris/ soil and cracks) result in excellent environments for *L. monocytogenes* biofilm formation, encouraging the persistence of the pathogen (Møretrø *et al.,* 2017; Leong *et al.,* 2017). Figure 2.2 does however neglect the application of a sanitizer after the initial cleaning stages, it is important to note that the sanitizer application after the preceding steps is of vital importance.

The application of a sanitizer is the final step in the factory cleaning process and it is important that after an optimal contact time between 10 and 15 minutes, the sanitizer is removed (either by water or mechanical action- this is factory preference based) as sanitizer residues may remain on the surface. This then results in an optimal environment for the development, inheritance, and ultimate selection for a sanitizer resistance mechanism such as those that code for QAC resistance (Møretrø *et al.,* 2017).

The removal of sanitizer with water after the minimum required contact time is debateable as some theories state that it actually decreases the efficacy of action, whilst others state it does not reduce the efficacy of the sanitizer and ultimately decreases the risk of sanitizer/ chemical residue being picked up on export goods. No published data was found to back up either of these claims. A study found that mechanically wiping the residual sanitizer off at production start-up and after the minimum contact may have helped with final removal of viral particles due to additional mechanical action (Bolton *et al.,* 2013). Mechanically wiping, to remove, the sanitizer at production start-up is still common practice in most industries however, historical preferences and chemical manufacturer's advice dictate a no-rinse treatment option after the application of the sanitizer.

2.4.3 Types of nozzles used as well as cleaning satellites

Controlling water pressure, as well as the shape of the spray of water or cleaning chemical is important as it ensures successful application so that interactions between the compounds and microorganism can occur or in the case of water, that as much soil and debris is removed from the area being cleaned (EcoWize, 2019). A novel way in which this is being done in South Africa is using cleaning satellites (EcoWize: Cape Town, South Africa). These cleaning satellites are built to house hosepipes with nozzle attachments that control the shape and pressure of the liquid being discharged. The cleaning satellites also assist by mixing the undiluted chemical with water to the desired concentration as well as creating a foam from the detergents to ensure optimal application (EcoWize, 2019).

Cleaning satellites are wall-mounted units which allow for greater efficiency in factory cleaning. Different hose attachments as well as the ability to allow for foaming to occur make separate, stand-alone cleaning devices for surfactant and sanitizer application redundant (Ecowize, 2019).

2.4.4 Chemical tests carried out during cleaning

In order to ensure that the concentrations of chemicals used throughout the cleaning process are consistent in routine applications, concentration tests are conducted. Tests conducted are not as accurate as laboratory analysis but still give a strong enough indication to ensure effective chemical application. Detergents are tested whilst being applied using a conversion factor and an optical density reading. Sanitizers are tested by a simple titration (to determine concentration), in a closed office space, before being applied (Ecowize, 2019).

2.5 The rise of sanitizer resistance amongst bacteria in food factories

2.5.1 Ineffective application of sanitizers

Sanitizer application efficacy can be hindered by various factors such as concentration and contact time as mentioned previously. It is however important to note that the presence of soil such as improperly removed fat, carcass matter, packaging, blood, vegetable and fruit peels hinders the efficacy of sanitizer application (EcoWize, 2019; Leu *et al.,* 1998; Meng *et al.,* 1998). Thus, the importance of following the ten steps of cleaning to the letter is important and ensuring that a suitably qualified person verifies each step of the process according to specifications. Continuing with cleaning without validating the cleanliness of each step puts the efficacy of the subsequent cleaning steps in jeopardy and compromises the integrity of the cleaning operation (EcoWize, 2019).

2.5.2 Types of quaternary ammonium compound (QAC) sanitizers

Every cleaning chemical (including sanitizers) needs to undergo laboratory testing before a claim can be made regarding its efficacy against microorganisms. The testing methodology required in South Africa, as required in the EU, follows the protocol set out by EN 1276: 2009 and is used to determine the log reduction capability of the sanitizer (EN 1276: 2009). As with all bacteria, *L. monocytogenes* grows in a logarithmic manner due to the phenomenon of binary fission (Giotis *et al.,* 2007). The efficacy of a microbial killing step (such as cooking or sanitization) is measured by how great of a logarithmic reduction occurs upon treatment. A typical sanitizer is expected to have a logarithmic reduction capability of 10^4 to 10^5 cfu/ g and would thus be given the designation “kills” 99.99% or 99.999% of bacteria respectively (SANS: 51276). It is important to note that sanitization is not a stand-alone method in factory cleaning and its success is dependent on the prior cleaning steps carried out to reduce the bacterial load from soiling to ensure that the sanitizer can in fact reduce the microbial load on factory surfaces to an acceptable level.

QACs are quaternary ammonium groups bound to benzene rings (Figure 2.3) which act as cationic surface agents, resulting in damage to the cell membrane of *L. monocytogenes* by causing dissociation of cell membrane lipid bilayers through changing permeability, and the leakage of the contents of the *L. monocytogenes* cell (McDonnel, 2007). Benzalkonium chloride (BAC) is the simplest and one of the most widely used QACs in industry and it has proved highly effective throughout its application period (when it is in contact with the surface requiring disinfection). However, should the QAC-based sanitizer be used at too low of a concentration (below the critical minimum concentration) the compound activity will be too slow, which regardless of application time, will result in ineffective eradication of the *L. monocytogenes* contamination (Gerba, 2015; McDonnel, 2007).

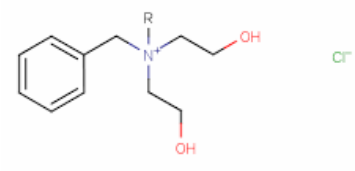


Figure 2.3 Chemical structure of a Quaternary Ammonium Compound.

The continuous development of QAC technologies has led to several generations of QACs being manufactured (Table 2.2) with each generation category being different from the last, they are classified as follows:

Table 2.2: Evolution of quaternary ammonium disinfectants from Gerba, 2015

Generation	Compound(s)
First	Benzalkonium, alkyl chains, C12 to C18
Second	Aromatic rings with hydrogen and chlorine, methyl and ethyl groups
Third	Dual QACs; mixture of alkyl dimethyl benzyl ammonium chloride (lower toxicity)
Fourth	Dialkylmethyl aminos with twin chains
Fifth	Synergistic combinations of dual QACs
Sixth	Polymeric QACs
Seventh	Bis-QACs with polymeric QACs

2.5.3 Alternatives to quaternary ammonium compound (QAC) sanitizers

Alternatives to QACs that are increasing in prevalence are PAA (Poimenidou, *et al.,* 2016), sodium hypochlorite-based sanitizers (Rios-Castillo, *et al.,* 2018) and L-lactic acid for Gram-positive bacteria (Boomsa

et al., 2015). Byotrol is a sanitizer manufacturing company which manufactures a QAC-containing and a QAC-free sanitizer with a unique formulation which is growing in popularity within the food industry, globally (Byotrol, 2020). The effects of the formulations created by Byotrol have been proven in the medical setting with its ability to remove established biofilms inside catheters and its high efficacy against planktonic cells being noted (Govindji *et al.,* 2013). Byotrol manufactures a QAC-based and a QAC-free sanitizer, both of which have been reported by industry to perform more efficiently than traditional QAC-based sanitizers (Hygiene Disposables, 2021).

Investigations into the effects of QAC-alternative sanitizers needs to be conducted due to the increasing prevalence of QAC-resistant *L. monocytogenes* (Møretrø *et al.,* 2017). An understanding of the alternatives and how their mode of action differs to QACs will be advantageous in determining new ways of treating both planktonic and biofilm communities within food factories and other processing environments. As mentioned above ineffective contact times and/or application contribute towards increased resistance (Flemming & Wingender, 2010).

In addition to what was noted previously, PAA is classified as an acidic, oxidizing disinfecting agent that can be created by blending acetic acid and hydrogen peroxide in an aqueous solution. Peracetic acid is a stronger oxidizing agent than chlorine and has been shown to work at removing biofilms from food contact surfaces (Block, 1991; Mosteller & Bishop, 1993; Marriot, 1999; Fatemi & Frank, 1999).

It has been reported that resistance to PAA has been encountered in *L. monocytogenes* via the *sig*B gene which is responsible for responses to environmental stresses (Van der Veen & Abee, 2010). Not all strains carry the *sig*B gene which encodes for the alternative sigma factor B, it is common however in lineage type III (Osri *et al*., 2011).

L-Lactic acid (LA) is one of the oldest known antimicrobial substances and has been evaluated by EN 1276 for its bactericidal properties. Whilst Gram-negative bacteria are more susceptible to it that Gram-positive bacteria, it is still considered a viable control for *L. monocytogenes* (Desriac *et al.,* 2013). It has a unique combination of a low acid dissociation constant (pKA) of 3.73 at 25 ^{O}C and a low hydrophobicity-allowing it to interact with bacteria residing in water which may be encountered in a food processing environment. It is good at shuttling protons and can enter the cell (Desriac *et al.,* 2013) whereby the acid stress disrupts cell regulation on a general level; bacteria spend energy trying to maintain pH, pumping out acid, bacteria change their metabolism to produce alkaline metabolites, and acid stress generates free radicals that damage all cellular mechanisms (Desriac *et al.,* 2013). Contact time can be as brief as 30 seconds, which does not allow adequate time for bacteria to adapt their structure or their metabolism for survival

which results in oxidative stress. Oxidative stress occurs at the cell membrane due to interference with the electron transport chain that destabilises the bacterial membrane (Glover *et al*., 1999).

Hypochlorite or "bleach" is a powerful oxidising agent, however its use in commercial processing and cleaning is rather limited due to more cost-effective terminal disinfectants (sanitizers) being available as well as its adverse effects on stainless steel. Certain applications are still found, however and they are mainly found in poultry processing facilities where its efficacy against *Salmonella spp.* is widely recognized; it is often applied as part of a "*Salmonella*" specific protocol for controlling incidents of the organism (Steenackers *et al*., 2012).

2.6 Resistance mechanisms of *L. monocytogenes*

2.6.1 Sanitizer resistance genes

L. monocytogenes as with most bacterial species have evolved various methods to counteract different types of antimicrobial (AM) treatments including drug inactivation, target alteration, prevention of drug influx and the active exclusion of a drug from a cell (Dever & Dermody, 1991; Lambert, 2005; Munita & Arias, 2016).

Efflux pumps are a transferable, active exclusion mechanism and are located within the outermost layer of the bacterial cell (the cell wall in the case of *L. monocytogenes*). *L. monocytogenes* is associated with two families of pumps, which have been found in strains isolated from industry (Putman *et al.,* 2000). Major facilitator super family (MFS) is classification of the benzalkonium chloride resistance cassette (*bcr*ABC) efflux gene and small multidrug resistant (SMR) family are responsible for the quaternary ammonium compound-resistance protein H gene (*qac*H) and the multidrug transporter E (*emr*E) genes (Putman *et al.,* 2000). Across bacteria there are four families of efflux pumps that have been discovered and are predominantly found in Gram-negative species, however SMR and MFS are found in both Gram-positive and Gram-negative bacteria. Resistant nodulation division (RND) as well as multidrug and toxic compound extrusion (MATE) families are found exclusively in Gram-negative bacteria (Putman *et al.,* 2000). The efflux pumps are found on mobile genetic elements (either plasmids or transposons) and can move between bacterial species including movement from a Gram-negative bacteria to a Gram-positive bacteria (and vice versa). This means that any organism containing a efflux pump mechanism belonging to either the SMR or MFS families poses a risk to the efficacy of a factory hygiene program as sanitizer resistance may be conveyed from a bacteria other than *L. monocytogenes* (Putman *et al.,* 2000). It was found that *L. monocytogenes* serotype 1 carried plasmids more frequently than serotype 4 isolates (Lebrun *et al.,* 1992; McLauchlin *et al.,* 1991). Plasmids were found more frequently in serotype 1/2 a isolates from food cases as opposed to clinical cases, and, conversely were more frequent amongst serotype 1/2 c isolates from human clinical cases as compared to isolates from food

(McLauchlin *et al.,* 1991). Cadmium resistance is often associated with resistant plasmids, which exhibits a type of cross-resistance against BAC sanitizers (Mullapudi *et al.,* 2010).

Gillings *et al.* (2009) described three mechanisms for the co-selection of resistant cassettes for QAC resistance. These include; the acquisition of genetic resistance mechanisms that function to exclude or remove both antibiotics and biocides, selection of AM determinants on the same plasmid or transposon and finally the use of biocides (sanitizers), which may promote the dissemination of mobile genetic elements i.e. resistance genes by promoting the selection of novel lateral gene transferable elements from environmental sources.

In food processing environments where QACs are routinely used, the presence of *qac*H and *bcr*ABC genes coding for QacH and (bcr A, bcrB and bcrC) transporters, respectively, which belong to the SMR family, is on the rise (Møretrø, *et al.,* 2017; Leong *et al.,* 2017). This increasing occurrence is being reported globally (Costa *et al.,* 2018; Leong *et al.,* 2017) and thus QAC-based sanitizer alternatives must be further studied.

Resistance to Benzalkonium chloride (BAC) can be mediated through a resistant gene cassette known as *bcr*ABC which was identified in a plasmid from a *L. monocytogenes* outbreak strain from the USA and has since been discovered in other *Listeria* genomes (Dutta *et al.,* 2013; Xu *et al.,* 2016). This BAC resistance cassette is composed of *bcr*A which is a Tetracycline resistance (TetR) family transcriptional regulator as well as MFS small multidrug resistant genes *bcr*B and *bcr*C. Sublethal concentrations of BAC (10 µg/ml) result in the transcription of *bcr*ABC (Dutta *et al.,* 2013). It can therefore be deduced that using BAC at its recommended concentrations should result in bacterial eradication as the *bcr*ABC cassette is unable to transcribe itself or in other words be activated prior to cell death. The ineffective and inappropriate application of QAC based sanitizers leads to selective pressure which favours mutations conferring resistance to the sanitizers used in a factory, thus yielding bacteria which are hardier and can survive the cleaning process. These surviving, resistant, bacterial strains go on to proliferate and contaminate products resulting in human infections, deaths and economic losses. This was demonstrated in Canada with *L. monocytogenes* strains containing the *emr*E resistance gene resulting in the deadly listeriosis outbreaks in Canada in 2008, as well as the *emr*C gene associated with numerous outbreaks within the Netherlands (Koopmans *et al.,* 2017), and *bcr*ABC in 1998-1999 across the USA (Elhanafi, 2010). Sanitizer resistant *L. monocytogenes* must therefore be managed and controlled (Kovacevic *et al.,* 2016).

Genes coding for QAC resistance have been identified and are varied. Several different genes can code for efflux pumps which affect QAC efficacy and they can be plasmid-borne which can further facilitate their spread across a food processing environment (Korsak, *et al.,* 2019). Genes associated with QAC resistance are described in Table 2.3.

Table 2.3: Genes associated with QAC resistance in *L. monocytogenes* including their genetic location

Gene	Codes for	Reference
*qac*H	Chromosomally located transposon Tn6188	Müller *et al.,* 2013 Müller *et al.,* 2014
*emr*C	Plasmid pLMST6	Kropac *et al.,* 2019
*emr*E	Genomic island LGI1	Kovacevic *et al.,* 2016
Multidrug resistance L (*mdr*L)	Located in bacterial chromosome	Mereghetti *et al.,* 2000; Romanova *et al.,* 2002; Romanova *et al.,* 2006
*bcr*ABC	Originally identified in plasmid 80 as part of a putative composite transposon	Dutta *et al.,* 2013; Elhanafi *et al.,* 2010

The following genes from Table 2.3 are described as follows. The *qac*H gene has been attributed to QAC resistance in various articles and can be found readily in strains isolated from industry in conjunction with the *bcr*ABC resistant gene (Møretrø *et al.,* 2017; Korsak *et al.,* 2019; Müller *et al.,* 2013; Elhanafi *et al.,* 2010). The *emr*C gene is plasmid pLMST6 borne and has been linked to BAC tolerance and meningitis in the *L. monocytogenes* ST-6 strain (Kropac *et al.,* 2019). The *emr*E has been shown to convey resistance to QAC-based sanitizers with no effect on the minimum inhibitory concentration (MIC) values of various antibiotics (Kovacevic *et al.,* 2016). The *emr*E gene was prevalent in many outbreaks across Canada (Knabel *et al.,* 2012). The *mdr*L gene has also been attributed to QAC resistance (Romanova *et al.,* 2006). The correlations between outbreaks of listeriosis and the presence of these QAC resistant genes within clinical isolates from these outbreaks (Kropac *et al.,* 2019; Møretrø *et al.,* 2017; Kovacevic *et al.,* 2016; Elhanafi *et al.,* 2010)- highlights the urgency for the need to better control sanitation within food processing environments.

2.6.2 Biofilms

Bacterial biofilms within the food industry are of growing concern due to their strong tolerance to antimicrobial agents. Biofilms of *L. monocytogenes* can persist and become long term residents within a factory, living on plant processing surfaces and throughout the plant (Olszewska *et al.,* 2016). The control and prevention of *L. monocytogenes* should be a factories top priority and special mention should be included as part of their cleaning pre-requisite program (PRP) (Muhterem-Uyar *et al.,* 2015).

The ability of bacteria to adhere to a vast array of surfaces is of growing concern in the food-processing environment; most notable the adherence of *L. monocytogenes* to stainless steel which is a common component of most factory equipment. Bacteria form biofilms as a part of a natural process when

they meet a surface. Biofilm (sessile) cells are shown to hold an increased resistance to external stresses such as desiccation, mechanical action/ scrubbing and even sanitizer treatments. The contact of the *L. monocytogenes* flagella (lipopolysaccharides within the cell walls of *L. monocytogenes* cells) is crucial in biofilm formation on stainless steel and other surfaces (Van Houdt & Michiels, 2010).

Identified areas of biofilm formation that lead to contamination of a food processing facility as well as the final product have been reported in literature and are well known throughout industry. If a biofilm is to form on one or more surfaces, it will be able to persist and resist applied antimicrobials more effectively than planktonic *L. monocytogenes* cells (Aarnisalo *et al.,* 2000; Gram *et al.,* 2007; Pan *et al.,* 2006; Robbins *et al.,* 2005; Van der Veen & Abee, 2010). Examples of where *L. monocytogenes* are commonly encountered are: NFCS such as the walls and floors of a factory, particularly where cracks or weaknesses are found, drains, trolley wheels, waste water pipes, bends in pipes, drain covers, conveyor belts, rubber seals and any stainless steel surfaces (Di Ciccio *et al.,* 2012). People and their boots (shoes) have been shown to be vectors for the transport of *L. monocytogenes* throughout a food processing facility (Di Ciccio *et al.,* 2012).

Biofilm formation (Figure 2.4) has been reported to occur in four sequential steps namely; a. reversible adhesion, b. irreversible adhesion, c. biofilm maturation and d. cell to cell signalling (Kocot, & Olszewska 2017). As mentioned earlier in this review, the formation of a biofilm can be attributed to *prf*A and the sigB stress factor which are temperature dependant transcription factors which prepare flagellum propelled (planktonic) *L. monocytogenes* for biofilm formation on an abiotic surface within a food production facility as illustrated in Figure 2.4 (Lemon *et al.*, 2010; Colagiorgi *et al*., 2017). *Prf*A can be attributed to playing a role in the initial surface attachment of a biofilm to a surface (Lemon *et al.,* 2010).

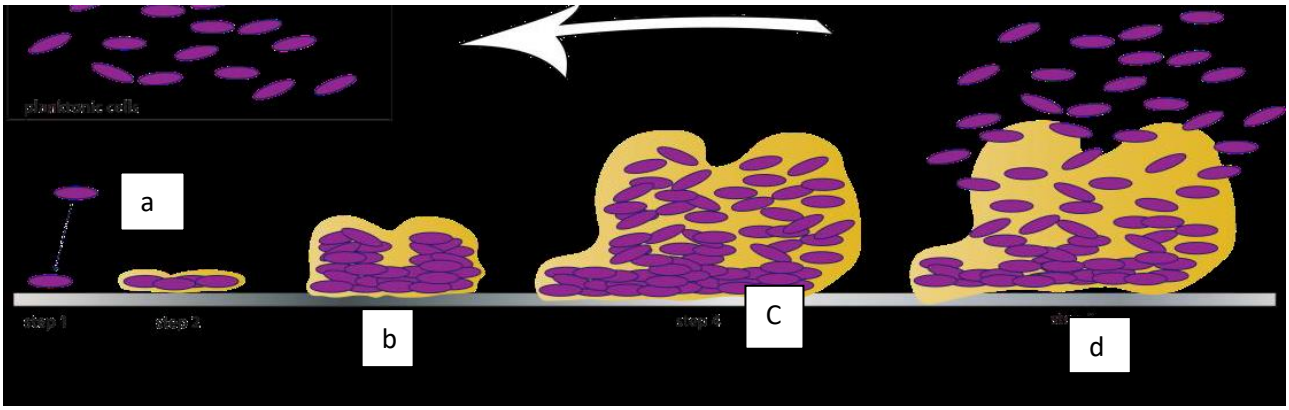


Figure 2.4: The four stages of biofilm development (Colagiorgi *et al*., 2017). Label a shows reversible adhesion, label b shows irreversible adhesion, label c shows biofilm maturation and d shows cell to cell signalling and the shedding of free planktonic cells.

Reversible adhesion is the process whereby cells of *L. monocytogenes* begin to interact with a surface from approximately 50 nm away via gravitational, electrostatic or van der Waals forces (Kocot & Olszewska, 2017). Irreversible adhesion is induced by stronger cell to cell interactions than those experienced in reversible adhesion. Lock and key bonds are formed between the cell and the surface and Extracellular Polymeric Substances (EPS) are secreted and surrounds the biofilm cells (Kocot & Olszewska, 2017). Biofilm maturation involves the formation of micro colonies and the increase of biofilm volume along with characteristic biofilm structures.

Finally, dispersion of bacterial cells to the environment sees cells of *L. monocytogenes* shedding and entering the environment where they either contaminate food or colonize new surfaces within the factory environment where subsequent biofilms may become established (Kocot & Olszewska, 2017).

Through a process known as quorum sensing (Mizan *et al.,* 2015), cells within a biofilm can be regulated and communicate with each other and are able to respond more effectively to environmental stimuli and stresses. An example of such a stress is sanitizer treatments through the production of autoinducers for *L. monocytogenes* biofilms which facilitate this cell to cell communication (Mizan *et al.,* 2015; Madsen *et al*., 2012). Over-stressed biofilms may detach from a surface entirely resulting in a larger amount of possible product contamination instead of the shedding of planktonic cells into the product. This is, however, commonly encountered when surfactants are used and thus common during cleaning operations only (Mizan *et al.,* 2015).

The enhanced resistance of sessile cells when compared to planktonic cells of the same strain can be influenced by a variety of factors including the physical and chemical properties of the surface on which they are attached, the growth phase of the cells when the sanitizer is applied, temperature and the presence of food residues (soiling) around the sessile community (Kocot & Olszewska, 2017). Biofilms have been found to be more resistant to extreme environments such as high temperatures and lower pH's than planktonic cells (Castro-Rosas & Escartínb, 2005). It has also been reported that these, sessile, communities have an increased resistance to sanitizers when compared to their planktonic counterparts (Healy *et al*., 2009). This increased resistance to sanitizers has been primarily reported in those sanitizers containing QAC compounds (Reij & Den Aantrekker, 2004; Tompkin, 2002).

Mixed species biofilms of *L. monocytogenes* have been recorded in food factories and show great diversity in their makeup (Desai *et al.*, 2012; Metselaar *et al.,* 2015). Biofilms of *L. monocytogenes* have been found to contain other species of *Listeria*, other Gram-negative bacteria and even have a multi-genera makeup: including both Gram-positive and Gram-negative bacteria (Heir *et al.,* 2018). *L. monocytogenes*

strains show large variations in competitive growth in mixed culture biofilms and bacterial suspensions with bacteria from within food processing environments. It was, however further reported by Heir *et al.* (2018) that the ability of *L. monocytogenes* strains to grow in mixed species biofilms was strain dependent with some growing better in mixed biofilms than others.

The enhanced resistance of *L. monocytogenes* biofilms to various sanitizers has been reported (Olszewska *et al.,* 2016; Fagerlund *et al.,* 2020). This can be attributed to the spread of resistance determinants, the shielding effect of the biofilm layers, or the improper completion of a cleaning cycle as mentioned earlier. Resistance to QACs, PAA and LA have been reported with older biofilms being more resistant than younger biofilms (Fagerlund *et al.,* 2020). This therefore highlights the need for earlier detection and monitoring of known biofilm niche locations to prevent the dissemination of *L. monocytogenes* across the food-processing environment.

Ineffective sanitizer applications are the result of rushed cleaning practices that lead to the presence of soil and other waste materials such as fat or plastic remaining on a surface which can shield the bacteria from the action of surfactants and ultimately the action of the sanitizer. This results in minimal exposure, thus positively selecting for those cells carrying resistant determinants. Without quality control at the end of each stage of the cleaning program, the efforts of the staff are futile. Inadequate cleaning often arises from production pressures and food manufactures must learn to put profit on hold, if needs be, to facilitate proper cleaning practices and thus ensure the overall safety of the products that they produce.

2.7 Proliferation throughout a factory, isolation and resistance monitoring

2.7.1 Colonisation and persistence of *L. monocytogenes* within the factory environment is an issue for human health

There are numerous aspects that policy-makers of a food factory can adhere to such as good workflows (linear in nature), effective sanitizer applications and monitoring the microbial quality of incoming raw materials (Tompkin, 2002). The microbial quality of incoming raw materials is of great concern.

It has been found in numerous published articles that there is a correlation between human clinical isolates of *L. monocytogenes* and those that inhabit food factories (Leong *et al.,* 2017; Kovacevic *et al.,* 2016; Xu *et al.,* 2016). This is great cause for concern as it shows that factories and the hygiene as well as Critical Control Point (CCP) management within factories has a direct influence on the health and well-being of humanity. The food group that is said to be a major contributor to *L. monocytogenes* spread belongs to the RTE category that has no further processing step such as further cooking or a further pH drop to render the product safe. RTE products are incredibly susceptible to post processing contamination, hence the importance of a well-managed sanitation pre-requisite program (PRP) (Gillespie, *et al.,* 2006). Management

of the cleaning PRP may become costly as microbiological tests and a robust swabbing and sampling regime are paramount to ensuring that the final food product meets the required government mandated microbiological criteria. An example of such criteria as the absence of *L. monocytogenes* in five samples of 25 g of product that can support the growth of *L. monocytogenes* (EU, 2005) using the ISO 11290 methodology. Furthermore, the final *L. monocytogenes* count should not exceed 100 cfu / g by the end of shelf life. The persistence of *L. monocytogenes* is an important consideration and can be monitored via environmental monitoring programs. Persistent *L. monocytogenes* strains are defined as repeatedly isolated strains with an indistinguishable pulse field gel electrophoresis (PFGE) profile at an interval of 6 months or more apart (Leong *et al*., 2017). The monitoring of pathogens, is however, shifting towards the use of whole genome sequencing (WGS) with a decreasing cost and more usable data regarding persistence, virulence and resistance; this WGS data becoming accessible to factories (Fox *et al.,* 2015). Contamination of *L. monocytogenes* within various food factories across Ireland was highlighted in a study by Leong *et al.* 2017, which showed that all facilities with persistent contamination also showed sporadic contamination. This resulted in a recommendation to update cleaning procedures to remove resistant strains as well as to improve hygiene barrier systems with the aim of preventing initial and sporadic contamination events through robust low-risk and high-risk designation in factories. Three contamination scenarios, within food factories, were observed in a study by Muhterem-Uyar *et al.,* (2015) that showed the following: sporadic contamination in the interface of raw material reception and high risk areas; hotspot contamination within high risk areas of a factory and widely disseminated contamination within the entire food factory. Moreover, Leong *et al.* (2017) highlighted the role that cross contamination could play in further proliferation of *L. monocytogenes* in food factories that could arise from the food-processing environment to the food or from the food to the processing environment. The management of segregation barriers for productions areas (low risk to high risk), worker hygiene, movements of personnel and food production workflows are therefore imperative towards preventing the proliferation of *L. monocytogenes* within a food factory. Leong *et al.* (2017) and Rip & Gouws (2020) showed the bias towards colonisation of food factories by *L. monocytogenes* lineage type I and II being the most prolific lineages. Lineages III and IV are absent from food production environments. Serotypes 1/2 a, 4b, 1/2 b and 1/2 c were all found (presented in descending order of prevalence) which was in line with what has been reported in numerous other studies (Leong *et al*., 2017).

The harbourage points within factories that *L. monocytogenes* has been known to colonise are differentiated in terms of FCS and NFCS. Examples of NFCS that *L. monocytogenes* may harbour within are floors, cracks within the floors, walls, skirting boards, drains, cracks within drains, drain sieves, the boots of workers and trolley wheels can spread the pathogen throughout the factory (Muhterem-Uyar *et al.,* 2015; Leong *et al.,* 2017; Stressl *et al.,* 2020). Drains provide an interesting vector for the spread of *L. monocytogenes* as they are easily contaminated and may even act as a vector for contamination from a low risk side of a food factory (where raw food is handled) to a high risk side (where cooked and finished product

are handled). Lining drains with copper has been shown to decrease the likelihood of *L. monocytogenes* contamination, through the actions of copper ions which penetrate the outermost layer of the bacteria cell, leading to cell destruction (Rogovskyy *et al.,* 2006). However, concern was shown by a manufacturer in a study by Rotariu, *et al.,* 2014 regarding sanitizers attacking the copper; this was however quickly refuted due to copper having a low reactivity and therefore only exceptionally strong oxidising agents will have the capability of reacting with it. FCS include conveyors belts, meat slicers, bowl chopper blades, utensils used throughout processing, tables, extrusion pumps and any dents or cracks within any of the before mentioned could provide a niche environment in which *L. monocytogenes* could harbour, proliferate and further contaminate food products (Muhterem-Uyar *et al.,* 2015; Stressl *et al.,* 2020).

The presence of *L. monocytogenes* is not consistent throughout industries, with meat primary processing sites having a higher prevalence of *L. monocytogenes* due to the cross contamination which may occur during slaughter, plucking and evisceration. This was also encountered in food factories with poor overall factory design being blamed for this cross contamination due to inadequate segregation between high risk and low risk areas of the factory (Chiarini *et al.,* 2009; Martin *et al.,* 2011; Sakardis *et al.,* 2018).

Sporadic contamination within the food-processing environment is still a major concern as highlighted by Muhterem-Uyar *et al.,* 2015. Their research showed that the contaminations were usually located on the interfaces of raw material receiving bays and processing areas (e.g. the barriers between low risk and high risk). This was further highlighted by Fox *et al.* (2015), who showed a high prevalence of *L. monocytogenes* in the drains of smokers, and other critical control points. Personal hygiene of staff and awareness regarding the risk that biofilms play and why all areas of a food factory must be correctly cleaned are also a very big factor in terms of decreasing contamination. The staff themselves are the ones on the factory floor each day, and their actions may have far reaching consequences (Dalmasso & Jordan, 2013; Schoder *et al.,* 2011).

2.7.2 Hotspot management and environmental swabbing procedures

A contaminated hotspot is defined as a single sampling site (such as a smoker drain) which is repeatedly proved to be positive for *L. monocytogenes* (Muhterem-Uyar *et al.,* 2015). Furthermore, infrequent, or lacklustre sampling may result in a hotspot being missed which may result in a factory outbreak and product recalls. *L. monocytogenes* coming from a NFCS such as a floor, drain, drain sieve, trolley wheel, box or pallet are not to be disregarded due to their lack of physical contact with a food product: cross contamination is a very real threat (Dalmasso & Jordan, 2013; Rückerl *et al*., 2014). The cross-contamination pressure of *L. monocytogenes* within large and fast-moving food factories is thought to be incredibly high and thus daily disinfection (through a cleaning PRP) is strongly encouraged for any food factory, but especially a large, fast moving one. The cleaning PRP is to be discussed with an outsourced service provider ensuring

that no rooms are missed such as work-in-progress chillers, or at the very least cleaned as part of a schedule to ensure that no contamination of product occurs either through earlier soiling deposits of stored product or by ceiling condensate or cooling fan drip (Rotariu *et al.,* 2014).

Disinfection of a food production facility is a dynamic process and advancements in techniques, procedures and chemicals should be constantly monitored and carried out by specially trained staff (such as from an outsourced company) to ensure that adequate decontamination occurs, and the prevention and control of biofilms occurs especially after cleaning through detective swabbing (with the intent of finding pathogens) (Ecowize, 2019). This proactive swabbing helps prevent the development of a hotspot within the food factory.

Drying, through a stop in production, of the facility is highly recommended as it helps to facilitate a reduction of *L. monocytogenes* via the creation of pooled water or vaporised aerosol spread (Berrang & Frank, 2010; Campdepadros *et al.,* 2012; Lehto *et al.,* 2011; Williams *et al.,* 2011). Moreover, it was recommended by Muhterem-Uyae *et al.,* 2015 that segregation barriers are well defined and enforced within factories as the ones examined in their study did not adequately prevent contamination of *L. monocytogenes* from becoming widespread or hotspot in nature.

The European Union Reference Laboratory for *L. monocytogenes* published guidelines in 2012 and it was found that 'standard ISO 18593 guidelines for the detection of microorganisms', for *L. monocytogenes* detection was flawed. The new guidelines made recommendations regarding how and when samples should be collected (European reference laboratory, 2012). They recommend sampling should not be carried out immediately after cleaning as it would not give any surviving, viable, cells time to recover to a culturable state. Furthermore, a combination of sponge and cotton swabs should be used. Sponge swabs should be used to sample large areas, whilst cotton stick swabs should be used to reach small areas such as in between rollers on conveyor belts and machine work boxes (Keeratipibul *et al.,* 2017; New South Wales Government Department of Primary Industries Food Authority, 2016).

A *L. monocytogenes* factory swabbing plan should encompass all areas of a factory and areas where *L. monocytogenes* is isolated, post cleaning, should be marked clearly on a factory map (FAO, 2007). Differentiation should be made between FCS and NFCS on the factory map, with areas of repeat offense being declared "hotspots" such as drains (New South Wales Government Department of Primary Industries Food Authority, 2016; FAO, 2007). Additional sanitation effort must be placed on those hotspot areas to ensure that resistance doesn't arise and that they do not become sources of *L. monocytogenes* contamination throughout a factory (New South Wales Government Department of Primary Industries Food Authority, 2016; FAO, 2007). Test frequency should be conducted as a minimum weekly for products that have a shelf life of

more than 5 days and that can support the growth of *L. monocytogenes* and fortnightly if their shelf life is less than five days (New South Wales Government Department of Primary Industries Food Authority, 2016). Should a positive *L. monocytogenes* result return from a test, the frequency of swabbing will be increased to daily until three consecutive days of negative results for *L. monocytogenes* are returned (New South Wales Government Department of Primary Industries Food Authority, 2016).

The need for rapid detection of *L. monocytogenes* for food producers is high, as rapid and accurate results for the presence or absence of *L. monocytogenes* enables food producers to act on incidences of *L. monocytogenes* contamination in either product or environment and thus prevent foodborne infection of the pathogen or the establishment of a biofilm (Kivirand & Rinken, 2018).

Agar based methods (Figure 2.5) have been used for some time with chromogenic agar growing in popularity. "Agar Listeria Ottaviani and Agosti" (ALOA) and "RAPID' L.mono" agars are the most popular amongst industry and present positive *L. monocytogenes* indicators as a result of colour changes occurring due to chemical reactions occurring between the media and bacteria (Becker *et al.,* 2006; Park *et al.,* 2014). ALOA media is dependent on the fact that all *Listeriae* produce β-glucosidase, resulting in *L. monocytogenes* occurring as a blue colony, however an addition of a turbid halo within 24 hours separates it from other species due to the unique formation of phosphatidylinositol phospholipase C (PI-PLC) of *L. monocytogenes* that causes the formation of a turbid halo around the blue colony on ALOA media. *L. ivanovii* also produces a turbid halo, however this only occurs after 48 hours (Becker *et al.,* 2006; Park *et al.,* 2014). RAPID' L.mono detection is based on the specific detection of PI-PLC and results in *L. monocytogenes* and *L. ivanovii* producing blue colonies (Becker *et al*., 2006). Further differentiation is achieved through a yellow halo surrounding colonies of *L. ivanovii* due to its ability to ferment xylose, resulting in plain blue colonies for *L. monocytogenes* (Becker *et al.*, 2006). It was however highlighted in a review by Stressl *et al.,* (2009) that Polymerase Chain Reaction (PCR) confirmation of these media-positive results should be carried out to confirm that these presumptive colonies are indeed those of *L. monocytogenes*. An example of a PCR primer test is suggested below using the *hly* housekeeping gene (Table 2.4).

Table 2.4 Showing PCR primer sequence and amplicon size for genotypic confirmation of *L. monocytogenes*

Primer set	Sequence (5'—'3)	Product size (bp)	Source
hly	CATTAGTGGAAA GATGGAATG (F) GTATCCTCCAGAGTGATCGA (R)	730	Blaise & Phillippe, 1993.

Experiments regarding the growth and susceptibility of *L. monocytogenes* to sanitizers is often conducted on either tryptic soy agar (TSA) with an enrichment taking place in tryptic soy broth (TSB) (Pan *et al.,* 2006). Another option is Brain Heart Infusion (BHI) agar with enrichment taking place in BHI broth (Heir *et al.,* 2018). Both media have shown to support the growth of fastidious organism such as *L. monocytogenes.*

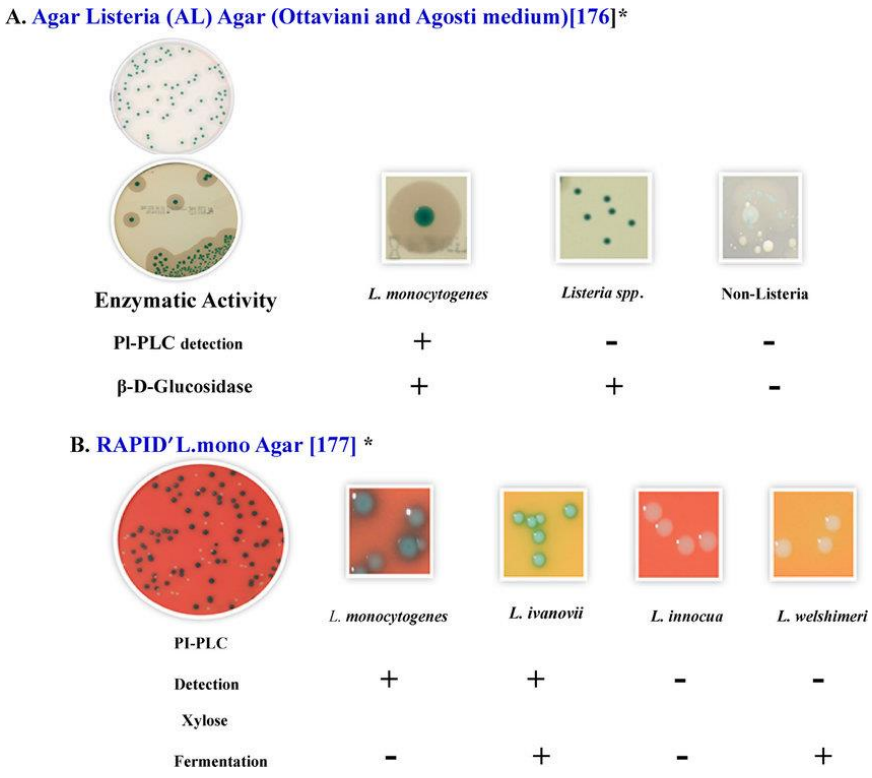


Figure 2.5 Colour-change differences of RAPIDL'mono and Ottaviani and Agosti media (https://www.researchgate.net/figure/AL-Agar-Listeria-according-to-Ottaviani-and-Agosti-medium-Agar-Adapted-from-Bio-Rad_fig1_318994661 by Zonglin Hu).

2.7.3 Resistance to sanitizers, monitoring and going forward

Bacterial cells in the sessile (biofilm state) are 10 to 1000 times more resistant to antimicrobial treatments like sanitizers than their planktonic counterparts (Mah, 2012). *L. monocytogenes* biofilms routinely inhabit drains of factories and can shed planktonic cells which can further contaminate a factory

environment and be transferred to final product (New South Wales Government Department of Primary Industries Food Authority, 2016).

Thus, a way to monitor a biofilm's response to a sanitizer in real time, would be advantageous to understanding sanitizer resistance within a food factory. A system known as the CO_2 evolution measurement system (CEMS) allows the user to see a biofilm's response to an antimicrobial through real-time measurement of CO_2 output (Loots, 2016).

The inner workings of the CEMS is highlighted in work by Kroukamp and Wolfaardt (2009), a simple illustration of the overview of the system is provided in Figure 2.6.

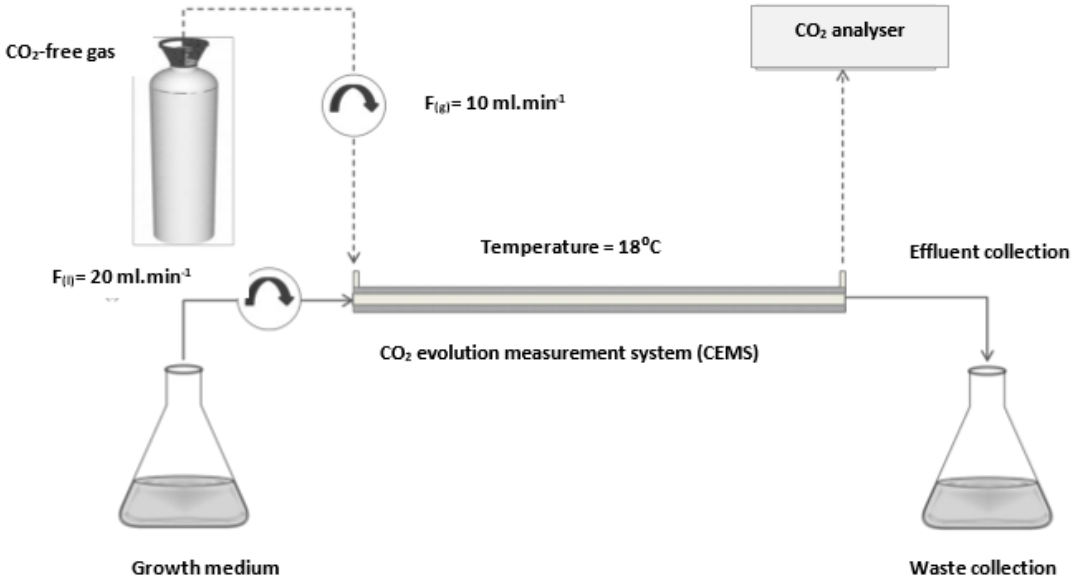


Figure 2.6 Set up of a single CEMS system tube (adapted from Loots, 2016). F(g) is representative of the flowrate of gas and F(i) is representative of the flowrate of growth media.

The flowrate of nutrients is faster than the doubling rate of the bacterial cells, thus ensuring that the responses of a pure biofilm towards an antimicrobial are exclusively recorded. This system has been used previously to test the responses of different bacterial species and their responses towards antimicrobial treatments such as *L. monocytogenes* towards a selection of commercial sanitizers (Ackerman, 2017; Loots, 2016).

The CEMS would allow for factories to investigate factory isolates and their responses to sanitizer treatments and thus select the most effective sanitizer for use in their factory, based on resistant patterns from historic uses of different sanitizers.

2.8 Conclusion

The prevalence of sanitizer resistant *L. monocytogenes* is of great concern due to its ability to survive factory cleaning procedures, contaminate food and infect food consumers. A better understanding and approach to combating the survivability of sanitizer resistant *L. monocytogenes* would thus be advantageous to the food producer.

Culturing of *L. monocytogenes* within the factory environment using traditional ISO methods (ISO 11290-1) has been used for a very long time and gives a good indication of final microbial quality of end products as well as identify hotspots of *L. monocytogenes* within a food factory (Fox *et al.,* 2015). Traditional culturing whilst indicating hotspots does not give a complete picture in terms of transmission routes of *L. monocytogenes* within a factory. A new approach is needed with targeted swabbing of NFCS as well as a solid understanding of production scheduling and flows within a food factory is therefore needed.

The use of sanitizers and targeted applications towards hotspots should be taken into consideration and a "one sanitizer fits all" approach for controlling *L. monocytogenes* within a factory should be strongly reconsidered. Cleaning should be closely monitored with emphasis on where sanitizers are working and where they are not to ensure control of the spread of *L. monocytogenes* throughout a food factory.

Without the implementation of a thorough and consistent cleaning schedule which allows deep-cleaning, including but not limited to the deconstruction, decontamination and re-assembly of food processing equipment, the decontamination of drains, drain covers, trolley wheels, flooring underneath machines, staff wellington boots, and the cleaning of cleaning equipment storage areas as well as the equipment itself; the control of *L. monocytogenes* within a factory will be a losing battle.

Resistance to sanitizers has been extensively reported in literature and the presence of genes encoding for sanitizer resistance (*brc*ABC, *emr*C, *emr*E and *qac*H) have been detected in isolates from human listeriosis cases and highlights the need for ensuring proper cleaning takes place. Furthermore, this eludes to the need to investigate alternative terminal disinfectants to ensure control of resistant strains going forward.

Whilst conventional and molecular techniques to detect the organism are available and in common use in factories, increased awareness regarding the spread and existence of sanitizer resistant determinants exists. The food safety success of any plant begins with a hygienic design followed by the handling and storage of equipment, followed by cleaning in place. Cleaning in place is a vital preceding step before any successful deep cleaning operation occurs and must therefore be closely monitored. These different steps as discussed must always be subjected to a meticulous quality control eye and buy-in/ support from factory management (by allowing adequate time for factory cleaning to occur), without which would result in the spread and

ultimate biofilm formation of *L. monocytogenes*. As seen in the 2017-2018 South African listeriosis outbreak, a failure in any of these aspects can have lethal consequences.

Biofilms have been shown to support the spread of resistance genes and protect cells from the effects of cleaning. The establishment of biofilms must therefore be prevented through allowing for adequate factory cleaning. Any existing biofilms must be eradicated to ensure safe food production.

2.9 References

Aalto-Areneda, M., Lunden, J., Markkula, A., Haola, S., Korkeala, H. (2019). Processing plant and machinery sanitiation and hygiene practices associated with *Listeria monocytogenes* occurrence in ready-to-eat fish products. *Food Microbiology*. **82**, 455-464.

Aarnisalo, K., Salo, S., Meittnen, H., Suihko, ML., Wirtanen, G., Autio, T., Korkeala, H., Sjöberg, AM. (2000). Bactericidal efficacies of commercial disinfectants against *Listeria monocytogenes* on surfaces. *Journal of Food Safety*. **20**(4). 237-250.

Alia, A., Andrade, M.J., Rodriguez, A., Martin, I., Perez-Baltar, A., Medina, M., Cordoba, J. (2020). Prevalence and characterization of *Listeria monocytogenes* in deboning and slicing areas of Spanish dry-cured ham processing. *LWT- Food Science and Technology*. **128**. 109498.

Allen, K.J., Walecka-Zacharska, E., Chen, J.C., Kataryna, KP., Devlieghere, F., Van Meervenne, E., Osek, J, Wieczorek, K., Bania, J. (2016). *Listeria monocytogenes*—An examination of food chain factors potentionially contributing to antimicrobial resistance. *Food Microbiology*,**54,**178-189.

Alonso, A.N., Perry, K.J., Regeimbal, J.M., Regan, P.M., Higgins, D.E. (2014). Identification *of Listeria monocytogenes* determinants required for biofilm formation. *PLoS One 9*. **12**. e113696

Alvaro, J.E., Moreno, S., Dianez, F., Santos, M., Carrasco, G., Urrestarazu, M. (2009). Effects of peracetic acid disinfectant on the postharvest of some fresh vegetables. *Journal of Food Engineering*. **95**. 11-15.

Baria, C., Singer, R.S., Bueno, I., Estrada, E., Rivera, D., Ulloa, S., Fernandez, J., Mardones, F.O., Moreno-Switt, A.I. (2020). Tracing *Listeria monocytogenes* contamination in artisanal cheese to the processing environments in cheese producers in southern Chile. *Food Microbiology*. **90**. 103449

Becker, B., Schuler, S., Lohneis, M., Sabrowski, A., Curtis, G.D.W., Holzapfel, W.H. (2006). Comparison of two chromogenic media for the detection of *Listeria monocytogenes* with the plating media recommended by EN/DIN 11290-1. *International Journal of Food Microbiology*. **109**(1-2). 127-131.

Benthin, S. & Villadsen, J. (1995). Production of optically pure D-lactate by *Lactobacillus bulgaricus* and purification by crystallisation and liquid/liquid extraction. *Applied Microbiology and Biotechnology*. **42**. 826-829.

Beresford, M.R., Andrew, P.W., Sharma, G. (2001). *Listeria monocytogenes* adheres to many materials found in food processing environments. *Journal of Applied Microbiology*. **90**(6). 1000-1005.

Berrang, M.E., Meinersman, R.J., Frank, J.F., Ladely, S.R. (2010). Colonization of a newly constructed commercial chicken further processing plant with *Listeria monocytogenes*. *Journal of Food Protection*. **73**. 286-291.

Blais, B. W., & Phillippe, L.M. (1993) A simple RNA probe system for analysis of *Listeria monocytogenes* polymerase chain reaction products. *Applied Environmental Microbiology*. **59**.2795–2800.

Block, SS. (1991). *Disinfection, Sterilization, and Preservation*. 4th Edition. Philadelphia: Lea & Febiger.

Boatemaa, S., McKenna, B., Drimie, S., Harper, J., Korsten, L., Pereira, L. (2019). Awakening from the listeriosis crisis: Food safety challenges, practices and governance in the food retail sector in South Africa. *Food Control*, **104**, 333-342.

Bolton, S.L., Kotwal, G., Harrison, M.A., Law, S.E., Harrison, J.A., Cannon, J.L. (2013). Sanitizer efficacy against murine norovirus, a surrogate for human norovirus, on stainless steel surfaces when using three application methods. *Applied and Environmental Microbiology*. **79**(4). 1368-1377.

Boomsma, B., Bikker, E., Lansdaal, E., Stuut, P. (2015). L-lactic acid- A Safe Antimicrobial for Home- and Personal Care Formulations. *SOFW Journal Home & Personal Care Ingredients and Formulations*. 141(**10)**. 1-5.

Borucki, M.K. & Call, D.R. (2003). *Listeria monocytogenes* Serotype identification by PCR. *Journal of Clinical Microbiology*,**41**(12),5573-5540.

Brinez, W.J., Roig-Sagues, A.X., Herrero, M.M.H., Lopez-Pedemonte, T., Guamis, B. (2006). Bactericidal effect of peracetic acid in combination with hydrogen peroxide against pathogenic and non pathogenic strains of *Staphylococcus* spp., *Listeria spp*., and *Escherichia coli*. *Food Control*. **17**. 516-521.

Bunch, P.K., Mat-Jan, F., Lee, N., Clark, D.P. (1997). The *IdhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. *Microbiology*. **143**. 187-195.

Butterfield, C. T., E. Wattie, S. Megregian, and C. W. Chambers. (1943). Influence of pH and temperature on the survival of coliforms and enteric pathogens when exposed to free chlorine. *Public Health Report*. 58:1837-1869.

Buzby, J.C. (2001). Children and microbial foodborne illness. *Food Review*. 24. 32–37.

Campdepadros, M., Stchigel, A. M., Romeu, M., Quilez, J., & Sol_a, R. (2012). Effectiveness of two sanitation procedures for decreasing the microbial contamination levels (including *Listeria monocytogenes*) on food contact and non-food contact surfaces in a dessert-processing factory. Food Control. **23**(1). 26-31.

Carpentier, B. & Cerf, O. (2011). Review- Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology*. 145. 1-8.

Castro-Rosas, J., Escartínb, E. (2005). Increased tolerance of *Vibrio cholerae* O1 to temperature, pH, or drying associated with colonization of shrimp carapaces. *International Journal of Food Microbiology*. 102, 195-201.

Ceitan, M.S., Zhang, C., Hutkins, R.W., Benson, A.K., (2004). Regulation of transcription of compatible solute transporters by the general stress sigma factor, sigmaB in *Listeria monocytogenes*. *Journal of Bacteriology*. 186.794-802.

Chan, Y. C., Boor, K. J., & Wiedmann, M. (2007). SigmaB-dependent and sigmaB independent mechanisms contribute to transcription of *Listeria monocytogenes* cold stress genes during cold shock. **73**(19). 6019-6029.

Chen, JQ., Healey, S., Regan, P., Laksanalamai, P., Hu, Z. (2017). PCR-based methodologies for detection and characterization of *Listeria monocytogenes* and *Listeria ivanovii* in foods and environmental sources. *Food Science and Human Wellness*. 6: 39-59.

Cheng, Y., Siletsky, R.M., Kathariou, S. (2008). Genomic divisions/ lineages, epidemic clones and population structure. In Liu, D., editior. *Handbook of Listeria monocytogenes Boca Raton*. CRC Press. 337-357.

Chiarini, E., Tyler, K., Farber, J., Pagotto, F., & Destro, M. (2009). *Listeria monocytogenes* in two different poultry facilities: manual and automatic evisceration. *Poultry Science*. **88**. 791-797.

Colagiorgi, A., Bruini, I., Di Ciccio, P.A., Zanardi, E., Ghidini, S., Ianieri, A. (2017). *Listeria monocytogenes* biofilms in the wonderland of food industry. *Pathogens*. 6 (41).

Costa, A., Lourenco, A., Civera, T., Brito, L. (2018). *Listeria innocua* and *Listeria monocytogenes* strains from dairy plants behave similarly in biofilm sanitizer testing. *LWT-Food Science and Technology*,**92**,477-483.

Da Luca, G., Sacchetti, R., Zanetti, F., Leoni, E. (2008). Comparative study on the efficacy of peracetic acid and chlorine dioxide at low doses in the disinfection of urban wastewaters. *Annals of Agricultural and Environmental medicine*. **15**. 217-224.

Da Silva Meira, Q.G., de Medeiros, Barbosa, I., Athayde, A.J.A.A., de Sequeira-Junior, J.P., de Souza, E.L. (2012). Influence of temperature and surface kind on biofilm formation by Staphylococcus aureus from food-contact surfaces and sensitivity to sanitizers. *Food Control*. **25**. 469-475.

Dalmasso, M., & Jordan, K. (2013). Process environment sampling can help to reduce the occurrence of *Listeria monocytogenes* in food processing facilities. *Irish Journal of Agricultural and Food Research*. **52**(1). 93-100.

Davis, M.L., Ricke, S.C., Donaldson, J.R. (2019). Establishment of *Listeria monocytogenes* in the Gastrointestinal Tract. *Microorganisms*. 7(**75**):1-10.

De Luca, G., Sacchetti, R., Zanetti, F., Leoni, E. (2008). Comparative study on the efficiency of peracetic acid and chlorine dioxide at low doses in the disinfection of urban wastewaters. *Annals of Agricultural and Environmental Medicine*. 15. 37-44.

Department of Health, National *Listeria* Incident Management Team. (2018). Situation Report. Department of Health: South Africa.

Desai, M.A., Soni, K.A., Nannapaneni, R., Schilling, M.W., Silva, J.L. (2012). Reduction of *Listeria monocytogenes* in raw catfish fillets by essential oils and phenolic constituent carvacrol. *Journal of Food Science*. **77**(9). 516-522.

Desriac, N., Broussolle, V., Postollec, F., Mathot, AG., Sohier, D., Coroller, L., Legeurinel, I. (2013). *Bacillus cereus* cell response upon exposure to acid environment: toward the identification of potential biomarkers. *Frontiers in Microbiology*. **4** (284). 00284.

Dever, L.A. & Dermody, T.S. (1991). Mechanisms of Resistance to Antibiotics. *JAMA Internal Medicine*. 151(**5**). 886-895.

Di Ciccio, P.; Conter, M.; Zanardi, E.; Ghidini, S.; Vergara, A.; Paludi, D.; Festino, A.R.; Ianieri, A. (2012). *Listeria monocytogenes*: Biofilms in food processing. *Italian Journal of Food Science*. 24. 203–213

Du, P., Liu, W., Cao, H., Zhao, H., Huang, CH. (2018). Oxidation of amino acids by peracetic acid: reaction kinetics, pathways and theoretical calculations. *Water Research X*. **1**.100002.

Dutta, V., Elhanafi, D., Kathariou, S. (2013). Conservation and distribution of the benzalkonium chloride resistance cassette bcrABC in *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **79** (19). 6067-6074.

Ecowize. (2019). *Introduction to factory cleaning*. 1-100. Cape Town: Ecowize.

Eifert, J.D., Curtis, P.A., Bazaco, M.C., Meinersmann, R.J., Berrang, M.E., Kernodle, S., Stam, C., Jaykus, L.A., Kathariou, S. (2005). Molecular characterization of *Listeria monocytogenes* 4b complex (4b, 4d, 4e) from two turkey processing plants. *Foodborne Pathogens and Disease*. **2**(3).

Elhanafi, D., Dutta, V., Kathariou, S. (2010). Genetic characterization of plasmid-associated benzalkonium chloride resistance determinants in a *Listeria monocytogenes* strain from the 1998-1999 outbreak. *Applied and Environmental Microbiology*. 8231-8238.

El-Kest, S., Marth, E.H. (1988). Inactivation of *Listeria monocytogenes* by Chlorine. *Journal of Food Protection*. **51**(7). 520-524.

Emerald Group Publishing. (2008). Byotrol the breakthrough in infection control. *Nutrition and Food Science*. **38**(1).

European Commission. (2019). Food Safety Maximum levels of Chlorate in Food. Internet document: https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/2075-Food-safety-maximum-levels-of-chlorate-in-food/F65317_en

European Food Safety Authority. (2013). Analysis of the Baseline Survey on the prevalence of *Listeria monocytogenes* in ready-to-eat foods in the EU, 2010-2011 Part A: *Listeria monocytogenes* prevalence estimates. *EFSA Journal*. **11**(6): 3241. 1-75.

European Reference Laboratory for *Listeria monocytogenes*. (2012). Guidelines for sampling the food processing area and equipment for the detection of *Listeria monocytogenes*.

Evans, D.A. (2000). Disinfectants. *Wiley Encyclopaedia of Food Science and Technology*. **1**. 501-509.

Fagerlund, A., Langsrud, S., Moretro, T. (2020). In-Depth Longitudinal Study of *Listeria monocytogenes* ST9 Isolates from the meat processing industry: resolving diversity and transmission patterns using Whole-Genome Sequencing. *Applied and Environmental Microbiology*. **86**(14). E00579-20.

Fatemi, P. & Frank, J.F. (1999). Inactivation of *Listeria monocytogenes*/ Pseudomonas biofilms by peracid sanitizers. *Journal of Food Protection*. **62**. 761-765.

Favaro, M., Sarmati, L., Sancesario, G., Fontana, C. (2014). First Case of Listeria innocua meningitis in a patient on steroids and etanercept. *JMM Case Reports*. 1-5.

Flemming, HC., Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*. **8**. 623-633.

Folsom, J.P., Frank, J.F. (2006). Chlorine resistance of *Listeria monocytogenes* Biofilms and relationship to Subtype, Cell density, and Planktonic Cell Chlorine Resistance. *Journal of Food Protection*. **69**(6). 1292-1296.

Food and Agricultural Organization of the United Nations (2007). Codex Alimentarius Guidelines on the application of the general principals of food hygiene to the control of *Listeria monocytogenes* in Foods. *CAC/GL* 61-2007. http://www.fao.org/fao-who-codexalimentarius/codex-texts/guidelines/en/ . Accessed 17/02/2021.

Forsythe, S.J. (2010). *The Microbiology of Safe Food Second Edition*. Pp 177-180. West Sussex: John Wiley & Sons ltd.

Fox, E.M., Wall, P.G., Fanning, S. (2015). Control of Listeria species food safety at a poultry production facility. *Food Microbiology*. **51**. 81-86.

Gamble, R. & Muriana, P.M. (2007). Microplate fluorescence assay for measurement of the ability of strains of *Listeria monocytogenes* from meat and meat-processing plants to adhere to abiotic surfaces. *Applied and Environmental Microbiology*. **73**. 5235-5244.

Gaul, L.K., Farag, N.H., Shim, T., Kingsley, M.A., Silk, B.J., Hyytia-Trees, E. (2013). Hospital-acquired listeriosis outbreak caused by contaminated diced celery—Texas 2010. *Clinical and Infectious Disesases*. **56**(1). 20-26.

Gazula, H., Scherem, H., Li, C., Takeda, F., Wang, P., Chen, J. (2019). Ease of biofilm accumulation, and efficacy of sanitizing treatments in removing the biofilms formed, on coupons made of materials commonly used in blueberry packing environment. *Food Control*. **104**. 167-173.

Gerba, C.P. (2015). Quaternary Ammonium Biocides: Efficacy in Application. *Applied and Environmental Microbiology*. 81(**2**):464-469.

Gibson, H., Taylor, J.H., Hall, K.E., Holah, J.T. (1999). Effectiveness of cleaning techniques used in industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology*. **87**. 41-48.

Gillespie, I.A., McLauchlin, J., Grant, K.A., Little, C.L., Mithani, V., Penman, C., Lane, C., Ragan M. (2006). Changing pattern of human listeriosis, England and Wales, 2001-2004. *Emerging Infectious Diseases*. **12**(9). 1361-1366.

Gillings, M.R., Xuejun, D., Hardwick, S.A., Holley, M.P., Stokes, H.W. (2009). Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons. *The ISME journal*. 3: 209-215.

Giotis, E.S., Blair, I.S., McDowell, D.A. (2007). Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *International Journal of Food Microbiology*. 120 (**3**): 250-258.

Glover, R.E., Smith, R.R., Jones, M.V., Jackson, S.K., Rowlands, C.C. (1999). An EPR investigation of surfactant action on bacterial membranes. *FEMS Microbiology letters*. **177**.57-62.

Godshall, C.E., Suh, G., Lorber, B., 2013. Cutaneous listeriosis. *Journal of Clinical Microbiology*. 51, 3591–3596.

Goulet V, Hebert M, Hedberg C, Laurent E, Vaillant V, De Valk H, (2012). Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. *Clinical Infectious Diseases*. **54**. 652–60.

Govindji, N., Wills, P., Upton, M., Tirelli, N., Yeates, S. (2013). The antibiofilm effect of Byotrol™ G32. *Journal of Applied Microbiology*. **114**. 1285-1293.

Gram, L., Bagge-ravn, D., Ng, Y.Y., Gymoese, P. & Vogel, B.F. (2007). Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. *Food Control*, **18**, 1165–1171.

Gravesen, A., Diao, Z., Voss, J., Budde, B.B., Knøchel, S. (2004). Differential inactivation of *Listeria monocytogenes* by D- and L-Lactic acid. *Letters in Applied Microbiology*. **39**. 528-532.

Greenspan, F.P., Margulies, P.H., 1950. Treatment of raw plant tissue. US Patent 2512,640. Buffalo Electro-Chemical Co.

Healy, B., Huynh, S., Mullane, N., O'Brien, S., Iversen, C., Lehner, A., Stephan, R., Parker, C.T., Fanning, S., (2009). Microarray-based comparative genomic indexing of the *Cronobacter* genus (*Enterobacter sakazakii*). *International Journal of Food Microbiology*. **136**. 159-164.

Hebraud, M. & ,Guzzo J.(2000). The main cold shock protein of *Listeria monocytogenes* belongs to the family of ferritin-like proteins. *FEMS Microbiology Letters*. **190**. 29–34.

Heir, E., Møretrø, T., Simensens, A., Langsrud, S. (2018). *Listeria monocytogenes* strains show large variations in growth in mixed culture biofilms and suspensions with bacteria from food processing environments. *International Journal of Food Microbiology*, **275**,46-55.

Holah, J.T., Bird, J., Hall, K.E. (2004). The microbial ecology of high-risk, chilled food factories; evidence for persistent Listeria spp. and Echerichia coli strains. *Journal of Applied Microbiology*. **97**. 68-77.

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P. & Rocourt, J. (2002). Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin. *Applied Environmental Microbiology*. **68**. 616–622.

Jiang, X., Yu, T., Liang, Y., Ji, S., Guo, X., Ma, J., Zhao, L. (2016). Efflux pump-mediated benzalkonium chloride resistance in *Listeria monocytogenes* isolated from retail food. *International Journal of Food Microbiology*. **217**. 141-145.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, Springer, M., Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell*. **110**. 551-561.

Johnson, J., Jinneman, J., Stelma, G., Smith, B.G., Lye, D., Messer, J., Ulaszek, J., Evsen, L., Gendel, S., Bennet, R.W., Swaminithan, B., Pruckler, J., Steigerwalt, A., Kathariou, S., Yildirim, S., Volokhov, D., Rasooly, A., Chizhikov, V., Wiedmann, M., Fortes, E., Duvall, R.E., Hitchens, A.D. (2004). Natural Atypical *Listeria innocua* Strains with *Listeria monocytogenes* Pathogenicity island 1 Genes. *Applied and Environmental Microbiology*.**70**(7). 4256-4266.

Kastbjerg, V.G. & Gram, L. (2012). Industrial disinfectants do not select for resistance in Listeria monocytogeens following long term exposure. *International Journal of Food Microbiology*. **160**. 11-15.

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection*. **65**(11). 1811-1829.

Keeratipibul, S., Laovittayanurak, T., Pornruangsarp, O., Chaturongkasumrit, Y., Takahashi, H., Techaruvichit, P. (2017). Effect of swabbing techniques on the efficiency of bacterial recovery from food contact surfaces. *Food Control*. **77**. 139-143.

Khalid, N.I., Saulaiman, U.S., Nasiruddin, N.A., Hatdran, M.M., Ab Aziz, N., Nor Khaizura, M.A.R., Hasna, N.Z.N., Taip, F.S., Sobri, S. (2019). Integrating cleaning studies with industrial practice: case study of an effective cleaning program for a frozen meat patties SME factory. *Journal of Cleaner Protection*. **235**. 688-700.

Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M., Hartl, FU. (2013). Molecular chaperone functions in protein folding and proteostasis. *Annual Reviews in Biochemistry*. **82**. 323-355.

Kivirand, K. & Rinkin, T. (2018). Introductory Chapter: Why Do We Need Rapid Detection of Pathogens?, *Biosensing Technologies for the Detection of Pathogens - A Prospective Way for Rapid Analysis*, IntechOpen, DOI: 10.5772/intechopen.74670. Available from: https://www.intechopen.com/books/biosensing-technologies-for-the-detection-of-pathogens-a-prospective-way-for-rapid-analysis/introductory-chapter-why-do-we-need-rapid-detection-of-pathogens-

Knabel, S.J., Reimer, A., Verghese, B., Lok, M., Ziegler, J., Farber, J., Pagotto, F., Graham, M., Nadon, C.A., the Canadian Public Health Laboratory Network (CPHLN), Gilmour, M.W. (2012). Sequence typing

confirms that a predominant *Listeria monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010. *Journal of Clinical Microbiology*. **50**. 1748-1751.

Kocot, A.M. & Olszewska, M.A. (2017). Biofilm formation and microscopic analysis of biofilms formed by *Listeria monocytogenes* in a food processing context. *LWT- Food Science and Technology*. **84**. 47-57.

Koopmans, M.M., Bijlsma, M.W., Brouwer, M.C., van de Beek, D., van der Ende, A. (2017). *Listeria monocytogenes* meningitis in the Netherlands,1985-2014: a nationwide surveillance study. *Journal of Infection*. **75**. 12-19.

Korsak, D. & Szuplewska, M. (2016). Characterization of non-pathogenic Listeria species isolated from food and food processing environment. *International Journal of Food Microbiology*. **238**. 274-280.

Korsak, D., Chimielowska, C., Szuplewska, M., Bartosik, D. (2019). Prevalence of plasmid-borne benzalkonium chloride resistance cassette *bcr*ABC and cadmium resistance *cad*A genes in nonpathogenic *Listeria* spp. isolated from food and food-processing environments. *International Journal of Food Microbiology*. **290**. 247-253.

Kovacevic, J., Ziegler, J. Walecka-Zacharska, E., Reimer, A., Kitts, D.D. Gilmour, M.W. (2016). Tolerance of *Listeria monocytogenes* to Quaternary Ammonium Sanitizers is mediated by a novel efflux pump encoded by emrE. *Applied and Environmental Microbiology*. **82**(3): 939-953.

Kropac, A.C., Eshwar, A.K., Stephan, R., Tasara, T. (2019). New insights on the role of the pLMST6 plasmid in *Listeria monocytogenes* biocide tolerance and virulence. *Frontiers in Microbiology*. **10**. 1-16.

Kuenne, C., Billion, A., Abu Mraheil, M., Strittmatter, A., Daniel, R., Goesmann, A., Barbuddhe, S., Hain, T., Chakraborty, T. (2013). Reassessment of the *Listeria monocytogenes* pan-genome reveals dynamic integration hotspots and mobile genetic elements as major components of the accessory genome. *BMC Genomic*. **14**(47). 1-19.

Lambert, P.A. (2005). Bacterial resistance to antibiotics: modified target sites. *Advanced Drug Delivery Reviews*. **57**(10).1471-1485.

Lamont, R.F., Sobel, J., Mazaki-Tovi, S., Kusanovic, J.P., Vaisbuch, E., Kim, S.K., Uldbjerg, N., Romero, R. (2011). Listeriosis in human pregnancy: a systematic review. *Journal of Perinatal Medicine*. 39(**3**). 227-236.

Lebreton, A. & Cossart, P. (2017). RNA- and protein-mediated control of *Listeria monocytogenes* virulence gene expression. *RNA biology*. 14(**5**): 460-470.

Lebrun, M., Loulergue, J., Chaslus-Dancla, E., Audurier, A. (1992). Plasmids in *Listeria monocytogenes* in relation to cadmium resistance. *Applied and Environmental Microbiology*. **58**(9). 3183-3186.

Leclerq, A., Moura, A., Vales,G., Tessaud-Rita,N., Aguilhon, C., Lecuit, M. (2019). *Listeria thailandensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. **69**: 74-81.

Lee, S.H.I., Barancelli, G.V. Corassin, C.H., Rosim, R.E., Coppa, C.F.S.C., de Oliveira, C.A.F. (2017). Effect of peracetic acid on biofilms formed by *Listeria monocytogenes* strains isolated from a Brazilian cheese processing plant. *Brazilian Journal of Pharmaceutical Sciences*. **53**(3). 1-7.

Lehto, M., Kuisma, R., Maaaattaa, J., Kymaalaainen, H., & Maki, M. (2011). Hygienic level and surface contamination in fresh-cut vegetable production plants. *Food Control*. **22**. 469-475.

Lemon, K.P., Freitag, N.E., Kolter, R. (2010). The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. *Journal of Bacteriology*. **192**(15): 3969-3976.

Leong, D., NicAogain, K., Luque-Sastre, L., McManamon, O., Hunt, K., Alvarez-Ordonez, A., Scollard, J., Schalenberger, A., Fanning, S., O'Byrne, C., Jordan, K. (2017). A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food business in Ireland. *International Journal of Food Microbiology*. **249**. 18-26.

Lepe, J.A. (2020). Current aspects of listeriosis. *Medicina Clinica (English Edition)*. **154**(11). 453-458.

Leu, M.C., Meng, P., Geskin, E.S., Tismeneskiy, L. (1998). Mathematical modelling and experimental verification of stationary waterjet cleaning process. *Journal of Manufacturing Science and Engineering*. 120(**3**). 571-579.

Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology*. **55**. 645-659.

Liu, D., Ainsworth, A. J., Austin, F. W. & Lawrence, M. L. (2003). Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of Medical Microbiology*. 52. 1066–1070.

Loots, R. (2016). *Biofilms as multifunctional surface coatings and adaptive systems : a biomimetic approach*. PhD thesis, Stellenbosch University, South Africa.

Low, J.C. & Donachie, W. (1997). A review of *Listeria monocytogenes* and listeriosis. *Vet Journal*. **153**. 9-29.

Madsen, J.S., Burmøle, M., Hansen, L.H., Sørsen, S.J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology*. **65**. 183-195.

Mah T.F. (2012). Biofilm-specific antibiotic resistance. *Future Microbiology.***7**.1061–72

Maia, D.S.V., Haubert, L., Kroning, I.S.,dos Santos Soares, K., Oliveira, T.L., da Silva, W.P. (2020). Biofilm formation by *Staphylococcus aureus* isolated from food poising outbreaks and effect of *Butia odarta* Barb. Rodr. Extract on planktonic and biofilm cells. *LWT- Food Science and Technology*. **117. 108685.**

Marriot, N. Principals of Food Plant Sanitation. 4th edition (1999). Aspen publishers, Gaithersberg, Maryland.

Martin, B., Garriga, M., Aymerich, T., 2011. Prevalence of Salmonella spp. and *Listeria monocytogenes* at small-scale Spanish factories producing traditional fermented sausages. *Journal of Food Protection*. **74**. 812-815.

McDonnel, G.E. (2020). Antisepsis, disinfection, and sterilization. *ASM press*: Washington DC.

McGann, P., Ivanek, R., Wiedmann, M., Boor, K.J. (2007). Temperature-dependent expression of *Listeria monocytogenes* internalin and internalin-like genes suggests functional diversity of these proteins amongst Listeriae. *Applied and Environmental Microbiology*. **73**(9). 2806-2814.

McLauchlin, J., Hall, S., Velani, S., Gilbert, R. (1991). Human listeriosis and pâté: a possible association. *British Medical Journal*. **303**. 773-775.

McWilliam Leitch, E.C. & Stewart, C.S. (2002). *Escherichia coli* O157 and Non-O157 isolates are more susceptible to L-lactate than to D-lactate. *Applied and Environmental Microbiology*. **68**(9).4676-4678.

Melero, B., Stessel, B., Manso, B., Wagner, M., Esteban-Carbonero, O.J., Hernandez, M., Rovira, J., Rodriguez-Lazaro, D. (2019). *Listeria monocytogenes* colonization in a newly established dairy processing facility. *International Journal of Food Microbiology*. **289**. 64-71.

Meng, P., Geskin, E.S., Leu, M.C., Li, F., Tismeneskiy, L. (1998). An analytical experimental study of cleaning with moving waterjets. *Journal of Manufacturing Science and Engineering*. 120(**3**). 580-589.

Mereghetti, L., Quentin, R., Marquet-van der Mee, N., Audurier, A. (2000). Low sensitivity of *Listeria monocytogenes* to Quaternary Ammonium Compounds. *Applied and Environmental Microbiology*. **66**(11). 5083-5086.

Merianos JJ. 2001. Surface-active agents, p 63–320. *In* Block SS (ed), Disinfection, sterilization, and preservation. Lippincott Williams & Wilkins, Philadelphia, PA.

Metselaar, K.I., Ibusquiza, P.S., Camargo, A.R.O., Krieg, M., Zwietering, M.H., den Besten, H.M.W., Abee, T. (2015). Performance of stress resistant variants of *Listeria monocytogenes* in mixed species biofilms with Lactobascillus plantarum. *International Journal of Food Microbiology*. **213**.24-30.

Miladi, H., Elabed, H., Ben Slama, R.,Rhim, R., Bakhrouf, A.(2017). A molecular analysis of analysis of the role of osmolye transporters opuCA and betL in *Listeria monocytogenes* after cold and freezing stress. *Archives of Microbiology.***199**. 259-265.

Mizan, M.F. R., Jahid, I.K., Ha, SD. (2015). Microbial biofilms in seafood: A food hygiene challenge. *Food Microbiology*. **49**. 41-55.

Montville, T.J., Matthews, K.R., Kniel, K.E. (2012). Food Microbiology: an introduction. Pp. 10-120. Washington DC: ASM Press

Møretrø, T., Schirmer, B.C.T., Heir, E., Fagerlund, A., Hjemli, P., Langsrud, S. (2017). Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. *International Journal of Food Microbiology*, **241**, 215-224.

Mosteller, T.M. & Bishop, J.R. 1993. Sanitizer efficacy against attached bacteria in a milk biofilm. *Journal of Food Protection*. **56**.4-41.

Muhterem-Uyar, M., Dalmasso, M., Bolocan, A.S., Hernandez, M., Kapetanakou, A.E., Kuchta, T., Manios, S.G., Melero, B., Minarovicova, J., Nicolau, A.I., Rovira, J., Skandamis, P.N., Jordan, K., Rodriguez-Lazaro, D., Stressl, B., Wagner, M. (2015). Environmental Sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. *Food Control*. **51**. 94-107.

Mullapudi S, Siletzky RM, Kathariou S. 2010. Heavy-metal and benzalkonium chloride resistance of *Listeria monocytogenes* isolates from the environment of turkey-processing plants. *Applied Environmental Microbiology*. **74**. 1464–1468.

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stressl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S. (2013). Tn6188- A Novel Transposon in *Listeria monocytogenes* Responsible for tolerance to Benzalkonium Chloride. *PLOS ONE*. **8**(10). 76835.

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stressl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S. (2014). The *Listeria monocytogenes* tansponson Tn6188 provides increased tolderance to various quaternary ammonium compounds and ethidium bromide. *FEMS Microbiology Research Letters*. **361**(2). 166-173.

Munita, J.M. & Arias, C.A. (2016). Mechanisms of antibiotic resistance. *Microbiology Spectrum*. **4**(2). 1-24.

Nadon, C.A., Bowen, B.M.,Wiedmann, M, Boor, K.J. (2002). Sigman B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. *Infectious Immunology*. **70**. 3948-3952.

National institute for communicable diseases (2018). Listeriosis incident report. Department of Health of the Republic of South Africa: Pretoria.

New South Wales Government Department of Primary Industries Food Authority. (2016). Controlling *Listeria monocytogenes* in the food processing environment guide for the development of an environmental monitoring plan.

Nguyen, H.D.N. & Yuk, HG. (2013). Changes in resistance of *Salmonella Typhimurium* biofilms formed under various conditions to individual sanitizers. *Food Control*. **29**. 236-240.

NicAogáin, K & O'bryne, C.P. (2016). The role of stress and stress adaptions determining the the fate of the bacterial pathogen Lidteria monocytogenes in the food chain. *Frontiers in Microbiology*. 7: 1865.

Nowak, J., Cruz, C.D., Palmer, J., Fletcher, G.C., Flint, S. (2015). Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditons. *Journal of Microbial Methods*. **119**.189-195.

Olanya, O.M., Hoshide, A.K., Ijabadeniyi Ukuku, D.O., Mukhopadhyay, S., Niemira, B.A., Ayeni, O.M. (2019). Cost estimation of listeriosis (*Listeria monocytogenes*) occurrence in South Africa in 2017 and its food safety implication. *Food Control*. **102**. 231-239.

Olszewska, M.A., Zhao, T., Doyle, M.P. (2016) Inactivation and induction of sublethal injury of *Listeria monocytogenes* in biofilm treated with various sanitizers. *Food Control*.70: 371-379.

Osri, R.H., den Bakker, H.C., Wiedmann, M. (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology*. 301(**2**). 79-96.

Pan, Y., Breidt, J.R., Kathariou, S.(2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology*. **72**(12). 7711-7717.

Parisi, A., Latorre, L., Normanno, G., Miccolupo, A., Fraccalvieri, R., Lorusso, V., Santagada, G. (2010). Amplified fragment length polymorphism and multi-locus sequence typing for high-resolution genotyping of *Listeria monocytogenes* from foods and the environment. *Food Microbiology*. **27**. 101–108.

Park, SH., Chang, PS., Ryu, S., Kang, DH. (2014). Development of a novel selective medium for the isolation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **80**(3). 1020-1025.

Perrin, M., Bemer, M., Delamare, C. (2003). Fatal Case of Listeria inoccua Bacteraemia. *Journal of Clinical Microbiology*. **41**(11). 5308-5309.

Piffaretti, J.C., Kressebuch, H., Aeschbacher, M., Billie, J., Bannerman, E., Musser, J.M., Selander, R.K., Rocourt, J. (1989). Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proceedings of The National Academy of Sciences of The United States of America*. **86**(10). 3818-3822.

Poimenidou, S.V., Chrysadakou, M., Tzakoniati, A., Bikouli, V.C., Nychas, G-J., Skanamis, P.N. (2016). Variability of *Listeria monocytogenes* strains on biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. *International Journal of Food Microbiology*. **237**,164-171.

Pouillot, R., Klontz, K.C., Chen, Y., Burral, L.S., Macarisin, D., Doyle, M., Bally, K.M., Strain, E., Datta, A.R., Hammack, T.S., Van Doren, J.M. (2016). Infectious Dose of *Listeria monocytogenes* in Outbreak Linked to Ice Cream, United States, 2015. *Emerging Infectious Diseases*. **22**(12). 2113-2119.

Putman, M., van Veen, H.W., Konings, W.N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiology and Molecular Biology Reviews*.**64**(4). 672-693.

Rasmussen, O.F., Skouboe, P., Dons, L., Roseen, L., Olsen, J.E. (1995). *Listeria monocytogenes* exists in three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology*. **141**. 2053-2061.

Ratani, S.S., Siletzsky, R.M., Dutta, V., Yildirim, S., Osborne, J.A., Lin, W., Hitchens, A.D., Ward, T.J., Kathariou, S. (2012). Heavy metal and disinfectant resistance of *Listeria monocytogenes* from foods and food processing plants. *Applied Environmental Microbiology*. **78**(19). 6938-6945.

Regan, M. (2006). Changing pattern of human listeriosis, England and Wales, 2001– 2004. *Emerging Infectious Diseases*. **12**. 1361–1366.

Reij, M. W., & Den Aantrekker, E. D. (2004). Recontamination as a source of pathogens in processed foods. *International Journal of Food Microbiology*. **91**. 1-11.

Rios-Castillo, A.G., Umana, F.F., Rodriguez-Jerez, J.J. (2018). Long-term antibacterial efficacy of disinfectants based on benzalkonium chloride and sodium hypochlorite tested on surfaces against resistant gram-positive bacteria. *Food Control*.**93**.219-225.

Robbins, J.B., Fisher, C.W., Moltz, A.G., Martin, S.E. (2005). Elimination of *Listeria monocytogenes* biofilms by ozone, chlorine, hydrogen peroxide. *Journal of Food Protection*. **68**(3). 494-498.

Rogovskyy, A. S. (2006). Control of Listeria species and other bacteria in crawfish and crab processing facilities, utilizing copper drains, coatings and concrete containing copper ions. MSc Thesis. Department of Food Sciences, Agricultural and Mechanical College, Louisiana State University. Available online at http://etd.lsu.edu/docs/available/etd-07112006-220909/unrestricted/Rogovskyy_thesis.pdf

Romanova, N., S. Favrin, and M. W. Griffiths. (2002). Sensitivity of *Listeria monocytogenes* to sanitizers used in the meat processing industry. *Applied Environmental Microbiology*. 68:6405–6409.

Rotariu, O., Thomas, D.J.I., Goodburn, K.E., Hutchinson, M.L., Strachan, N.J.C. (2014). Smoked salmon industry practices and their association with *Listeria monocytogenes*. *Food Control*. **35**. 284-292.

Rückerl, I., Muhterem-Uyar, M., Muri-Klinger, S., Wagner, K.-H., Wagner, M., Stessl, B. (2014). *L. monocytogenes* in a cheese processing facility: learning from contamination scenarios over three years of sampling. *International Journal of Food Microbiology*. **189**. 98–105.

Sakardis, I., Ellis, R.J. Cawthraw, S.A., van Vliet, A.H.M., Stekel, D.J., Penell, J., Chambers, M., La Ragione, R.M., Cook, A.J. (2018). Investigating the association between the caecal microbiomes of broilers and *Campylobacter* burden. *Frontiers in Microbiology*. **9**. Article 927.

Santos, T., Viala, D., Chambon, C., Esbelin, J., Hebraud, M. (2019). *Listeria monocytogenes* biofilm adaptation to different temperatures seen through shotgun proteomics. *Frontiers in Nutrition*.6: Article 89.

Scavia, G., Baldinelli, F., Busani, L., Caprioli, A., 2012. The burden of self-reported acute gastrointestinal illness in Italy: a retrospective survey, 2008–2009. *Epidemiological Infections*. **140**. 1193–1206.

Schlech, W.F., Haldane, H., Mailman, T.L., Warhuus, M., Crouse, N., Haldane, D.J.M. (2005). Does sporadic Listeria Gastroenteritis exist? A 2-Year Population-Based Survey in Nova Scotia, Canada. *Clinical Infectious Diseases*. **41**(6). 778-784.

Schoder, D., Melzner D., Schmalwieser, A., Zangana, A., Winter, P., Wagner, M. (2011). Important vectors for *Listeria monocytogenes* transmission at farm dairies manufacturing fresh sheep and goat cheese from raw milk. *Journal of Food Protection*. **74**(6). 919-924.

Shi, H., Trinh, Q., Xu, W.,,Luo, Y., Tian, W., Huang, K. (2013). The transcriptional response of virulence genes in *Listeria monocytogenes* during inactivation by nisin. *Food Control*. 21(**2**): 519-524.

Silk, B.J., Date, K.A., Jackson, K.A., Pouillot, R., Holt, K.G., Graves, L.M. (2012). Invasive Listeriosis in the Foodborne Diseases Active surveillance network (FoodNet), 2004-2009: further targeted prevention needed for higher-risk groups. *Clinical Infectious Diseases*. **54**(5). 396-404.

Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*. **43**(4), 573–583.

Skowron, K., Hulisz, K., Gryn, G., Olszewska, H., Wiktorczyk, N., Paluszak, Z. (2018). Comparison of selected disinfectants efficacy against *Listeria monocytogenes* formed on various surfaces. *International Microbiology*. **21**. 23-33.

South African National Standard. (2017). SANS 587 Canned fish, canned marine molluscs and canned crustaceans, and products derived therefrom. Pretoria: SABS Standards Division.

Steenackers, H., Hermans, K., Vanderleyden, J., De Keersmaecker, S.C.J. (2012). Salmonella Biofilms an overview on occurrence, structure, regulation, and eradication. *Food Research International*. **45**. 502-531.

Stressl, B., Luf, M., Schoder, D. (2009). Performance testing of six chromogenic ALOA-type media for the detection of *Listeria monocytogenes*. *Journal of Applied Microbiology*. **106**. 651-659.

Stressl, B., Szakmary-Brandle, K., Vorberg, U., Schoder, D., Wagner, M. (2020). Temporal analysis of the *Listeria monocytogenes* population structure in floor drains during reconstruction and expansion of a meat processing plant. *International Journal of Food Microbiology*. **314**. 108360.

SurTec Deutschland GmbH. (2014). Criteria for Selecting the correct cleaning system. *JOT International Surface Technology*. **1**. 34-36.

Swaminathan, B. & Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and Infection*. **9**(10):1236-1243.

Thomas, J., Govender, N., McCarthy, K.M., Erasmus, L.K., Doyle, T.J., Allam, M., Ismail, A., Ramalwa, P., Ntshoe, G., Shonhiwa, A., Essel, V., Tau, N., Smouse, S., Ngomane, H.M., Disenyeng, B., Page, N.A., Govender, N.P., Duse, A.G., Stewart, R., Thomas, T., Mahoney, D., Tourdjman, M., Disson, O., Thouvenot, P., Maury, M.M., Leclercq, M., Smith, A.M., Blumberg, L.H. (2020). Outbreak of Listeriosis in South Africa Associated with Processed Meat. *The New England Journal of Medicine*. 382: 632-643.

Tobin, H.M., Lele, S.R., Cutter, C.N., Anantheswaran, R.C., LaBorde, L.F. (2020). Hot water sanitization of a commercial mushroom disk slicer to inactivate *Listeria monocytogenes*. *Food* Control. **109**. 106900.

Tompkin, R.B. (2002). Control of *Listeria monocytogenes* in the Food Processing Environment. *Journal of Food Protection*. **65**. 709-725.

Tonney, F. O., F. E. Greer, and T. F. Danforth. (1928). The minimal "chlorine death points" of bacteria. 1. Vegetative forms. *American Journal of Public Health*. **18**. 1259-1263.

Udompijitkul, P., Alnoman, M., Sarker, M. (2013). Inactivation strategy for Clostridium perfringens spores adhered to food contact surfaces. *Food Microbiology*. **34**(2). 328-336.

Van der Veen, S. & Abee, T. (2010). Importance of SigB for *Listeria monocytogenes* Static and continuous flow biofilm formation and disinfection resistance. *Applied and Environmental Microbiology*. **76**(23). 7854-7860.

Van Houdt, R., Michiels, C.W. (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology*. **109**. 1117-1131.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*. 14. 584–640.

Vines, A., Reeves, M.W., Hunter, S., Swaminathan, B. (1992). Restriction fragment length polymorphism in four virulence-associated genes of *Listeria monocytogenes*. *Research in Microbiology*. **143**. 281-294.

Walker, S.J., Archer, P., Banks, J.G. (1990). Growth of *Listeria monocytogenes* at refrigeration temperatures. *Journal of Applied Bacteriology*.**68**. 157-162.

Wang, H., Wang, X., Yu, L., Gao, F., Jiang, Y., Zu, X. (2020). Resistance of biofilm formation and formed biofilm of *Escherichia coli* O157:H7 exposed to acid stress. *LWT- Food Science and Technology*.**118**. 108787.

Wang, Y., Zhao, A,, Zhu, R., Lan, R., Jin ,D., Cui, Z., Wang, Y., Li, Z., Wang, Y., Xu, J.,Ye, C. (2012) Genetic diversity and molecular typing of *Listeria monocytogenes* in China. *BMC Microbiology* **12**(119). 1-9.

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., Bielawski, J.P. (2008). Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. *Applied Environmental Microbiology*. **74**. 2046-2054.

Wemekamp-Kamphuis, H.H., Sleator, R.D., Wouters, J.A. Hill, C., Abee, T.(2004). Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu and OpuC in growth of *Listeria monocytogenes* at low temperatures. *Applied Environmental Microbiology*. **70**.2912-2918.

Wiedmann, M., Bruce, J. L., Knorr, R., Bodis, M., Cole, E. M., McDowell, C. I., McDonough, P. L. & Batt, C. A. (1996). Ribotype diversity of *Listeria monocytogenes* strains associated with outbreaks of listeriosis in ruminants. *Journal of Clinical Microbiology*. **34**. 1086–1090.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infectious Immunology*. **65**. 2707-2716.

Wiley, J.M., Sherwood, L.M., Woolverton, C.J. (2014). *Prescott's Microbiology Ninth Edition*. Pp 167 & 193. New York: McGraw-Hill Education.

Willet, H. P. (1980). *Listeria* and *Erysipelothrix,* p. 627-638. *In* W. K. Joklik, H. P. Willett, and D. B. Amos (eds.), Zinsser microbiology. Appleton Century Crofts, New York.

Williams, S. K., Roof, S., Boyle, E. A., Burson, D., Thippareddi, H., Geornaras, I (2011). Molecular ecology of *Listeria monocytogenes* and other *Listeria* species in small and very small ready-to-eat meat processing plants. *Journal of Food Protection*. **74**. 63-77.

Wilson, A., Gray, J., Scott Chandry, P., Fox, E.M. (2018). Phenotypic and genotypic analysis of antimicrobial resistance among *Listeria monocytogenes* isolated from Australian food production chains. *Genes*. **9**(80).

Xu, D., Nie, Q., Wang, W., Shi, L., Yan, H. (2016). Characterization of a transferable bcrABC and cadAC genes-harbouring plasmid in *Listeria monocytogenes* strain isolated from food products of animal origin. *International Journal of Food Microbiology*. **217**. 117-122.

Chapter 3

Identification and classification of sanitizer resistant *Listeria monocytogenes*

3.1 Summary

Listeria monocytogenes is a ubiquitous, Gram positive, bacteria which causes the human disease listeriosis. It can survive in food processing environments and has been shown to survive the application of certain classes of terminal disinfectants (sanitizers) and in particular, the QAC class of sanitizer. These isolates have been shown to be responsible for outbreaks of listeriosis globally with certain genes that confer resistance towards QACs being found within the clinical isolates. This study investigates 50 *L. monocytogenes* isolates from six different South African FPEs using RapidL'mono plates and *hly*A gene screening confirmation to confirm its identity. Further tests for the presence of three genes (*emr*C, *emr*E and *qac*H) and one gene cassette (*bcr*ABC) were also conducted. Finally phenotypic expression of resistance by the 50 isolates to nine different commercial sanitizers, four of which belonged to the QAC class of sanitizer was investigated. The results painted a picture of the problem of QAC sanitizer resistant *L. monocytogenes* in South African FPEs and compared it to results from studies across the globe. The findings showed that resistance to QACs was prolific across the sample set.

3.2 Introduction

L. monocytogenes is a ubiquitous, Gram-positive bacterium that can grow at refrigerated temperatures, adhere to stainless steel, and form biofilms throughout a factory environment. *L. monocytogenes* can cause the human-disease listeriosis which can be fatal (Costa *et al.,* 2018). Recently, cases of listeriosis have shown that the outbreak strains often possess genes that encode for resistance to a certain class of sanitizer called Quaternary Ammonium Compounds (QACs) which are commonly used in food factories (Kropac *et al.,* 2019; Møretrø *et al.,*2017; Kovacevic *et al.,* 2016; Müller *et al.,* 2014; Müller *et al.,* 2013; Dutta *et al.,* 2013; Elhanafi *et al.,* 2010).

Factory cleaning is a multistep process that requires full cooperation from all facets of the company including (but not limited to): technical; production; maintenance and the cleaning service provider (be it an external contractor or an inhouse team). If these factory role players do not communicate effectively ineffective cleaning is likely to take place (Khalid *et al.,* 2019; Leu *et al.,* 1998). This can prove disastrous as it means pathogens can survive and further proliferate but also be exposed to sublethal concentrations of sanitizer applied as a terminal disinfectant. This sublethal exposure could result in the rise of resistance to an antimicrobial (in this case a disinfectant like a QAC-based sanitizer). QAC resistance has been attributed to multiple genes, however four of the most prevalent genes in literature (all of which have been attributed to a listeriosis outbreak) were chosen. Namely *bcr*ABC, *qac*H, *emr*C and *emr*E genes (Kropac *et al.,* 2019; Kovacevic *et al.,* 2016; Müller *et al.,* 2014; Müller *et al.,* 2013; Dutta *et al.,* 2013; Elhanafi *et al.,* 2010).

QAC sanitizers have undergone multiple generational changes since their inception (Gerba, 2015). Resistance to first generation QACs have been reported extensively in literature, and industry role players have been moving towards later generations of QACs or QAC cocktails as an economic way of overcoming resistance to first generation QACs (Kropac *et al.,* 2019; Møretrø *et al.,*2017; Kovacevic *et al.,* 2016; Müller *et al.,* 2014; Müller *et al.,* 2013; Dutta *et al.,* 2013; Elhanafi *et al.,* 2010).

L. monocytogenes has been classified into four distinctive lineage types with lineages I and II being the only lineage types isolated from the factory environment (Rip & Gouws, 2020; Leong *et al.,* 2017). Lineage II has been shown to be prevalent in the factory environment whilst Lineage I isolates have been attributed to most human cases of listeriosis (Osri *et al.,* 2011). From lineage I isolates, ST-6 isolates have been shown to harbour the *emr*C resistance gene which confers resistance towards QACs and increases pathogenicity (Kropac *et al.*, 2019; Koopmans *et al.*, 2017). Lineage II isolates have been shown to contain the *bcr*ABC QAC resistance gene (Chmielowska *et al*., 2021). It will be interesting to note weather or not the transfer of these resistance genes has taken place in South African FPEs.

Classifying *L. monocytogenes* into its distinctive lineages is advantageous as it can give an indication of possible downstream implications of infection as well as give a top-down view on the issue of *L. monocytogenes* contamination within a factory. For example, it gives an indication of the proliferation of in-house strains versus the introduction of new strains.

Polymerase Chain Reaction (PCR) is a powerful and accurate tool that can be used to identify the presence or absence of a gene of interest within a sample's Deoxyribose Nucleic Acid (DNA) sequence. This technique was subsequently used to confirm genotypic identity of the *L. monocytogenes* isolates through the absence or presence of the listeriolysin gene (*hly*). The presence or absence of the *bcr*ABC, *emr*C, *emr*E and *qac*H genes was then determined using conventional PCR with primers attributed to those genes.

The presence of genes is not enough to state whether it is being transcribed or not (Henderson *et al.*, 1999). This is due to expression of genes within bacteria being controlled via either a graded response (through promoter control mechanisms) or via an "all-or-none" response and these conditions are often modulated by the conditions that a bacterial cell finds itself in (Henderson *et al.*, 1999). A method adapted from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2017) was applied to test whether the isolates were expressing resistance to the different sanitizers or not. This was done using disks impregnated with the sanitizers used in the study, much-like how antibiotic impregnated disks are used for EUCAST methods (EUCAST, 2012).

In this study, *L. monocytogenes* isolates (N=50) were tested from six different food processing plants across the Republic of South Africa. Various tests were carried out to confirm the genotypic identity of the isolates, absence, or presence of resistance genes and to ascertain their phenotypic sanitizer susceptibility. The results of these tests would be beneficial to industry as it would highlight the prevalence of sanitizer resistance amongst *L. monocytogenes* isolates across South African food factories. It is hoped that with this increased awareness that action will be taken to combat irresponsible antimicrobial (sanitizer) applications and promote the importance of following through with adequate hygiene and factory cleaning operations.

3.3 Materials and methods

3.3.1 Bacterial strains

L. monocytogenes isolates from six South African food processing plants from the environment (representative of different environments within the processing plants) were isolated using ISO 16140 methodology for the enumeration and detection of *L. monocytogenes* (International Standards Organization). RapidL'mono (BIORAD, FRA) selective media plates, that were positive for *L. monocytogenes*, where shipped (by means of refrigerated containers) to The Department of Food Science at Stellenbosch University.

In total, 50 *L. monocytogenes* isolates were obtained. The isolates came from factory drains (specified as drain), FCS such as conveyor belts, bowl choppers and work surfaces (specified as food contact) or from areas such as the factory floor, boots or trolley wheels (specified as environmental). No *L. monocytogenes* isolates from final product were tested.

American Type Culture Collection (ATCC) strains *L. monocytogenes* (ATCC 7644, serotype 1/2 c lineage II) and *L. innocua* (ATCC 33090) were also included as a pathogenic and non-pathogenic control strain respectively. The organisms were stored in in 25% glycerol brain heart infusion (BHI) broth cryovials at -80 ^{O}C until required for use.

For the lineage typing confirmation three positive *L. monocytogenes* controls were used. The lineage I control was a serotype 1/2 b (CIP 105.449) from the Institut Pasteur Collection, the lineage II control was a serotype 1/2 c (ATCC 7644) and the lineage III was a serotype 4 a isolate (ATCC 1914).

3.3.2 Sample processing and glycerol stocks

Once the RapidL'mono (BioRad, FRA) plates were received: presumptive positive *L. monocytogenes* colonies were aseptically transferred to BHI broth (CM 1135, Oxoid, UK) and incubated at 37 ^{O}C for 24 hours before being re-streaked onto fresh RapidL'mono plates. From these RapidL'mono plates a 10 µL loopful of pure colonies were placed into fresh BHI broth (CM 1135, Oxoid, UK) and incubated for 24 hours at 37 ^{O}C.

Stock cultures were then prepared by taking 0.75 mL of vortexed broth culture into a 2 mL cryovile containing 0.75 mL of 50% sterile glycerol and were stored at -80 ^{O}C (Fluka Analytical, Germany) (Gorman & Adley, 2004).

3.3.3 DNA extraction

After pure overnight cultures of the *L. monocytogenes* were grown for 24 hours at 37 ^{O}C on BHI agar plates, a Quick-DNA^{TM} Fungal/ Bacterial Miniprep DNA extraction kit (ZymoResearch, USA) was used to extract whole bacterial DNA: according to the manufacturer's instructions.

In brief, a 10 µL loopful of colonies was picked up from the BHI agar plate and suspended in 750 µL BashingBead Buffer in a ZR BashingBead Lysis Tube. The tubes were then secured in a bead beater, Disruptor Genie (ZymoResearch, USA) and processed on maximum speed for 10 minutes. The ZR BashingBead Lysis Tubes were then centrifuged at 10 000 x *g* for 1 minute. Then 400 µL of the supernatant was transferred into a Zymo-Spin III-F Filter in a collection tube and centrifuged at 8000 x *g* for 1 minute. Then 1200 µL of Genomic Lysis Buffer was added to the filtrate in the collection tube from previous step. Thereafter 800 µL of the mixture was transferred into a Zymo-Spin IICR Column in a collection tube and centrifuged at 10 000 x *g* for 1 minute. The flow through of the collection tube was then discarded and the previous step repeated. Then 200 µL of DNA Pre-Wash Buffer was added to the Zymo-Spin IICR Column in a new Collection Tube and centrifuged at 10 000 x *g* for 1 minutes. G-DNA Wash Buffer (500 µL) was added to the Zymo-Spin IICR Column and centrifuged at 10 000 x *g* for 1 minute. The Zymo-Spin IICR Column was then transferred to a clean 1.5 mL microcentrifuge tube and 100 µL DNA elution buffer was added directly to the column matrix. It was then centrifuged at 10 000 x *g* for 1 minute to elute the DNA. The DNA was then stored at – 20 ^{O}C until required for use (Jones & D'Orazio, 2014).

3.3.4 Polymerase Chain Reaction

Primer selection

The *hly*A housekeeping gene, which encodes for listeriolysin which forms pores inside cells of a host facilitating infection, was selected due to its almost ubiquitous presence in *L. monocytogenes* (Blaise & Phillippe, 1993). Three genes and one cassette conferring resistance to QACs were selected based on their association with clinical isolates from outbreaks of listeriosis (*bcr*ABC cassette, *qac*H, *emr*C and emr*E* genes) (Kropac *et al.,* 2019; Møretrø *et al.,*2017; Kovacevi, *et al.*, 2016; Elhanafi, *et al.*, 2010). All primer sets and product sizes are depicted in Table 3.1.

Table 3.1 Description of gene primers used in this study and their respective sequences and PCR product size

Primer set	Sequence (5'—'3)	Product size (bp)	Source
hly	CATTAGTGGAAA GATGGAATG (F) GTATCCTCCAGAGTGATCGA (R)	730	Blaise & Phillippe, 1993.
*bcr*ABC	GAATGGATCCTTCAATTAGATCGAGGCACG (F) GTATGAATTCGTATAATCCGGATGCTGCCC (R)	1130	Elhanafi *et al.,* 2010
*qac*H	CCGCCATGGCATATCTATATTTAGCA (F) CGGTCTAGAGACTCATACGTATATAAATAA (R)	439	Müller *et al.,* 2014
*emr*C	TTA TTC CAT TTT ATT ACT GGC AAT G (F) CGT ATT TAT ATT TAA CAC TAG CCA (R)	387	Kropac *et al.,* 2019
*emr*E	GCC ACA AAA GGG CAG GTT (F) TAA AGC TCT CCC GCA GTA CC (R)	1588	Kovacevic *et al.,* 2016

F= Forward, R=Reverse

PCR reaction conditions for the genes used in this study

The *hly* gene required the following amplification conditions: initial denaturation for 3 minutes at 94 ^{O}C; 30 cycles of denaturation at 94 ^{O}C for 40 seconds; annealing at 55 ^{O}C for 40 seconds; extension at 72 ^{O}C for 40 seconds; followed by final extension at 72 ^{O}C for 5 minutes (Blaise & Phillipe, 1993)

The *bcr*ABC gene required the following PCR amplification conditions: 15 minutes of initial denaturation at 95 ^{O}C; 30 cycles of denaturation at 94 ^{O}C for 30 seconds, annealing at 60 ^{O}C for 90 seconds, extension at 72 ^{O}C for 90 seconds; followed by a final extension of 10 minutes at 72 ^{O}C (Møretrø *et al.,*2017).

The *qac*H gene required the following amplification conditions: 15 minutes of initial denaturation at 95 ^{O}C; 30 cycles of denaturation at 94 ^{O}C for 30 seconds, annealing at 56 ^{O}C for 30 seconds, extension at 72 ^{O}C for 90 seconds with a final extension of 10 minutes at 72^{O}C (Møretrø *et al.,*2017).

The *emr*C gene required the following amplification conditions: 2 minutes of initial denaturation at 94 ^{O}C ; 36 cycles of denaturation at 94 ^{O}C for 15 seconds; annealing at 50 ^{O}C for 15 seconds, extension at 72 ^{O}C for 30 seconds with a final extension at 72 ^{O}C for 5 minutes (Kropac *et al.,* 2019).

The *emr*E gene required the following PCR amplification conditions: 2 minutes of initial denaturation at 95 ^{O}C; followed by 30 cycles of denaturation at 95 ^{O}C for 30 seconds; annealing at 50 ^{O}C for 30 seconds, extension for 102 seconds at 72 ^{O}C. Final extension was carried out at 72 ^{O}C for 10 minutes (Kovacevic *et al*., 2016).

PCR master mix parameters

PCR reactions were performed in 25 µL reaction volumes with the following makeup: 0.2 µM of the forward and the reverse primers (for all reactions), 10.5 µL of DreamTaq Hot Start Master Mix (ThermoFisher Scientific, Lithuania) (as per the manufacturers guidelines). The DreamTaq Hot Start Master mix contained DreamTaq DNA polymerase, 2x DreamTaq buffer,0.4mM dATP, 0.4mM dCTP, 0.4mM dGTP, 0.4mM dTTP and 4 mM $MgCl_2$. Finally, nuclease free water (ThermoFisher Scientific, Lithuania) to make up the final volume.

Positive controls

Unfortunately, due to the Corona virus (SARS COVID-19) pandemic, bacterial controls for resistance gene (*brc*ABC, *emr*C, *emr*E and *qac*H) could not be brought into the country. Thus, all samples were screened by PCR for the resistance genes and if positives were found, the presumed positive underwent SANGER sequencing at InqabaBiotec in Pretoria. The *bcr*ABC and *emr*C gene controls were created in this manner by confirming the identity of the amplified sequence and ensuring that it matches with the sequence reported in literature.

3.3.5 Gel electrophoresis

The PCR products with loading dye (New England BioLabs, USA) were first separated and then visualized, using SafeView Classic (ABM, USA) on a 1.1% TAE agarose gel, alongside a 100 bp ladder (ThermoFisher Scientific, Lithuania). Gel electrophoresis in 1x TAE buffer took place for 2 hours at 65 V to allow for adequate band separation.

Gel imaging and visualization took place using a Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) along with its Image Lab Software (version 5.2.1). Results were then recorded, and samples discarded. These gel electrophoresis conditions were applied for all the genotyping and lineage typing as these conditions allowed for adequate separation of the DNA ladder and thus accurate identification based on base pair size.

3.3.6 Lineage typing/ RFLP analysis

Using the method described by Rip & Gouws (2020) the abovementioned *hly* gene PCR product was used in the following manner to categorize each *L. monocytogenes* isolate into either lineage group I, II or III based on conserved single nucleotide polymorphisms (SNPs).

Using a 25 µL PCR tube, a 10µL solution, containing: 5µL PCR product, 1 µL of FastDigest Buffer (Final concentration was set up) (ThermoFisher, Lithuania) and 3 µL nuclease free water (ThermoFisher Scientific,

Lithuania). First, 1 µL of FastDigest Enzyme (ThermoFisher, Lithuania) specific to lineage type was added. For lineage I screening, *Nde*I FastDigest enzyme (ThermoFisher, Lithuania) was used. For lineage II screening, *BfoI* restriction enzyme (ThermoFisher, Lithuania) was used. For lineage III screening, *Bsh*12851 restriction enzyme (ThermoFisher, Lithuania) was applied to the samples in question. Samples were first screened with *Nde*I to ascertain if they belonged to lineage I and if so they were excluded from screening with *BfoI* to ascertain if they belonged to lineage II. If not, then they were finally screened with *Bsh*12851. Each enzyme leaves a band size indicative for each lineage type when cutting occurs. Lineage I's band sizes are 390 and 341 bps; lineage II's band sizes are 294, 277 and 160 bps; and lineage III's band sizes are 384 and 347 bps (Rip & Gouws, 2020).

Table 3.2 RFLP restriction enzyme reaction parameters and amplicon base pair (bp) size

Lineage type	Enzyme	Reaction time (minutes)	Reaction temperature (^{O}C)	Enzyme inactivation condition	bp
I	*Nde*I	25	37	On ice	390 & 341
II	*BfoI*	30	37	65 ^{O}C	294; 277 & 160
III	*Bsh*12581	25	37	On ice	384 & 347

Once the 10 µL restriction digestion mixture was made up, different digestion conditions were used to ensure adequate digestion took place, as per the manufacturer's instructions. Digestion with *Nde*I occurred for 25 minutes at 37 ^{O}C after which the tubes were placed on ice. Digestion with *Bfo*I occurred for 30 minutes at 37 ^{O}C followed by thermal inactivation at 65 ^{O}C for 10 minutes, after which tubes were stored on ice. Digestion with *Bsh*12851 occurred for 25 minutes at 37 ^{O}C followed by cooling on ice.

The following lineage type controls were included for the lineage type classification: Lineage I - *L. monocytogenes* 1/2 b (CIP 105.449); Lineage II - *L. monocytogenes* 1/2 c (ATCC 7644); Lineage III -*L. monocytogenes* 4 a (ATCC 1914).

3.3.7 Phenotypic resistance gene testing

Sanitizers used

The different strains of *L. monocytogenes* were subjected to phenotypic resistance testing by means of blank diffusion disks (Oxoid, GBR) impregnated with sanitizers as described in Table 3.2. The sanitizers used in the study were comprised of QAC-based sanitizers and non QAC-based sanitizers. All sanitizers were made up to 1% concentration. The only exception was L-Lactic acid which was used at 3% which was the

minimum recommended application concentration, as per the manufacturer's guidelines. All sanitizers are approved for commercial use and have undergone EN 1276 and SANS 12786 testing.

Table 3.3: Table of sanitizers used in this study and their active compounds

Sanitizer	Active compounds	Manufacturer
QC50	First generation QAC (Benzalkonium Chloride)	Ecowize (South Africa)
TCQ	A fourth generation QAC	Ecowize (South Africa)
QC80	Combination of a first and fourth generation QAC (QAC cocktail)	Ecowize (South Africa)
ByoSan concentrate surface sanitizer	QAC cocktail	Byotrol (Great Britain)
QFS	QAC-free alternative to Byosan	Byotrol (Great Britain)
AN8	Non-foaming peracetic acid	Ecowize (South Africa)
AF8	Foaming peracetic acid	Ecowize (South Africa)
SH-12	Sodium hypochlorite	Ecowize (South Africa)
L-Lactic Acid	L-Lactic Acid	Ecowize (South Africa)

3.3.8 Disk impregnation and plating

Blank diffusion cartridges (disks) (Oxoid, UK) were impregnated by soaking in 1 % concentration of the different sanitizers except L-Lactic acid. L-Lactic acid at 3% was used as per the manufacture's guidelines (Ecowize, South Africa). The blank diffusion disks were soaked for one hour within their respective mixtures and dried for half an hour within a sterile biosafety level II cabinet inside separate, open, petri dishes to prevent sanitizers interacting with each other. Inoculums of each of the *L. monocytogenes* used in the study were prepared by means of overnight BHI broth culture and diluted to an OD600 reading of 0.260. This value correlated to 10^8 cfu/ mL through direct plating and this aligned with values from an international study (Yap & Trau, 2019) to allow for consistent bacterial load on the each of the plates.

3.3.9 Concentration confirmation of the inoculum

Using a UV/VIS spectrophotometer (Beckman, USA) a standard curve was constructed at OD600 value readings of different dilutions of BHI *L. monocytogenes* cultures with a lineage II, serotype 1/2 c isolate (ATCC 7644). Once an absorbance reading was obtained from all cultures, a dilution series was then carried out, to facilitate counting, and 100 µL of the culture was plated onto BHI agar plates in triplicate using the spread plate method. These plates were incubated for 24 hours at 37 ^{O}C and colonies were counted. An OD600 value at 0.260 correlated to approximately 10^8 cells.

3.3.10 Placement of the diffusion disks

The lawn of cells (corresponding to approximately 10^8 cfu/ mL) was spread onto the BHI agar plates and sterilized tweezers were used to place the sanitizer impregnated diffusion disks on the BHI agar plates as depicted in Figure 3.1, flaming of the tweezers occurred between each placement.

The BHI agar (CM 1136, Oxoid, UK) plates were then incubated at 37 ^{O}C for 24 hours to ensure optimal growth. Diffusion zone results were then examined after 24 hours of incubation. Figure 3.1 explains the layout of the blank diffusion disks on plate 1 (P1) and plate 2 (P2) which ensured that the correct zones of diffusion were attributed to their respective sanitizer. The same strain was plated on plate 3 (P3) which was the negative control containing a blank diffusion disk and one soaked in dH_2O (the negative controls) as well as on plate 4 (P4) which contained no disks.

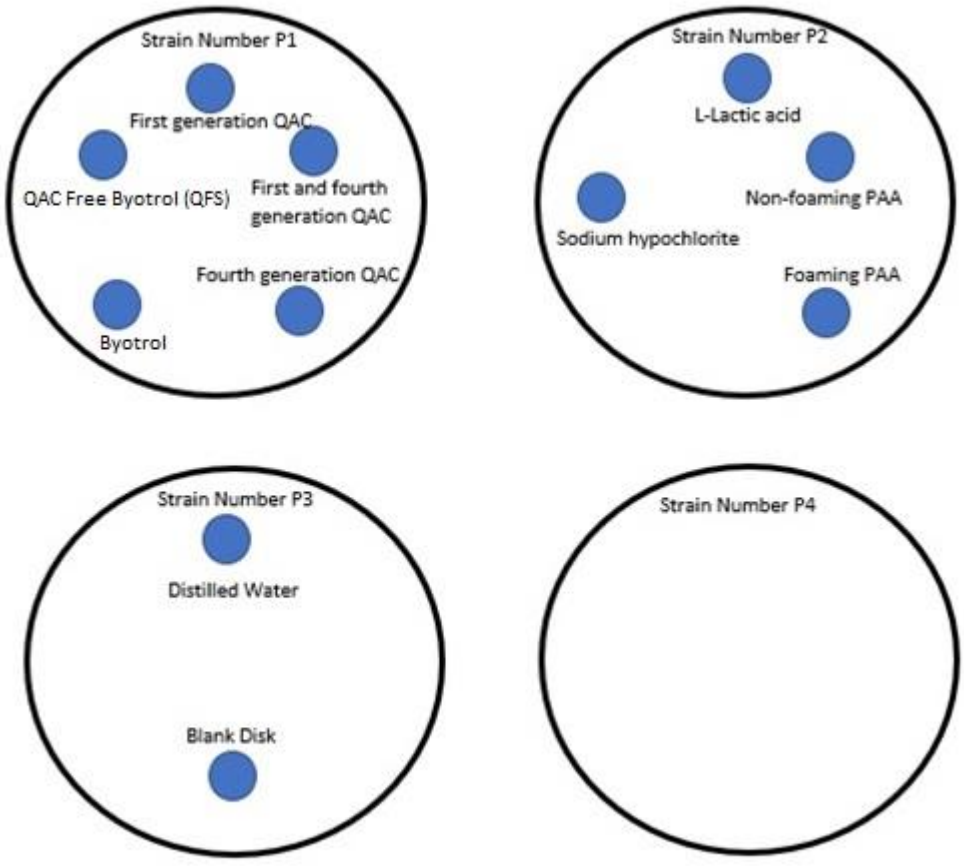


Figure 3.1 Placement of impregnated diffusion disks on a lawn of *L. monocytogenes* on BHI agar.

3.3.11 Storage of the plates whilst in the incubator

In-line with EUCAST guidelines: within 15 minutes that the impregnated-diffusion disks were placed, the plates were incubated to prevent the formation of abnormally large diffusion zones due to pre-diffusion. This would have occurred due to impregnated disks diffusing before microbial growth had occurred. The plates were stacked in fours to ensure adequate heat circulation and distribution (Andrews, 2005).

3.3.12 Interpretation of diffusion results

After 24 hours incubation, diffusion zones were measured using a digital vernier calliper (0-150mm) and compared to the results of the ATCC 7644 (serotype 1/2 c) *L. monocytogenes* reference strain and if found to be smaller: resistance was declared and if larger the isolate was considered susceptible to the sanitizer. This ATCC strain was chosen because it had no genes that conferred resistance to QACs, did not come from a factory environment and was therefore expected to be susceptible towards the sanitizers -with no history of exposure towards commercially used sanitizers, unlike the FPE isolates used in the study.

3.4 Results and Discussion

Globally, the resistance of *L. monocytogenes* towards QAC-based sanitizers has been reported with limited research data from the South African food processing environments (Kropac *et al.*, 2019; Ackerman, 2017). When looking into contributing factors towards QAC-resistance: a strong association between a variety of resistance genes such as *bcr*ABC, *qac*H, *emr*C and *emr*E can be drawn. All these genes have been detected from clinical isolates in outbreaks (Dutta *et al.,* 2013; Elhanafi, *et al.,* 2010; Jiang, *et al.,* 2016; Korsak & Szuplewska, 2016; Mereghetti *et al.,* 2000; Møretrø *et al.,* 2017; Mullapudi *et al.,* 2008; Ratani *et al.,* 2012; Xu *et al.,* 2016). With this in mind, the prevalence of these resistance genes amongst isolates of *L. monocytogenes* from South African food processing environments was investigated.

Four lineage types make up *L. monocytogenes* with lineage I and II being the only lineages isolated from food processing environments thus far (Tsai *et al.*, 2011). Lineage group identification is of importance because within each lineage groups are different serotypes and ultimately sequence types which have human-health consequences. Lineage I contain the following serotypes of human health concern: 1/2 b; 3 b; 4 b; 4 d; 4 e, and 4 b contains the ST-6 sequence type (serotype 4 b) which is the causative sequence type of most human cases of listeriosis (Kremer *et al*., 2017). ST-121 (serotype 1/2 a) is the dominant sequence type of lineage II (1/2 a, 1/2 c, 3 a, 3 c) as is often associated with incidents of environmental contamination of *L. monocytogenes* (Schmitz-Esser *et al.,*2015).

Table 3.3 summarises the results of lineage typing of the 50 isolates included in this study from the South African FPE isolates and indicates the presence (+) or absence (-) of the QAC resistance genes screened for in this study. The table identifies a relationship between lineage type and resistance genes as well as give

an overall indication of resistance gene prevalence amongst isolates from six different FPEs in various geographical regions of RSA. In terms of sample set makeup, 27 of the 50 isolates came from drains across the six different factories, 9 were environmental samples and 14 were from food contact surfaces. All 50 isolates and the *L. monocytogenes* control (ATCC 7644) tested positive for the *hly*A gene.

Table 3.4 Results showing relationship between strain (N=50) and its isolation location, lineage type and presence (+) or absence (-) of a resistance gene

Strain Code	Isolation	Lineage	*bcr*ABC	*emr*C	*emr*E	*qac*H
YF	Drain	1	+	+	-	-
CIRCLE T14	Drain	1	-	+	-	-
17JBX53	Drain	1	+	+	-	-
YA	Drain	1	+	+	-	-
17JBX33	Drain (next to conveyor belt)	1	+	+	-	-
17JBX31	Drain (next to water tank)	1	-	-	-	-
17JBX44	Environment	1	+	+	-	-
E1	Environment (Air Conditioner)	1	-	+	-	-
17J86	Environment (Floor)	1	+	+	-	-
17J86	Environment (LR floor)	1	-	+	-	-
CIRCLE 32	Environment (Trolley)	1	+	+	-	-
39	Food Contact (Chopping board)	1	-	-	-	-
17JBX36	Food Contact (Vienna filler)	1	+	+	-	-
17JBX54	Food Contact (Vienna filler)	1	+	+	-	-
17JBX12	Drain	2	-	+	-	-
17JBX11	Drain	2	-	+	-	-
17JBX42	Drain	2	+	+	-	-
17JBX41	Drain	2	+	+	-	-
17JBX47	Drain	2	+	+	-	-
17JBX9	Drain	2	+	-	-	-
17JBX2	Drain	2	+	-	-	-
CIRCLE 22	Drain	2	+	-	-	-
CIRCLE 3	Drain	2	+	+	-	-
17JBX9	Drain	2	+	+	-	-
43	Drain (High Risk Area)	2	+	-	-	-
44	Drain (High Risk Area)	2	+	-	-	-
47	Drain (High Risk Area)	2	-	-	-	-
48	Drain (High Risk Area)	2	-	-	-	-
YB	Drain (Low Risk area)	2	+	+	-	-
17JBX2	Drain (next to ice machine)	2	+	+	-	-
17J41	Drain (next to mincer)	2	-	+	-	-
CIRCLE 17	Drain (next to mixer)	2	+	-	-	-
17JBX46	Drain (next to Vienna filler)	2	+	+	-	-
17J87	Drain (next to waste area)	2	+	-	-	-
17J62	Drain (Passage)	2	-	+	-	-
29JB719	Environment (Floor scale)	2	+	-	-	-
45	Environment (Plastic lug/ crate)	2	+	-	-	-

46	Environment (Plastic lug/ crate)	2	-	-	-	-
YD	Environment (Trolley washer)	2	+	+	-	-
A	Food Contact (Brine autoinjector)	2	+	+	-	-
B	Food Contact (Brine autoinjector)	2	+	+	-	-
C	Food Contact (Brine autoinjector)	2	+	-	-	-
29JB721	Food Contact (Mixing bowl)	2	-	-	-	-
17J64	Food Contact (Polony pump)	2	+	+	-	-
17JBX32	Food Contact (Table)	2	+	+	-	-
E23	Food Contact (Vienna filler)	2	-	-	-	-
E20	Food Contact (Vienna filler)	2	-	-	-	-
E13	Food Contact (Vienna filler)	2	+	+	-	-
E7	Food Contact (Vienna filler)	2	-	-	-	-
17JBX13	Food Contact (Vienna hopper)	2	+	+	-	-
ATCC7644	*L. monocytogenes* control (1/2 c)	2	-	-	-	-
ATCC33090	*L. innocua* control	NA	-	-	-	-

3.4.1 Lineage typing results

Examining the lineage type distribution across the different factories a higher percentage (72%) (N=36) of the isolates were assigned to lineage II and (28%) (N=14) to lineage I. It is important to note that these isolates were obtained from different areas of the factories, on different days and ultimately from different factories. Thus, a good composite understanding of lineage type distributions in South African food processing environments can be obtained. Knowing lineage type breakdowns within a factory (and prevalence) is advantageous as it is an easy way of basic differentiation between different isolates and gives a top-down view of whether similar isolates may be responsible for factory contamination and identify "problematic" or hotspot forming lineage types which may aid in earlier control.

The three major isolation area groupings for the different factory isolates were as follows: drains; environmental (anywhere from the floor to ceiling that does not come in direct contact with food) and food contact (any surface or object that comes in direct contact with food).

The isolates used in this study came from a diverse array of factory areas and equipment. Examples such as drains from all areas of a factory (from the low-risk side where raw materials are handled and on the high-risk side where final product is handed). As well as environmental isolates (floors, ceilings, air conditioners, doors, walls, table-legs and washing machines); and food contact surfaces (such as lugs, bring auto-injectors, vienna sausage fillers and hoppers and table countertops).

Of the lineage I isolates (N=14): 43% were from drains with 36% from environmental origin and 21% from food contact surfaces. Of the lineage II isolates (N=36): 58% were from various factory drains; 11% were from environmental origin; and 31% from food contact surfaces.

According to Chmielowska *et al*. (2021), *L. monocytogenes* isolated from Polish fish-product factories, serotypes 1/2 a and 3a (belonging to Lineage II) were the most prevalent followed by a combination of serotypes belonging to lineage I (serotypes 4 b, 4 d, 4 e). As stated by Chmielowska *et al*. (2021) these results were also in line with what was reported in numerous other studies on *L. monocytogenes* contamination within food factories (Abdollahzadeh *et al*., 2016; Ebner *et al*., 2015; Gianfranceschi *et al*., 2009; Korsak *et al*., 2012; Kramarenko *et al*., 2013; Martín *et al*., 2014; O'Connor *et al*., 2010; Parisi *et al*., 2010; Wilson *et al*., 2018). Work by Pan *et al*. (2009) found that serotype 1/2 a accounts for more than half of the serotypes of *L. monocytogenes* isolated from food producers so it may be a possibility that a large proportion of the lineage II samples in this study, could be assigned to serotype 1/2 a.

The research by Chmielowska *et al*., (2021) differs from the results of this study in that their samples came primarily from food product and raw materials, rather than from the environment. Work carried out by Pan *et al*. (2009) confirms that lineage II isolates from raw material can in fact proliferate within a factory environment.

Factory drains provide a good niche for biofilm formation and habitation as a whole and should thus be the subject of more rigorous monitoring and cleaning to prevent the shedding of free *L. monocytogenes* cells into the factory environment. When looking at the percentages of isolates in this study, that were obtained from various factory drains alone (Table 3.3) it becomes clear that drains do in fact house a, worryingly, large proportion of *L. monocytogenes* found within a factory.

In terms of an overview of an association between lineage type and resistance gene prevalence Table 3.4 provides an insight into how many of the isolates had resistance genes conferring resistance towards QACs (further explained in section 3.4.2).

Table 3.5 Association between *L. monocytogenes* lineage type and resistance gene presence

Lineage	n= number of isolates	N = No *brc*ABC and *emr*C genes detected (%)	N= both genes (%)	N = one of either gene (%)
I	N=14	N=2 (14)	N=9 (64)	N=3 (21)
II	N=36	N=7 (19)	N=15 (36)	N=14 (39

Table 3.5 shows that larger percentage of lineage I isolates had both the *bcr*ABC and *emr*C genes and a larger percentage of the lineage II isolates had no QAC resistance genes. As discussed earlier: lineage II isolates were more commonly isolated from FPEs than lineage I isolates (Schmitz-Esser *et al*., 2015). This suggests that lineage II isolates may have more unique adaptions that lineage I, which facilitates lineage II isolates enhanced survival within an FPE. Lineage I on the other hand does not seem to survive as well as

lineage II within an FPE which can be seen as a positive occurrence because lineage I isolates are of most concern towards human health. Table 3.4 illustrates that they have a higher percentage of resistance gene prevalence within them, within this study, which indicates that they are acquiring adaptive measures to survive within FPEs. This is worrying as they could further proliferate, contaminate product and ultimately harm consumers, thanks to these resistance genes. Further investigations should be carried out into the adaptability and survivability of *L. monocytogenes* lineage I within an FPE upon acquiring these resistance genes.

3.4.2 Resistance gene presence

Screening for the three resistance genes and one resistance cassette (*bcr*ABC, *emr*C, *emr*E and *qac*H) in this study was to determine whether or not genes that have been associated with isolates from clinical origin in the Netherlands, Canada and The United States of America, are in fact present in South African FPEs. These genes encode for QAC-resistance as has been reported internationally are, based on results from this study, prolific in South African FPE (Korsak & Szuplewska, 2016; Mereghetti *et al.,* 2000; Møretrø *et al.,* 2017; Mullapudi *et al.,* 2008). The *emr*C gene increase virulence through an unknown mechanism in zebra fish larvae tests (Kropac *et al*., 2019). This further enhances the concern regarding the spread of sanitizer resistance genes throughout an FPE. Moreover, QAC resistance genes have been suggested to confer an increase in resistance towards certain classes of antibiotics (Kropac *et al.*, 2019; Guérin *et al.*, 2021). This finding that QAC resistance genes may have further implications with regards to human infections and the treatment thereof as an increased resistance towards ciprofloxacin was prevalent Guérin *et al.* (2021). Keet & Rip (2021) found that South African *L. monocytogenes* isolates from the Western Cape Province differed in antibiotic resistant trends when compared to global resistance patterns. It would be interesting to investigate this further when looking at a correlation between QAC (and other commercial sanitizer) resistance and antibiotic resistance in future studies.

From the results in Table 3.3, two of the four resistance genes were detected in the samples evaluated in this study, namely the *bcr*ABC and *emr*C resistance genes which encode for QAC resistance mechanisms. The presence of resistance genes amongst *L. monocytogenes* isolates is worrying, not only because they can encode for QAC resistance (Kropac *et al.,* 2019 and Muller *et al.*, 2014) but also because they can be spread from resistant strains to susceptible strains with ease via mobile genetic elements. Examples of these mobile genetic elements are transposons or plasmids which are shared via a process known as horizontal gene transfer (HGT) from a resistant bacterium to a susceptible bacterium. This leads to more bacteria (*L. monocytogenes* in this case) becoming resistant to, and surviving, the application of the terminal disinfectant in the case of factory cleaning (Kovacevic, *et al*. 2016). Cases of HGT for QACs in *L. monocytogenes* have only been observed in laboratory conditions for the *bcr*ABC resistance gene (Korsak, *et al.*, 2019; Katharios-Lanwermeyer *et al*., 2012) but highlights that this process could be taking place in a

factory. Moreover Chmielowska *et al.* (2021) found that multiple resistance genes (*bcr*ABC and *emr*C) have been found within the same isolate of *L. monocytogenes* in FPEs in Poland. Similar results can be seen in Table 3.3.

The four QAC-resistance genes that were chosen for this study are located on different types of mobile genetic elements. The *qac*H resistance gene is encoded by a transposon known as TN6188 (Müller *et al.*, 2013), whilst the *bcr*ABC resistance cassette is found on a transposon found within a plasmid (Elhanafi, *et al.*, 2010). The *emr*C resistance gene is found on a plasmid known as pLMST6 and *emr*E gene is found within a genomic island (Kovacevic, *et al.*, 2016).

Interestingly, work carried out in Polish FPEs by Chmielowska *et al.* (2021) found that 91% of their *L. monocytogenes* sample set had the *qac*H gene, whilst only 4% had the *emr*C gene and only 7 out of the 287 samples had the *bcr*ABC cassette. Conversely, results from this study showed a high percentage of both the *bcr*ABC (68%) and *emr*C (62%) genes across the sample set.

As is evident from the *L. monocytogenes* isolates representing the 50 factory strains, 34 (68%) tested positive for the *bcr*ABC resistance gene (Table 3.3), 31 (62%) of the isolates tested positive for the *emr*C resistance gene and 24% of the isolates had both the *bcr*ABC and *emr*C genes. Nine isolates (18%) contained neither the *bcr*ABC nor *emr*C (Table 3.3). The presence of a gene encoding for resistance to a QAC is simply not enough to determine whether resistance will be expressed or not. Thus, tests for phenotypic expression of resistance should be conducted before conclusions are made regarding QAC-resistance.

There are other genes that also encode for resistance to QACs and the absence of any of the resistance genes (mentioned above and tested for in this study) does not necessarily mean that the isolate is susceptible to a QAC-based sanitizer. Moreover, the possibility remains that an isolate has become so de-sensitised to QACs in its environment (due to possible inappropriate use) and that it has become accustomed to taking in QACs alongside their nutrients from the environment. Sublethal concentrations of any antimicrobial may facilitate the spread or rise of resistance mechanisms or factors which, if kept unchecked would have drastic consequences in terms of populating a factory, and in turn for human health.

The sanitizer resistance genes referred to in this study encode for efflux pumps which are transferable, active exclusion mechanisms of which two families have been associated with *L. monocytogenes* (Putman, 2010). The two families are the Major Facilitator Superfamily (MFS) and Small Multidrug Resistant family (SMR). The *bcr*ABC resistance gene belongs to the MFS family, whilst *emr*C, *emr*E and *qac*H belong to the SMR family. All the above-mentioned efflux pumps are found on mobile genetic

elements which means that they can be spread to other *L. monocytogenes* strains that lack them, should they encounter each other- such as in a biofilm.

The *bcr*ABC cassette was first isolated on the pLM80 plasmid and putative composite transposon, meaning that it can be spread via a process known as horizontal gene transfer (HGT) through a biofilm, whilst *emr*C is spread via the pLMST6 plasmid which was found to confer BAC resistance in ST-6 strains in cases of listerial meningitis (Kropac *et al.,* 2019).

The EmrC efflux pump, that *emr*C encodes for, was found in 1.6 % of the *L. monocytogenes* isolates in a study by Kropac *et al.* (2019) from human and food isolates across Switzerland and Finland (N=439) and in 4.1% in a study by Chmielowska, *et al.* (2021) from a Polish fish factory (N=287). Results from this South African study show 62% of the isolates carry this gene (Table 3.3). A study by Korsak *et al.* (2019) found 95.5% of *L. monocytogenes* isolates in their study carried the *bcr*ABC resistance gene versus a study by Müller *et al.* (2013), reporting 5.5% of isolates carrying this gene. This South African study reported a *bcr*ABC gene prevalence of 68% and *emr*C resistance gene prevalence of 62%. This shows an alarmingly high prevalence of QAC sanitizer resistance genes amongst these *L. monocytogenes* (N=50) isolates from six different South African FPEs.

Moreover, work by Chmielowaka, *et al.* (2021) found that *L. monocytogenes* isolates from Polish fish factories contained both the *emr*C and *qac*H genes: both encoding for resistance towards QACs. This suggests that these resistance genes may be having a synergistic effect against the QAC treatments, especially in terms of later-generation QAC formulations (or “cocktails”) that are becoming more popular in industry in an attempt to find a cost-effective solution towards this resistance. Another possibility suggests that only one or both of the genes could have been acquired at some point and are now forming a part of the *L. monocytogenes* core genome.

Another possibility remains that both these detected genes are not in fact being transcribed but that other, not screened for, QAC-resistance genes could be coding for resistance and survival (Elhanafi *et al.*, 2010; Müller *et al.*, 2013; Xu *et al*., 2014). Thus, without WGS, one could not, with certainty, list all the resistance genes that these isolates have. Moreover, testing for phenotypic expression of QAC resistance needs to be carried out to determine if these isolates are indeed resistant to QACs by other methods or genes.

3.4.3 Phenotypic expression of sanitizer resistance

Based on plating results, an OD600 value of 0.260 (10^8 cfu) (Yap & Trau, 2019) was chosen to best represent a totally soiled surface within a food factory. This completely contaminated surface combined with

the application of a sanitizer at its lowest recommended concentration would give ample-opportunity for gene encoded sanitizer resistance mechanisms to be expressed and thus give a good overall indication on the prevalence of QAC-sanitizer resistance amongst *L. monocytogenes* isolates from South African Food Processing Plants.

3.4.4 Interpretation of diffusion disk zones and examples of resistant isolates

Diffusion disks, as their name suggests, diffuse compounds such as antimicrobial agents (or in this case sanitizers) into the agar medium which causes a ring of diffusion into the lawn of bacterial cells. Should there be growth within the zone of diffusion or should the zone of diffusion be smaller than a known reference (such as tables for antibiotics, made available by EUCAST or Clinical and Laboratory Standards Institute (CLSI)) then the isolate can be classified as resistant.

Zones of diffusion were measured for each of the sanitizers (in triplicate) and their diffusion zone diameters were compared to the averages of the results between the two *Listeria* ATCC reference strains (ATCC 7644 and ATCC 33090): which have no resistance genes towards QAC-based sanitizers. However, if growth was observed within the zone of diffusion (as seen in Figure 3.2) then that isolate was classified as resistant towards that particular sanitizer. It must be noted that these results were consistent throughout each of their repeat sampling (i.e. they were either consistently under the "breakpoint" diffusion zone size, compared to the ATCC reference strains, or consistently all over the zone size or all had growth within the zones). Figure 3.3 and Figure 3.4 give a good indication of different diffusion zone sizes as observed for two different isolates. It is important to note that no inhibition was noted on any of the control plates, as indicated in Figure 3.1, which shows the placement of disks. This means that any diffusion was occurring as a result of the sanitizer's antimicrobial activity.

The results of this section of the study are illustrated in Table 3.4 below. A (+) sign indicates resistance to a sanitizer and (-) indicates susceptibility to a particular sanitizer

Figure 3.2 Growth of *L. monocytogenes* isolate, 17JBX13, within zone of diffusion.

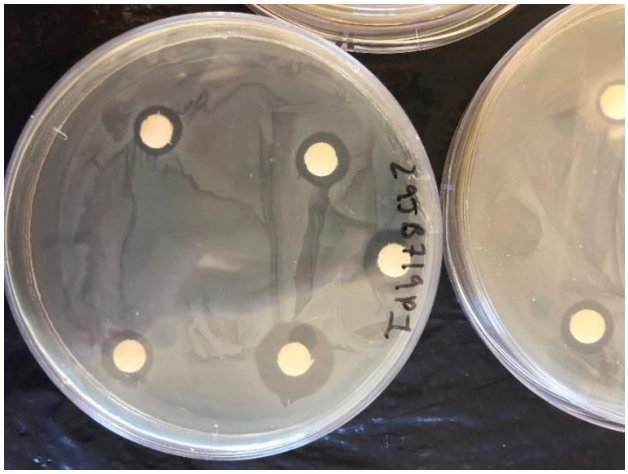


Figure 3.3 Showing different zones of diffusion for different sanitizers for isolate 29JB719.

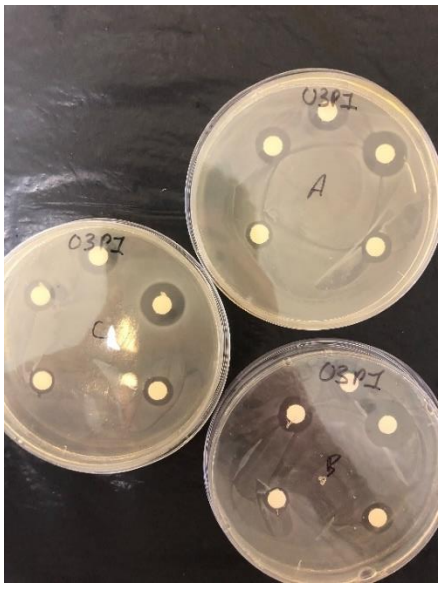


Figure 3.4 Showing consistency between diffusion zones across a single isolate.

Table 3.6: Table illustrating phenotypic resistance of *L. monocytogenes* isolated in South African food factories towards sanitizers at 1% concentration and 3% L-Lactic acid

Strain Code	Isolation	QC50[1]	QC80[2]	TCQ[3]	BYOTROL[4]	QFS[5]	L-LACT	SH-12	AN8	AF8
YB	LR drain	+	+	-	-	-	+	+	+	+
YD	Trolley washer Environment	-	+	-	-	-	+	+	+	+
17JBX12	Drain	+	+	+	+	-	+	+	+	+
17JBX11	Drain	+	+	+	-	-	+	+	+	+
17J86	LR floor Environment	+	+	+	+	-	+	+	+	+
17J41	Drain next to mincer	+	+	+	+	-	+	+	+	+
17J62	Passage drain	+	+	+	+	-	+	+	+	+
A	Brine auto injector	-	+	+	+	-	+	+	+	+
B	Brine auto injector	-	+	+	+	-	+	+	+	+
CIRCLE 32	Trolley (underneath) Environment	+	+	+	+	-	+	+	+	+
29JB721	Mixing bowl	+	+	+	+	-	+	+	+	+
YF	Drain	+	+	+	+	-	+	+	+	+
17JBX36	Vienna filler	+	+	+	+	+	+	+	+	+
17JBX42	Drain	+	+	+	+	+	+	+	-	+
17JBX54	Vienna filler	+	+	+	+	+	+	+	+	+
17JBX13	Vienna hopper	+	+	+	+	-	+	+	+	+
17JBX41	Drain	+	+	+	+	-	+	+	+	+
17JBX47	Drain	+	+	+	+	-	+	+	+	+
17JBX9	Drain	+	+	+	+	+	+	+	+	+
17JBX2	Drain	+	+	+	+	-	+	+	+	+
CIRCLE 22	Drain	+	+	+	+	+	+	+	+	+
CIRCLE 17	Drain next to mixer	+	+	+	+	-	+	+	+	+
CIRCLE T14	Drain	+	+	+	+	+	+	+	+	+
C	Brine auto injector	+	+	+	-	-	-	-	-	-
17JBX53	Drain	+	+	+	+	-	+	+	+	+
17JBX44	Environment	-	+	-	+	-	+	+	+	+
17JBX46	Vienna Drain	+	+	+	+	+	+	+	+	+

17JBX32	Table (food contact surface)	+	+	+	+	+	+	+	+	+
17JBX33	Drain next to conveyor belt	+	+	+	+	-	+	+	+	+
YA	Drain	+	+	+	+	+	+	+	+	+
CIRCLE 3	Drain	+	+	+	+	+	+	+	+	+
17JBX9	Drain	+	+	+	+	-	+	+	+	+
17JBX2	Drain next to ice machine	+	+	+	+	-	+	+	+	+
17J86	Floor	+	+	+	+	+	+	+	+	+
E23*	Vienna filler	+	+	+	+	-	+	+	+	+
E20*	Vienna filler	+	+	+	+	-	+	+	+	+
17J87	Drain next to waste are	+	+	+	+	-	+	+	+	+
29JB719	Floor scale	+	+	+	+	-	+	+	+	+
E13	Drain	+	+	+	+	-	+	+	+	+
17J64	Polony pump	+	+	+	+	+	+	+	+	+
E1	Air conditioner	+	+	-	+	+	+	+	+	+
E7*	Vienna filler	+	+	+	+	-	+	+	+	+
17JBX31*	Drain next to water tank	+	+	+	+	-	+	+	+	+
39*	Chopping board	-	-	-	+	+	+	+	-	+
43	High Risk Drain	+	+	+	+	+	+	+	+	+
44	High Risk Drain	+	+	+	+	+	+	+	+	+
45	Plastic lug	-	+	+	+	+	+	+	+	+
46*	Plastic lug	+	+	+	+	+	+	+	+	+
47*	High Risk Drain	+	+	+	+	+	+	+	+	+
48*	High Risk Drain	+	+	+	+	-	+	+	+	+
ATCC7644	*L. monocytogenes* control	-	-	-	-	-	+	+	+	+
ATCC33090	*L. innocua* control	-	-	-	-	-	+	+	+	+

*isolates (N=8) that did not contain both the *bcr*ABC resistance cassette or the *emr*C resistance gene. [1]First generation QAC; [2]A QAC cocktail containing a combination of a first and a fourth generation QAC,[3] A fourth generation QAC , [4]A QAC cocktail (by Byotrol), [5]Novel QAC free sanitizer from Byotrol

L. monocytogenes isolates from different factory environments differed in their phenotypic responses to different generations of QAC sanitizers (Table 3.4). The differences between QAC generations can be explained by the following table (Table 3.5).

Table 3.7: Evolution of quaternary ammonium disinfectants from Gerba, 2015

Generation	Compound(s)
First	Benzalkonium, alkyl chains, C12 to C18
Second	Aromatic rings with hydrogen and chlorine, methyl and ethyl groups
Third	Dual QACs; mixture of alkyl dimethyl benzyl ammonium chloride (lower toxicity)
Fourth	Dialkylmethyl aminos with twin chains
Fifth	Synergistic combinations of dual QACs
Sixth	Polymeric QACs
Seventh	Bis-QACs with polymeric QACs

For this study only a first generation QAC (BAC), a fourth generation QAC (Dialkylmethyl aminos with two chains) and a QAC cocktail containing a first and a fourth generation QAC (Aromatic rings and Dialkylmethly aminos with two chains) were used. From the genotypic results in Table 3.3 it is evident that genes conferring resistance to QAC sanitizers are present in the *L. monocytogenes* isolates. Strains of *L. monocytogenes* harbouring resistance genes that encode for resistance to QAC-based sanitizers have been responsible for numerous global outbreaks of listeriosis (Møretrø *et al.,* 2017; Korsak & Szuplewska, 2016; Jiang, *et al.,* 2016; Xu *et al.,* 2016; Dutta *et al.,* 2013; Ratani *et al.,* 2012; Elhanafi, *et al.,* 2010; Mullapudi *et al.,* 2008; Mereghetti *et al.,* 2000). This suggests that *L. monocytogenes* isolates that can survive the application of a terminal disinfectant (sanitizer) are able to proliferate and contaminate final food product which may in turn cause disease.

L. monocytogenes can form biofilms and adhere to stainless steel surfaces within a food factory. Bacterial cells in a biofilm or sessile state show an enhanced resistance to external stresses such as desiccation, pH extremes and the application of antimicrobial agents such as antibiotics and sanitizers. Biofilms can be single-specie or single isolate (same strain) biofilms, or mixed strains or species as is commonly reported in the food industry. Bacterial cells within biofilms are exposed to sublethal concentrations of antimicrobials such as sanitizers (due to the protective properties of the extra polymeric substance (EPS) layer), which leads to the development or selection of resistance

mechanisms. Biofilms facilitate the spread of resistance determinants like genes and plasmids through HGT. This means that biofilms are a key cause for the spread of sanitizer resistance throughout a factory and resistance determinants gained from a biofilm can spread to the rest of the factory via the natural shedding of cells, as part of the biofilm growth. This means that biofilms need to be properly managed and controlled within food factories.

QAC-based sanitizers used in this study were comprised of a first generation QAC, a fourth generation QAC and a sanitizer consisting of a combination of a first and a fourth generation QAC. A foaming and a non-foaming peracetic acid were also tested in addition to a 3% concentration of L-Lactic acid, a sodium hypochlorite-based sanitizer and two sanitizers from a company called Byotrol which have a unique formulation (a QAC-based sanitizer and a non-QAC based sanitizer).

3.4.5 Differences in expression of resistance to QACs amongst strains possessing the same resistance gene composition

When looking at the phenotypic resistance expression, differences were noted in expression of resistance towards different generations of QACs, despite the same resistance genes being present. This may indicate that other mechanisms of resistance are at play such as other resistance genes or perhaps the isolates from environmental sources are adapting to nutrient uptake in the presence of the various sanitizers. It was still clear that QFS (Byotrol, UK), which is a novel QAC-free sanitizer proved to be the most consistently effective against isolates from environmental and drain origin. Resistance genes can be found on either plasmids or transposons and most plasmids encode for mechanisms that increase a bacteria's response to an environmental stress (favourable attribute) such as multidrug resistance, osmotic stress tolerance, heat shock proteins or transcriptional regulation adaptations towards cold adaption and cold growth (Korsak, *et al.,* 2019; Schmitz-Esser *et al.,* 2015; Kuenne *et al.,* 2010). Transposons play a role in dissemination of foreign DNA of adaptive value to different isolates such as resistance to heavy metals and sanitizers (Korsak, *et al.,* 2019). Inter-specie spread of resistance determinants has also been well documented and reported such as amongst different species of *Listeria*. To better understand why certain strains responded differently to the same sanitizers, despite having the same resistance gene composition (*bcr*ABC, *emr*C, *emr*E and *qac*H), one must understand a bit more about the genes themselves and what they encode for and how the efflux mechanisms differ.

Based on the findings of previous studies on the inefficiencies of BAC- based QAC sanitizers, industry role players have begun to develop combinations of different generations of QACs as

"cleaning cocktails" which provide an affordable solution to the resistance whilst still using QAC technology. Two different QAC cocktails, with differing QAC makeups, used in this study were a combination of a fourth and first generation QAC (QC80) and QAC-based Byotrol. From the results in Table 3.4, cocktails (QC80 and Byotrol) had success in eliminating otherwise QAC resistant isolates e.g. strain code E1, YB, YD, C, and 17JBX44, despite the presence of sanitizer resistance genes. This may be due to other mechanisms such as a strains unique susceptibility or resistance towards components within a cocktail resulting in these differences. They were also successful in eliminating isolate 39 which did not have both the *bcr*ABC cassette and *emr*C gene. Despite a widespread presence of the *bcr*ABC resistance cassette: some isolates still showed susceptibility towards BAC (QC50), for which the *bcr*ABC cassette has been shown to encode resistance. This may be due to isolates having different tolerances towards BAC due to prolonged exposure and the niche that they find themselves in such as different food contact surfaces (strain code-A, B, 39 and 45) (Table 3.4) and different environmental points within a factory (YD and 17JBX44). These different locations may have been subjected to slightly different exposures within a factory than other environmental isolates based on the factory design or cleaning pressures: thus, not being subjected to sublethal concentrations and remaining susceptible towards the applied sanitizer. The MICs of the sanitizers towards individual isolates may differ across an FPE, thus an increased concentration of application could be suggested to counteract this increased tolerance, which would explain this difference.

3.4.6 The future of QACs may be in cocktail combinations

Industry has begun to combine various generations of QACs into mixtures that they colloquially call "cocktails" such as QC80 (Ecowize, RSA) and Byotrol (Byotrol, GBR) which is a way in which they attempt to eliminate QAC-resistant bacteria in a cost-effective manner.

Outputs from this study, Table 3.4, have shown that cocktails do work and that they do pose a solution to the problem with QAC resistance in *L. monocytogenes* as seen in isolates- strain code- YB, YD, 17JBX11, C, 17JBX44 and E1. There are differences between an isolate's responses to the two different cocktails used which shows that their responses are not universal. This highlights weaknesses in resistance that can be carefully exploited using QAC cocktails.

3.4.7 Byotrol QFS provides an effective option against QAC-resistant isolates

The QFS (Byotrol, GBR) sanitizer showed the highest efficacy of any of the sanitizers used in this chapter with 62% of the isolates showing susceptibility to the sanitizer. This shows that QFS

(Byotrol, GBR), a QAC free sanitizer, may be a key component in the fight against QAC resistant isolates.

3.4.8 Unsatisfactory results from peracetic acid, L-lactic acid, and Sodium Hypochlorite

Unfortunately, due to the reactivities of the peracetic acid, L-lactic acid and sodium hypochlorite solutions, their results gave the impression of not being effective, despite their efficacies being well documented in literature. By the time the bacterial lawn had begun to grow and was interpreted: the sanitizer solutions had dissipated. This means that their results as shown in Table 3.4 should be disregarded. What this does show is the importance of applying sanitizers at the correct time, immediately after cleaning to eradicate any surviving cells effectively. More research should be carried out on their efficacies of these acid-based sanitizers, particularly on their real-time effects in the CO_2 evolution measurement system (CEMS) (Ackerman, 2017).

3.4.9 More needs to be done

Despite QFS (Byotrol, GBR) being the most effective sanitizer against the *L. monocytogenes* isolates, some isolates (38%) in this study showed resistance to QFS (Byotrol, GBR). To the author's knowledge, this has not been reported previously and warrants further investigation. Moreover, this observation shows that non-chemical means of control such as a biological agent (bacteriophages) could be advantageous in controlling these sanitizer resistant isolates. Studies have been carried out on the use of these agents in food processing environments and they indicate that they can be successful (Reinhard *et al.,* 2020a; Reinhard *et al.,* 2020b; Gutiérrez *et al.*, 2016).

3.5 Conclusion

This research shows that *L. monocytogenes* sanitizer resistance trends mediated by resistance genes, in the South African setting, differ from what is seen internationally. This study also differentiated between the efficacies of commercially used QAC-based sanitizers (in terms of generation) and investigated other commercially used options. Many QAC-sanitizer research papers focus solely on BAC, and its ineffectiveness has subsequently been widely reported. To re-iterate, QFS a novel QAC free sanitizer, showed much promise for controlling isolates that were resistant to a large array of different QAC-based sanitizers.

It is important to note that the inoculation loads used do not represent loads that would be appearing on factory equipment to which the sanitizer would be applied. The actual loads would be significantly

lower due to the application of previous cleaning steps as described in chapter 2. These loads were used to recreate a “worst-case” scenario and try and facilitate the expression of resistance.

A total of 68% and 62% of isolates carried the *bcr*ABC and *emr*C genes respectively. Both lineage I and II isolates of *L. monocytogenes* were shown to carry either the *bcr*ABC gene, *emr*C gene or both of the genes. Interestingly, some isolates that held neither the *bcr*ABC or *emr*C resistance genes still expressed resistance towards different QAC-based sanitizers. This warrants further investigation as different QAC resistance genes, that were not screened for, may be present. A higher prevalence of lineage I isolates (64%) had both the *emr*C and *bcr*ABC genes. Isolates from lineage I are most often associated with human disease which further highlights the concern regarding their enhanced survivability of cleaning and QAC application within an FPE.

Without a synergistic relationship between food producer and cleaning provider, the problem of sanitizer resistance will continue to arise which will further compromise food safety going forward. Studies into the efficacies of new QAC-based cocktails should be conducted where trade-offs between costs and usability of different non-QAC options should be investigated such as PAA. Moreover, bacteriophages should be further investigated as an option to overcome sanitizer resistance within factories and their use, in combination with a sanitizer treatment regime should also be further investigated to find a balance between fiscal requirements and efficacy.

3.6 References

Abdollahzadeh, E., Ojagh, S.M., Hosseini, H., Irajian, G., Ghaemi, E.A. (2016). Prevalence and molecular characterization of *Listeria* spp. and *Listeria monocytogenes* isolated from fish, shrimp, and cooked ready-to-eat (RTE) aquatic products in Iran. *LWT- Food Science and Technology*. **73**. 205–211.

Ackerman, E. (2017). Survival and proliferation of *Listeria monocytogenes* in a South African ready-to-eat factory. MSc Thesis. Stellenbosch University, South Africa.

Andrews, J.M. (2005). BSAC standardized disc susceptibility testing method (version 4). *Journal of Antimicrobial Chemotherapy*. **56**. 60-76.

Anonymous. (2017). *ISO 11290-1:2017 Microbiology of the food chain- Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. – Part 1: Detection method.* [Internet document] URL https://www.iso.org/standard/60313.html.

Blais, B. W., & Phillippe, L.M. (1993) A simple RNA probe system for analysis of Listeria monocytogenes polymerase chain reaction products. *Applied Environmental Microbiology*. 59:2795–2800.

Chmielowska, C., Korsak, D., Szuplewska, M., Grzelecka, M., Maćkiw, E., Macion, A., Skowron, K., Bartosik, D. (2021). Benzalkonium chloride resistance and heavy metal resistance profiles of *Listeria monocytogenes* strains isolated from fish, fish products and food processing factories in Poland. *Food Microbiology*. **98**. 103756.

Costa, A., Lourenco, A., Civera, T., Brito, L. (2018). *Listeria innocua* and *Listeria monocytogenes* strains from dairy plants behave similarly in biofilm sanitizer testing. *LWT-Food Science and Technology*,**92**,477-483.

Dutta, V., Elhanafi, D., Kathariou, S. (2013). Conservation and distribution of the benzalkonium chloride resistance cassette bcrABC in *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **79** (19). 6067-6074.

Ebner, R., Stephan, R., Althaus, D., Brisse, S., Maury, M., Tasara, T. (2015). Phenotypic and genotypic characteristics of *Listeria monocytogenes* strains isolated during 2011-2014 from different food matrices in Switzerland. *Food Control*. **57**. 321–326.

Elhanafi, D., Dutta, V., Kathariou, S. (2010). Genetic characterization of plasmid-associated benzalkonium chloride resistance determinants in a *Listeria monocytogenes* strain from the 1998-1999 outbreak. *Applied and Environmental Microbiology*. 8231-8238.

Gerba, C.P. (2015). Quaternary Ammonium Biocides: Efficacy in Application. *Applied and Environmental Microbiology*. 81(**2**):464-469.

Gianfranceschi, M.V., D'Ottavio, M.C., Gattuso, A., Bella, A., Aureli, P. (2009). Distribution of serotypes and pulsotypes of *Listeria monocytogenes* from human, food and environmental isolates (Italy 2002-2005). *Food Microbiology.* **26**. 520–526.

Gorman, R., Adley, C.C. (2004). An evaluation of five preservation techniques and conventional freezing temperatures of -20 °C and -85 °C for long term preservation of *Campylobacter jejuni*. *Letters in Applied Microbiology*. **38**(4). 306-310.

Guérin, A., Bridier, A., Le Grandois, P., Sévellec, Y., Palma, F., Félix, B., LISTADAPT Study Group., Roussel, S., Soumet, C. (2021). Exposure to quaternary ammonium compounds selects resistance to ciprofloxacin in listeria monocytogenes. *Pathogens*. ***10***(2). 220

Gutiérrez, D., Rodríguez-Rubio, L., Martínez, B., Rodríguez, A., García, P. (2016). Bacteriophages as a weapon against bacterial biofilms in the food industry. *Frontiers in Microbiology*. **7**. 1-15.

Henderson, I.R., Owen, P., Nataro, J.P. (1999). Molecular switches- the on and off of bacterial phase variation. *Molecular Microbiology*. **33**(5). 919-932.

Jiang, X., Yu, T., Liang, Y., Ji, S., Guo, X., Ma, J., Zhao, L. (2016). Efflux pump-mediated benzalkonium chloride resistance in *Listeria monocytogenes* isolated from retail food. *International Journal of Food Microbiology*. **217**. 141-145.

Jones, G.S. & D'Orazio, S.E.F. (2014). *Listeria monocytogenes*: cultivation and laboratory maintenance. *Current Protocols in Microbiology*. **31**. 1-9.

Katharios-Lanwermeyer, S., Rakic-Martinez, M., Elhanafi, D., Ratani, S., Tiedje, J.M., Kathariou, S. (2012). Co-selection of cadmium and benzalkonium chloride resistance in conjugative transfers from non-pathogenic *Listeria* spp. to other *Listeriae*. *Applied Environmental Microbiology*. 78. 7549–7556.

Keet, R., & Rip, D. (2021). Listeria monocytogenes isolates from Western Cape, South Africa exhibit resistance to multiple antibiotics and contradicts certain global resistance patterns. *AIMS Microbiology*, ***7***(1), 40.

Khalid, N.I., Saulaiman, U.S., Nasiruddin, N.A., Hatdran, M.M., Ab Aziz, N., Nor Khaizura, M.A.R., Hasna, N.Z.N., Taip, F.S., Sobri, S. (2019). Integrating cleaning studies with industrial practice: case study of an effective cleaning program for a frozen meat patties SME factory. *Journal of Cleaner Protection*. **235**. 688-700.

Koopmans, M.M., Bijlsma, M.W., Brouwer, M.C., van de Beek, D., van der Ende, A. (2017). *Listeria monocytogenes* meningitis in the Netherlands,1985-2014: a nationwide surveillance study. *Journal of Infection*. **75**. 12-19.

Korsak, D. & Szuplewska, M. (2016). Characterization of non-pathogenic Listeria species isolated from food and food processing environment. *International Journal of Food Microbiology*. 238: 274-280.

Kovacevic, J., Ziegler, J. Walecka-Zacharska, E., Reimer, A., Kitts, D.D. Gilmour, M.W. (2016). Tolerance of *Listeria monocytogenes* to Quaternary Ammonium Sanitizers is mediated by a novel efflux pump encoded by emrE. *Applied and Environmental Microbiology*. 82(**3**): 939-953.

Kramarenko, T., Roasto, M., Meremäe, K., Kuningas, M., Põltsama, P., Elias, T., 2013. *Listeria monocytogene*s prevalence and serotype diversity in various foods. *Food Control*. 30. 24–29.

Kropac, A.C., Eshwar, A.K., Stephan, R., Tasara, T. (2019). New insights on the role of the pLMST6 plasmid in *Listeria monocytogenes* biocide tolerance and virulence. *Frontiers in Microbiology*. **10**. 1-16.

Leong, D., NicAogain, K., Luque-Sastre, L., McManamon, O., Hunt, K., Alvarez-Ordonez, A., Scollard, J., Schalenberger, A., Fanning, S., O'Byrne, C., Jordan, K. (2017). A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food business in Ireland. *International Journal of Food Microbiology*. **249**. 18-26.

Leu, M.C., Meng, P., Geskin, E.S., Tismeneskiy, L. (1998). Mathematical modelling and experimental verification of stationary waterjet cleaning process. *Journal of Manufacturing Science and Engineering*. 120(**3**). 571-579.

Martín, B., Perich, A., Gómez, D., Yangüela, J., Rodríguez, A., Garriga, M., Aymerich, T. (2014). Diversity and distribution of *Listeria monocytogenes* in meat processing plants. *Food Microbiology*. **44**. 119-127.

Mereghetti, L., Quentin, R., Marquet-van der Mee, N., Audurier, A. (2000). Low sensitivity of *Listeria monocytogenes* to Quaternary Ammonium Compounds. *Applied and Environmental Microbiology*. 66(11). 5083-5086.

Møretrø, T., Schirmer, B.C.T., Heir, E., Fagerlund, A., Hjemli, P., Langsrud, S. (2017). Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. *International Journal of Food Microbiology*, **241**, 215-224.

Mullapudi S, Siletzky RM, Kathariou S. 2010. Heavy-metal and benzalkonium chloride resistance of *Listeria monocytogenes* isolates from the environment of turkey-processing plants. *Applied Environmental Microbiology*. 74:1464–1468.

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stressl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S. (2013). Tn6188- A Novel Transposon in *Listeria monocytogenes* Responsible for tolerance to Benzalkonium Chloride. *PLOS ONE*. **8**(10). 76835.

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stressl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S. (2014). The *Listeria monocytogenes* tansponson Tn6188 provides increased tolderance to various quaternary ammonium compounds and ethidium bromide. *FEMS Microbiology Research Letters*. **361**(2). 166-173.

O'Connor, L., O'leary, M., Leonard, N., Godinho, M., O'Reilly, C., Egan, J., & O'Mahony, R. (2010). The characterization of Listeria spp. isolated from food products and the food-processing environment. *Letters in Applied Microbiology*, **51**(5), 490-498.

Osri, R.H., den Bakker, H.C., Wiedmann, M. (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology*. 301(**2**). 79-96.

Pan, Y., Breidt, F., Kathariou, S. (2009). Competition of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed-culture biofilms. *Applied and Environmental Microbiology*. **75**(18). 5846-5852.

Putman, M., van Veen, H.W., Konings, W.N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiology and Molecular Biology Reviews*. 672-693.

Ratani, S.S., Siletzsky, R.M., Dutta, V., Yildirim, S., Osborne, J.A., Lin, W., Hitchens, A.D., Ward, T.J., Kathariou, S. (2012). Heavy metal and disinfectant resistance of *Listeria monocytogenes* from foods and food processing plants. *Applied Environmental Microbiology*. **78**(19). 6938-6945.

Reinhard, R.G., Kalinowski, R.M., Bodnaruk, P.W., Eifert, J.D., Boyer, R.R., Duncan, S.E., Bailey, R.H. (2020a). Fate of *Listeria* on various food contact and noncontact surfaces when treated with bacteriophage. *Journal of Food Safety*. **40**(3). 1-6.

Reinhard, R.G., Kalinowski, R.M., Bodnaruk, P.W., Eifert, J.D., Boyer, R.R., Duncan, S.E., Bailey, R.H. (2020b). Practical application of bacteriophage in food manufacturing facilities for the control of *Listeria sp. Journal of Food Safety*. e12871.

Rip, D., & Gouws, P. A. (2009). Development of an internal amplification control using multiplex PCR for the detection of Listeria monocytogenes in food products. *Food Analytical Methods*. **2**(3). 190-196.

Schmitz-Esser, S., Müller, A., Stressl, B., Wagner, M. (2015) Genome sequence type 121 *Listeria monocytogenes* strains harbor highly conserved plasmids and prophages. *Frontiers in Microbiology*. **6**(360).

Tsai, Y-H. L., Maron, S.B., McGann, P., Nightingale, K.K., Wiedmann, M., Osri, R.H. (2011). Recombination and positive selection contributed to evolution of *Listeria monocytogenes* lineages III and IV, two distinct and well supported uncommon *L. monocytogenes* lineages. *Infectious Genetic Evolution*. **11**(8). 1881-1890.

Xu, D., Nie, Q., Wang, W., Shi, L., Yan, H. (2016). Characterization of a transferable bcrABC and cadAC genes-harbouring plasmid in *Listeria monocytogenes* strain isolated from food products of animal origin. *International Journal of Food Microbiology*. **217**. 117-122.

Yap, P.Y. & Trau, D. (2019). Direct *E. coli* cell count at OD600*. Trip Biosystems Application Note*. Singapore.

Chapter 4

Response of a Quaternary Ammonium Compound-resistant *Listeria monocytogenes* strain to commercially available sanitizers in the biofilm state

4.1 Summary

Resistance towards QAC-based sanitizers has been shown to be an emerging risk to food production and safety, with genes encoding for QAC-resistance (e.g. *bcr*ABC and *emr*C) isolated from clinical isolates of human origin. *L. monocytogenes* has the ability to form biofilms in various niches within a factory (conveyor belts to drains) and requires careful monitoring and effective cleaning practices to keep it under control and prevent proliferation throughout a factory and into a final product. In this study the responses of one lineage II *L. monocytogenes* isolate from a factory's drain (17JBX41), harbouring the *bcr*ABC and *emr*C genes was subjected to three separate, commercial, sanitizer treatments in the novel CO_2 Evolution Measurement System (CEMS). The CEMS system allowed for real-time monitoring of CO_2 output and elucidated findings regarding a benzalkonium chloride (BAC), Peracetic Acid (PAA), and QAC-free sanitizer called Byotrol QFS. The CEMS results showed that biofilm recovery was observed in the biofilm treated with BAC within 20 hours of treatment and the biofilms treated with PAA and Byotrol QFS showed no recovery within the 20 hour treatment. This study shows the potential that QAC-free sanitizers have in overcoming and controlling *L. monocytogenes* isolates which have become resistant to QAC-based sanitizers.

4.2 Introduction

Listeria monocytogenes is a ubiquitous, Gram-positive, rod shaped bacterium that causes the human diseases listeriosis. It can form biofilms and adhere to stainless within food factories (Keet & Rip, 2021; Poimenidou *et al.*, 2017).

Cells in the biofilm (sessile) state are between 10-1000 times more resistant to disinfectants, like sanitizers, than cells in the planktonic state (Mah, 2012). This can prove incredibly problematic for food producers, where the aim is to ensure safe and consistent quality food on a mass scale. Biofilms pose huge risks for product contamination due to the shedding/ release of planktonic cells from the biofilm (Poimenidou *et al.*, 2017).

Biofilms can also be problematic for the food industry as they contribute to cell survivability in the presence of stresses such as factory cleaning and the application of the terminal disinfectant (sanitizer). Biofilm formation takes place in 4 distinct steps: reversible adhesion, irreversible adhesion,

biofilm maturation, and cell to cell signalling (Kocot *et al*., 2017). Biofilms are surrounded by a polysaccharide rich (slimy) layer known as an extracellular polymeric substance (EPS) which is comprised of polysaccharides and other microbially derived substances (proteins, phospholipids, teichoic nucleic acids and extracellular DNA (eDNA) (Sharma *et al.*, 2019).

The EPS has been shown to provide a protective barrier around the sessile bacterial cells and plays a role in water retention (preventing dehydrations), antimicrobial/ sanitizer protection, sorption of organic or inorganic compounds (energy reservoir) and affecting enzymatic activity (Flemming & Wingender, 2010). Figure 4.1 gives a good illustration of the makeup of the EPS of a biofilm, the extracellular DNA (eDNA) is visible as double helixes in the image.

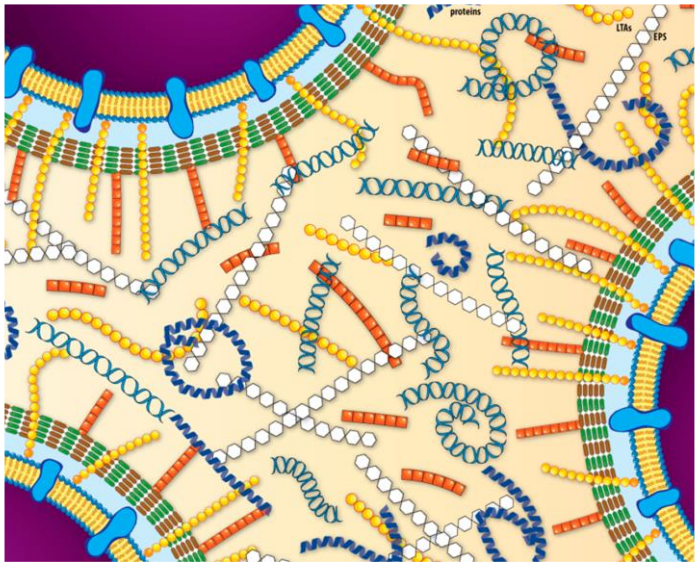


Figure 4.1 An illustration of the EPS of a biofilm with all major components being distributed heterogeneously (Adapted from Colagiorgi *et al*., 2016).

Understanding a biofilm's response to a sanitizer is of incredible importance as they play a role in the postproduction contamination of food. Critical Control Points (CCPs) are steps that are in place in food factories to eliminate or reduce a food safety risk, for example cooking a product to a desired core temperature for a certain time; should contamination after such a step occur, the results would prove disastrous (Sharma & Anaand, 2002). Thus, a good understanding of the risks of biofilm-sanitizer-survivability is of the utmost importance.

Literature highlights the importance of not becoming complacent during factory cleaning as the build-up of soil can facilitate biofilm formation and provide a protective layer on top of the EPS which will further inhibit disinfectant and sanitizer application (Colagiorgi *et al*., 2016).

The structure of *L. monocytogenes* biofilms differs based on the environmental conditions that they are exposed to within a drain such as flow conditions, or within the welds of food contact surfaces i.e. static conditions (Rieu *et al.*, 2008; Da Silva & De Martinis, 2013). Under static conditions, biofilms are found in heterogenous layers whilst under flow conditions they form cocci-like microcolonies in a knitted-chain like structure (Rieu *et al.*, 2008; Da Silva & De Martinis, 2013). Based on these structural difference's biofilms in flow conditions would respond differently to stresses than those in static conditions. Thus, a sound understanding of how responses to sanitizers differ between the different structures of biofilms would help elucidate better control measures which would prove beneficial to factory operations and facilitate safer food production.

4.2.1 Cells within the biofilm state communicate via a process known as quorum sensing

Quorum sensing plays a major role in the structure of a biofilm as individual bacterial cells communicate with each other about stresses that they are encountering, therefore allowing the other cells within the biofilm to respond accordingly (Sharma, *et al.*, 2019). Quorum sensing also plays a role in the upregulation of certain genes and complete disassembly of certain biofilms as well as total maturation of the biofilm (Solano *et al.*, 2014). The phenomenon of quorum sensing is a powerful adaptation of biofilms and is thus, more proof that controlling biofilms early on is the best weapon against preventing their establishment within a food processing environment.

4.2.2 Spread of resistance determinants

Horizontal gene transfer (HGT) is a process whereby mobile chromosomal elements known as transposons are exchanged between different bacterial strains (Madsen et al., 2012). The *bcr*ABC is an example of a resistance gene that is located and can be transferred to bacterial cells within a community (Madsen *et al*., 2012). Figure 4.2 gives an illustration of the process of HGT. This also takes place with the *emr*C, *emr*E and *qac*H genes which are located on plasmids and transferred via the same, HGT mechanism (Kropac *et al*., 2019; Korsak *et al*., 2019).

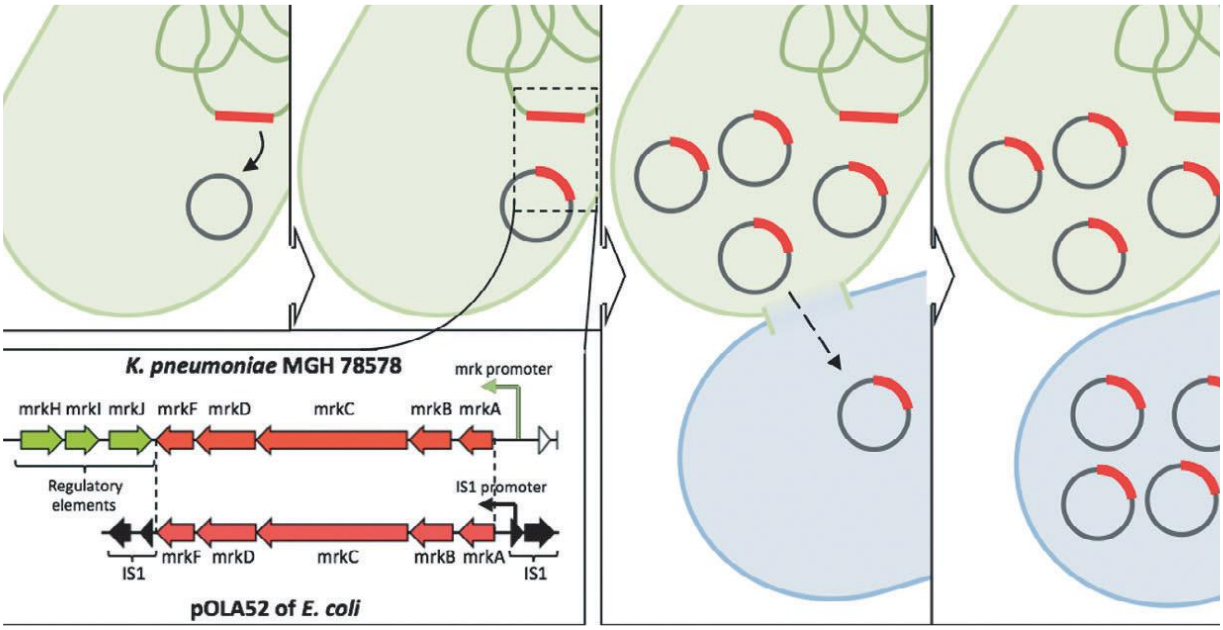


Figure 4.2 Illustration of the movement of a mobile genetic element from the chromosome of a resistant cell, into a plasmid and then into a susceptible cell that then acquires resistance (adapted from Madsen *et al*., 2012).

4.2.3 Mixed-species biofilms

Mixed species biofilms comprising of other bacterial species, living alongside *L. monocytogenes*, have been identified in food processing environments and are shown to be more resistant to antimicrobials than single-species or single-*L. monocytogenes* biofilms (Jahid & Ha, 2014). Furthermore, mixed species biofilms are shown to be dominant in factories and nature, however understanding a *L. monocytogenes*-specie's response can be advantageous to better control measures within food processing environments.

4.2.4 Biofilms and CO_2 production

Biofilms produce CO_2 as they grow and mature and this CO_2 can be measured to ascertain a mature biofilm's response to a stressor such as a sanitizer. Using a novel machine, the CEMS system, a QAC resistant *L. monocytogenes* strain (Chapter 3-17JBX44, drain origin, lineage II) was used to highlight responses to benzalkonium chloride, Byotrol QFS and non-foaming peracetic acid. With this information it is hoped to highlight resistance occurring in both the sessile and planktonic state and show which QAC- alternatives hold the most promise for controlling QAC- resistant *L. monocytogenes*.

4.3 Materials and methods

4.3.1 The CEMS system

The CEMS system was setup for isolate inoculation following a method described by Kroukamp & Wolfaardt (2009) and was disinfected prior to use according to Loots (2016) as seen in Figure 4.3. The following figure describes the experimental conditions for each CEMS line. The flow rate is faster than the doubling time of *L. monocytogenes* and thus only sessile responses to the sanitizer applications were recorded which eliminates any interference from planktonic responses.

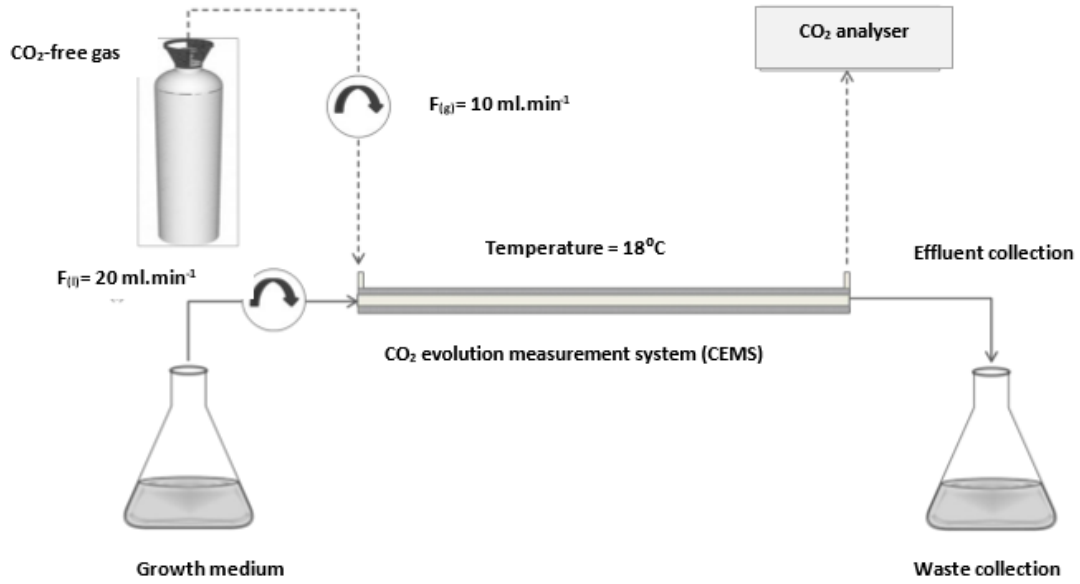


Figure 4.3 Set up of a single CEMS system tube (adapted from Loots, 2016). F(g) is representative of the flowrate of gas and F(i) is representative of the flowrate of growth media (1% TSB).

4.3.2 Inoculum preparation

A *L. monocytogenes* isolate was cultured from a South African food factory drain (categorized in Table 3.1 as belonging to lineage II). This isolate, 17JBX41, was prepared for usage in the CEMS system through streaking onto a BHI agar plate from a 25% glycerol stock stored at -20 ^{O}C. This isolate was chosen because it harboured both the *bcr*ABC and *emr*C genes which confer resistance to QACs and it showed phenotypic resistance to QACs in the previous chapter. Incubation was carried out at 37 ^{O}C, the isolate was then grown in a 1% TSB (CM0129,Oxoid, UK) broth which would be the growth medium for this CEMS study. This isolate was also chosen because it represented the dominant lineage group found in this study (Table 3.1) The 1% TSB (Oxoid, GBR) was used to prevent overloading the CEMS lines with nutrients. After 24 hours of growth at 37 ^{O}C in a shaking incubator, the broth was

further suspended in 1% TSB as to allow the culture to acclimatise and adapt to the new nutrient source.

4.3.3 Loading of the CEMS lines with inoculum

Using a sterile needle and a 3 mL syringe; 1 mL of the overnight 1% TSB (Oxoid, GBR) *L. monocytogenes* culture, was transferred into each of the CEMS lines, once the inoculum was standardised to an OD600 value of 0.260 which correlates to 10^8 cfu. The culture was allowed to adhere to the tubes for an hour before the peristaltic pump was turned on to pump the culture with broth at a rate of 20 mL/ hour through the system.

4.3.4 Preparation of the sanitizers

The three sanitizers (Table 4.1) used in this study were prepared in accordance with the manufacturer's guidelines to the recommended, minimum, concentrations for usage in the factory environment operation.

4.3.5 Treatment application

Sanitizer treatment was only applied to the system once a "steady state" of biofilm growth had been established at 50 hours. This steady state was characterized by a non-fluctuating stationary phase of the log curve produced by the CO_2 production-recording graph. Immediately before sanitizer application, the peristaltic pump- which feeds the 1% TSB (Oxoid, GBR) broth from the reservoirs, was turned off. A volume, 1 mL, of each sanitizer was then loaded into its respective CEMS tube and allowed to diffuse into the biofilm for a 15 minute period (recreating the minimum contact time of the sanitizer, as prescribed by the manufacturers). The peristaltic pump was then turned on again to 20 mL/ hour and CO_2 outputs were recorded.

Table 4.1 Table highlighting package instructions and active ingredients for the sanitizers used in the study

Sanitizer name	Recommended application	Active ingredients
QC 50 Benzalkonium Chloride	Dilute to between 1-5% and apply to all areas of clean surface for a minimum of 5 minutes. No rinsing required if applied at less than 1%.	5-10% Benzalkonium Chloride
AN8 Peracetic Acid	Dilute to between 0.5-4% and apply to all areas of a clean surface for a minimum of 15 minutes. Do not rinse with hot water.	>5% Peracetic acid
Byotrol QFS	Dilute to required concentration (1% for food contact surfaces and 2% for drains), ensure area is clean and apply to all areas. Allow to dry. No need to rinse.	Dodecyl dipropylenetriamine Polyhexamethalene Biguanide hydrochloride (10-15% of all active ingredients)

4.4 Results and discussions

Resistance to BAC has been widely reported in literature (Kropac *et al*., 2019; Korsak *et al*., 2019; Müller *et al.*, 2014) and in 1998 to 1999 an outbreak of listeriosis was linked to isolates of *L. monocytogenes* possessing the *bcr*ABC resistance cassette that encodes for QAC resistance. Moreover, a strain containing the *emr*C resistance gene was also responsible for an outbreak of listeriosis in the Netherlands associated with sequence type (ST) / clonal complex (CC) 6 belonging to lineage type I (Kropac *et al*., 2019; Koopmans *et al*., 2017).

CEMS has shown to be a reliable, real-time, measure of a biofilm's response to various antimicrobial stresses in a non-destructive and non-invasive manner (Kroukamp & Wolfaardt, 2009).

To the knowledge of the author: only one other study (Ackerman, 2017) used the CEMS system to test a biofilm's response to commercial sanitizers.

Graphs (Figures 4.4-4.7) demonstrating CO_2 production (µmol. $CO_2.h^{-1}$) over time were generated with the arrow indicating when dosing took place.

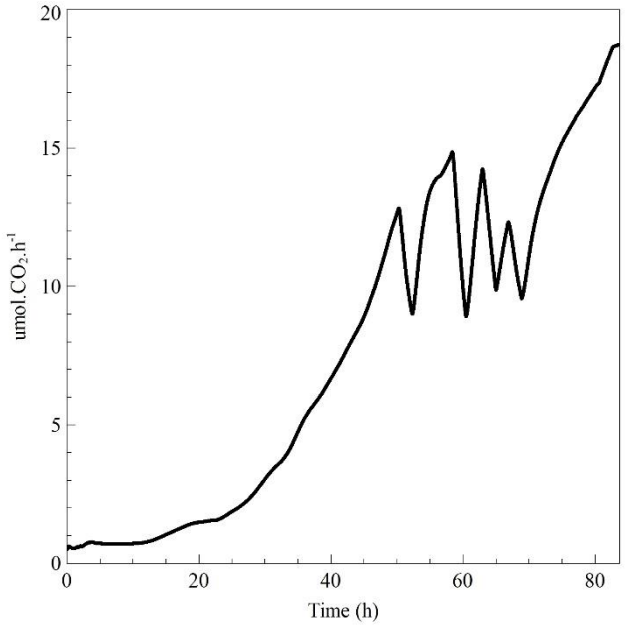


Figure 4.4 Growth control of the *L. monocytogenes* culture in 1% TSB within a CEMS tube with no sanitizer treatment application.

As is evident from the graph in Figure 4.4, no dosing took place and this graph is a representation of a biofilm that has not been treated with a sanitizer. There is an exponential increase in CO_2 output towards the 45 hour position before a fluctuating CO_2 output which represents a stable-overall stationary phase before a sudden increase in CO_2 output from the 70 hour mark. This may be as a result of the pump being turned off followed by a sudden rush of nutrients which stimulated CO_2 production. The pump was turned off to allow dosing of the sanitiser to occur.

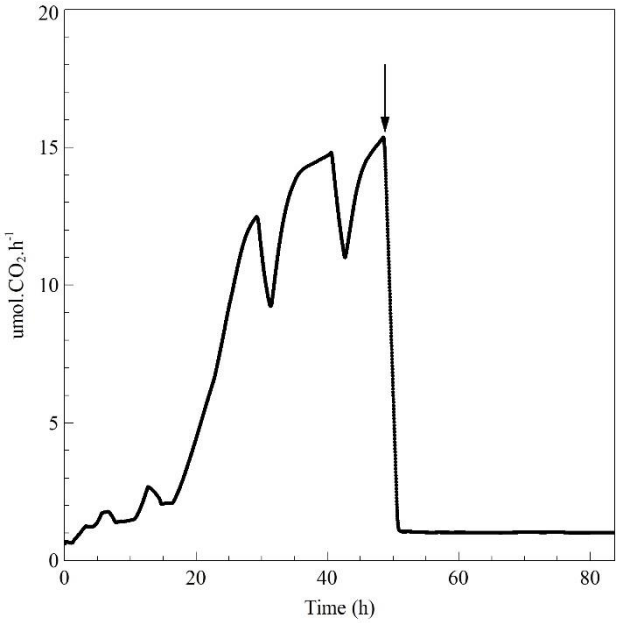


Figure 4.5 Biofilm response to QFS (1%) demonstrating CO_2 production (µmol. $CO_2.h^{-1}$) over time with the arrow representing when the dose was applied.

Figure 4.5 shows a similar trend as Figure 4.4 with an exponential growth phase (exponential growth of CO_2 output). There is a clear drop in CO_2 production immediately following the sanitizer application and no increase in CO_2 production was observed for 30 hours after application. This suggests that this isolate was susceptible towards the 1% QFS sanitizer (Byotrol, GBR) application. Byotrol QFS is free from QACs and relies on Dodecyl dipropylenetriamine, Polyhexamethalene and Biguanide hydrochloride (Table 4.1) for its antimicrobial actions. Much like QAC-based Byotrol, QFS is long lasting and leaves a residual coating on the surface and offers extended antimicrobial protection (PES Africa, 2021). This longer-lasting antimicrobial action may be contributing towards the lack of subsequent growth, post treatment application.

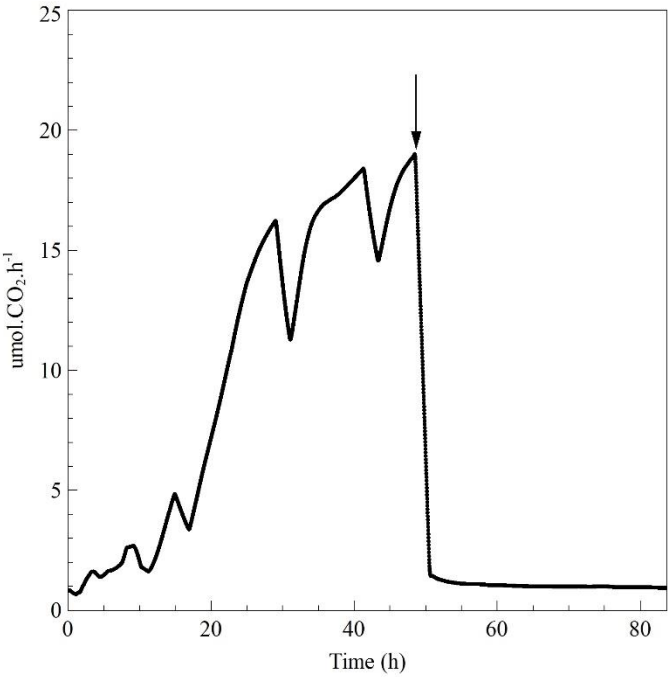


Figure 4.6 Biofilm response to peracetic acid (AN8) (1%) demonstrating CO_2 production (µmol. $CO_2.h^{-1}$) over time with the arrow representing when the dose was applied.

In Figure 4.6 the exponential increase in CO_2 production followed by an easing-off to an overall steady state, was observed from around 35 hours. Dosing took place at the 50 hour mark followed by, as indicated by the arrow, and a sudden drop in CO_2 production. No recovery, indicated by rising CO_2 output levels, was observed. Peracetic acid acts as most oxidising agents and denatures the cell proteins, disrupts cell wall permeability, and oxidises sulfhydryl and sulphur bonds of cell proteins (Center for Disease Control and Prevention, 2008).

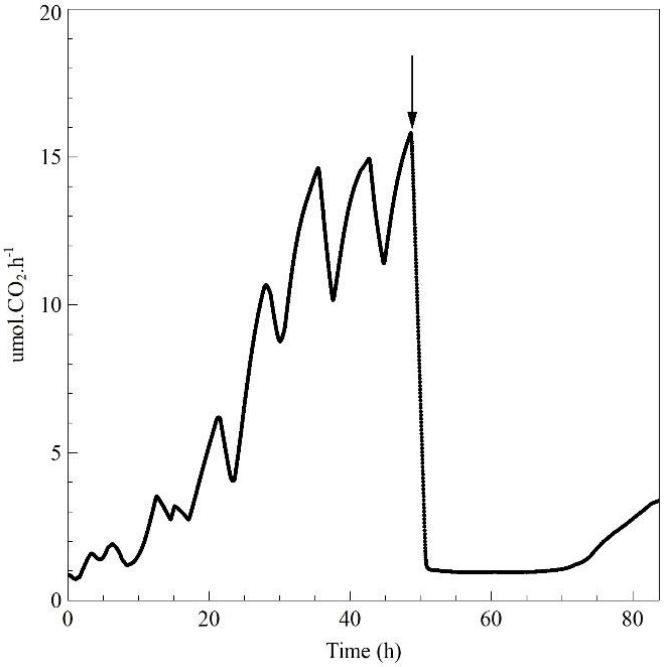


Figure 4.7 Biofilm response to benzalkonium chloride (BAC) (1%) demonstrating CO_2 production (µmol. $CO_2.h^{-1}$) over time with the arrow representing when the dose was applied.

Figure 4.7 shows the same, initial trends as seen in Figures 4.4, 4.5 and 4.6, with a sharply rising exponential phase followed by a relatively stable stationary phase. Once dosage took place at the 50 hour mark, a sharp decrease in CO_2 output was observed. Soon after the 65-hour mark, a rise in CO_2 production was observed. This indicated that the *L. monocytogenes* isolate was in fact resistant towards BAC. This resistance towards BAC has been extensively reported in literature (Kropac *et al*., 2019; Korsak *et al*., 2019; Müller *et al.*, 2014). BAC acts as a cationic membrane-active agent, as it is a QAC, and thus interacts with the cell membrane of *L. monocytogenes*. QACs have also been shown to interact with intracellular components and bind to DNA (Gerba, 2015). According to McDonell & Russel (1999), QACs have the following flow mode of action: firstly: QAC adsorption and penetration of the cell wall. Secondly: reaction with the cytoplasmic membrane which causes the disorganization of the membrane. Finally: leakage of intracellular material and finally cell wall lysis.

The isolate 17JBX41 was shown to possess the *bcr*ABC and *emr*C genes (Table 3.3) and belonged to lineage II (Table 3.3). The *emr*C gene was found to be characteristic in ST-6 isolates which are part of lineage I (Kropac *et a*l., 2019). Thus, exchange of genetic components, that encode for a survival advantage, must have been transferred within a biofilm community within the drain or from somewhere else in the factory. This most probably occurred via HGT.

HGT and biofilm formation are connected processes as rates of HGT are higher in biofilm populations than they are in planktonic ones (Madsen *et al*., 2012). HGT takes place in one of three ways. The first being cell to cell contact (conjugation) which takes place in Gram-positive bacteria (like *L. monocytogenes*) through the detection of specific pheromones whereby conjugative plasmids encode their genes and ensure segregation to both daughter cells during division (Madsen et al., 2012). The second being by means of a bacteriophage mediated DNA transfer (transduction) and finally the uptake of naked DNA (transformation) (Madsen *et al*., 2012).

The *bcr*ABC gene has been found across lineage I and II within food factories and also confers a survival advantage to *L. monocytogenes* isolates against QACs. The presence of two different QAC resistance genes may suggest that a synergistic effect is taking place and both genes may be playing a role in the resistance of the organism. To gain better insight into the resistance of this isolate one must examine the expression of resistance of the planktonic cells against the various sanitizer treatments. The isolate 17JBX41 was resistant to all the sanitizers except QFS. It is important to be reminded that the results of phenotypic resistance towards L-Lactic acid, Sodium hypochlorite, non-foaming (AN8) and foaming peracetic acid (AF8) were unsatisfactory based on the half-life of the compounds being less than that of the bacterial growth rates, thus the appearance of resistance was observed (with no clear evident zone of diffusion).

Based on this and the knowledge that both the *bcr*ABC and *emr*C resistance genes can confer resistance to various QACs (Kropac *et al*., 2019; Korsak *et al*., 2019; Müller *et al*., 2014), it could be concluded that these genes were contributing to the survivability of the isolate 17JBX41 within a food factory's drain. Unfortunately, without gene knock-out tests it would be impossible to determine to what extent either of the two genes is contributing to the QAC resistance of 17JBX41. Moreover, it may be possible that neither of the two genes is in fact conferring resistance towards the QACs as there are many genes that encode for QAC resistance that were not screened for in this study. Furthermore, without WGS analysis it cannot be conclusively stated that either *bcr*ABC or *emr*C is contributing towards the resistance of the organism, as they may be present, and not translating for their respective efflux pumps. They may also be inherent part of this isolates makeup.

When looking at the CEMS system results of this study, the presence of QAC resistance genes and their effect on the survivability of the isolate becomes apparent. When looking at Figure 4.7 compared to Figures 4.5 and 4.6, one can see an increase in the rate of CO_2 production when the CO_2 production curve returns to baseline. This was not observed for the QFS and peracetic acid, thus

indicating the expression of resistance to BAC by the isolate, whilst susceptibility is shown towards QFS and peracetic acid.

4.5 Conclusion

Resistance towards QACs amongst *L. monocytogenes* is an issue of global importance and whilst alternatives do in fact exist (such as PAA and QFS), resistance to these sanitizers still remains a possibility and careful monitoring should be carried out to ensure that sanitizer resistance is not arising. The rise in resistance towards commercial disinfectants is a growing issue which can be attributed to ineffective cleaning practices.

The response of a biofilm towards commercial sanitizers was investigated and found that the results of planktonic tests (chapter 3) mostly aligned with the CEMS (cells in the biofilm or sessile state) results for the tested isolate. It was shown by the CEMS results that PAA was in fact effective against isolate 17BX41, and this method confirmed the effectiveness of QFS against this drain isolate. The results for the planktonic resistance testing for acid-based sanitizers and sodium hypochlorite proved inconclusive in Chapter 3 due to the sanitizers breaking down before bacterial growth occurred, thus giving the appearance of resistance. The CEMS system mitigates this issue due to the already established bacterial growth, and through the real time measurement of the sanitizer's efficacy. This form of testing should be carried out for the other sanitizers, as used in chapter 3, in the future to further expand the understanding of their efficacies against sessile *L. monocytogenes* cells.

Moreover, it highlighted just how quickly a QAC resistant isolate can recover from a standard factory application of BAC and suggests that factories should in fact be moving away from QACs should resistance be encountered. Further tests should be conducted on later generations of QACs to see if this observation is repeated. The effect of an increased concentration of the QAC sanitizer should be investigated to see if it could overcome the QAC resistance mechanisms to avoid it becoming commercially unviable.

QAC cocktails should be further investigated as alternatives towards conventional QACs, especially BAC, to which *L. monocytogenes* resistance is well documented. QAC cocktail efficacies, QAC-free alternatives such as PAA and QFS as well as bacteriophages and enzyme technologies should be investigated going forward.

Antimicrobial resistance in the food industry, shows potential to become a prominent threat to food safety and supply, and should receive the utmost attention. There are also public health implications to this data as an increased survivability within an FPE may result in more product contamination and ultimately more infections, which may become harder to treat with conventional antimicrobials due to cross-resistance brought about from the sanitizer resistant mechanisms. As demonstrated with the *emr*C gene, these surviving isolates, may be more virulent and further increase the toll of listeriosis.

In closing, BAC resistance has been encountered in a *L. monocytogenes* biofilm from a South African FPE. This leads to the question: what alternatives can be recommended? Byotrol QFS and PAA should be recommended as replacements based on the findings of this chapter. In addition: carefully selected QAC cocktails do hold promise in eliminating QAC-resistant *L. monocytogenes*. QACs also show potential in eliminating other bacterial species such as *Salmonella*. In the multispecies environments, that are FPEs, the bigger picture and subsequent knock-on effects, need to be carefully considered before changes in sanitizers are implemented. In the words of Dutch Botanist, Lourens Baas Becking: “Everything is everywhere, but the environment selects”. This is especially true for FPEs where selection pressures applied have consequences that will need to be considered as time progresses.

4.6 References

Ackerman, E. (2017). Survival and proliferation of *Listeria monocytogenes* in a South African ready-to-eat factory. MSc Thesis. Stellenbosch University, South Africa.

Centers for Disease Control and Prevention. (2008). *Peracetic Acid Sterilization*. [Internet document]. URL https://www.cdc.gov/infectioncontrol/guidelines/disinfection/sterilization/peracetic-acid.html. Accessed 8/03/2021.

Colagiorgi, A., Bruini, I., Di Ciccio, P.A., Zanardi, E., Ghidini, S., Ianieri, A. (2017). *Listeria monocytogenes* biofilms in the wonderland of food industry. *Pathogens*. 6 (41).

Da Silva Meira, Q.G., de Medeiros, Barbosa, I., Athayde, A.J.A.A., de Sequeira-Junior, J.P., de Souza, E.L. (2012). Influence of temperature and surface kind on biofilm formation by Staphylococcus aureus from food-contact surfaces and sensitivity to sanitizers. *Food Control*. **25**. 469-475.

Flemming, HC. & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*. **8**. 623-633.

Gerba, C.P. (2015). Quaternary Ammonium Biocides: Efficacy in Application. *Applied and Environmental Microbiology*. **81**(2):464-469.

Jahid, I.K. & Ha, S-D. (2014). The paradox of mixed-species biofilms in the context of food safety. *Comprehensive Reviews in Food Science and Food Safety*. **13**. 990-1011.

Keet, R. & Rip, D. (2021). *Listeria monocytogenes* isolates from Western Cape, South Africa exhibit resistance to multiple antibiotics and contradicts certain global resistance patterns. *AIMS Microbiology*. **7**(1). 40-58.

Kocot, A.M. & Olszewska, M.A. (2017). Biofilm formation and microscopic analysis of biofilms formed by *Listeria monocytogenes* in a food processing context. *LWT- Food Science and Technology*. **84**. 47-57.

Koopmans, M.M., Bijlsma, M.W., Brouwer, M.C., van de Beek, D., van der Ende, A. (2017). Listeria monocytogenes meningitis in the Netherlands, 1985-2014: A nationwide surveillance study. *Journal of Infection*. **75**. 12-19.

Korsak, D., Chimielowska, C., Szuplewska, M., Bartosik, D. (2019). Prevalence of plasmid-borne benzalkonium chloride resistance cassette *bcr*ABC and cadmium resistance *cad*A genes in nonpathogenic *Listeria* spp. isolated from food and food-processing environments. *International Journal of Food Microbiology*. **290**. 247-253.

Kropac, A.C., Eshwar, A.K., Stephan, R., Tasara, T. (2019). New insights on the role of the pLMST6 plasmid in *Listeria monocytogenes* biocide tolerance and virulence. *Frontiers in Microbiology*. **10**. 1-16.

Kroukamp, O. & Wolfaardt, G. (2009). CO_2 production as an indicator of biofilm metabolism. *Applied Environmental Microbiology*. **75**. 4391-4397.

Loots, R. (2016). *Biofilms as multifunctional surface coatings and adaptive systems: a biomimetic approach*. Phd thesis, Stellenbosch University, South Africa.

Madsen, J.S., Burmøle, M., Hansen, L.H., Sørsen, S.J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology*. **65**. 183-195.

Mah T.F. (2012). Biofilm-specific antibiotic resistance. *Future Microbiology.***7**.1061–1072.

McDonnel, G.E. (2020). Antisepsis, disinfection, and sterilization. *ASM press*: Washington DC.

McDonnell & Russell. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews*. **12**. 147–179.

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stressl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S. (2014). The *Listeria monocytogenes* tansponson Tn6188 provides increased tolerance to various quaternary ammonium compounds and ethidium bromide. *FEMS Microbiology Research Letters*. **361**(2). 166-173.

PES, Africa. (2021). Byotrol QFS Surface Sanitiser Concentrate 25L. Internet Document: https://pesafrica.net/product/byotrol-qfs-surface-sanitiser-concentrate-25lt/

Poimenidou, S.V., Chrysadakou, M., Tzakoniati, A., Bikouli, V.C., Nychas, G-J., Skanamis, P.N. (2016). Variability of *Listeria monocytogenes* strains on biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. *International Journal of Food Microbiology*.**237**,164-171.

Rieu, A., Lemaitre, J.P., Guzzo, J., Piveteau, P. (2008). Interactions in dual species biofilms between Listeria monocytogenes EGD-e and several strains of Staphylococcus aureus. *International Journal of Food Microbiology*. **126**. 76–82.

Sharma, D., Misba, L., Khan, A.U. (2019). Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antibiotic Resistance and Infection Control*. **8**(76). 1-10

Sharma, M. & Anand, S.K. (2002). Biofilms evaluation as an essential component of HACCP for food/dairy processing industry- a case. *Food Control*. **13**. 469-477.

Solano, C., Echeverz, M., Lasa, I. (2014) Biofilm dispersion and quorum sensing. *Current Opinions in Microbiology*. **18**. 96–104.

Chapter 5

General Discussion and Conclusion

The main aim of this study was identifying the prevalence of resistance towards commercial sanitizers amongst *L. monocytogenes* isolates from six different FPEs across the RSA. Commercial sanitizer resistance amongst *L. monocytogenes* has been well documented in international literature, particularly to the QAC-class of sanitizer. These sanitizer resistant isolates, and their associated genes, have been found in clinical cases of listeriosis and this suggests that, in surviving the application of the terminal disinfectant they are continuing to proliferate and contaminating the final product, infecting humans and causing them to succumb to listeriosis. Given South Africa's recent brush with listeriosis in the 2018 outbreak: an investigation of this thesis's nature was long overdue.

The first objective of this study was to identify and confirm the identification of single colonies of *L. mono*cytogenes from the six South African FPES using RapidL'mono selective agar plates (BIORAD, FRA) and conventional PCR screening for the *hly* gene.

The second objective of this study was carried out to provide industry with information pertaining to the efficacies of commercially used sanitizers, particularly QAC-based sanitizers, against commercially isolated stains. This was done in conjunction with screening for three genes and one cassette (*bcr*ABC, *emr*C, *emr*E and *qac*H) that confers resistance on *L. monocytogenes* towards QACs. All four of these genetic elements have been previously isolated from human cases of listeriosis.

The third, a biofilm's responses to the various commercial sanitizers was investigated to compare its responses towards the results obtained in planktonic cell-diffusion disk testing and to further validate the CEMS system for sanitizer testing.

For the first objective, this study found that all 50 factory isolates gave a phenotypic expression of growth, on RapidL'mono plates, that is consistent with that of *L. monocytogenes*, furthermore it was found that all 50 isolates contained the *hly*A gene which is consistent with confidently confirming their identity as *L. monocytogenes*.

The second research objective of this study found a high prevalence of both the *bcr*ABC (68%) and *emr*C (62%) QAC resistance genes amongst the 50 factory *L. monocytogenes* isolates. A higher percentage on lineage I isolates (64%) possessed both the *bcr*ABC and *emr*C genes compared to 36% of lineage II isolates possessing both those genes. There was a notable absence of the *emr*E and *qac*H genes from the sample set, which differs from international findings. Furthermore, finding in terms of phenotypic responses towards the commercial sanitizers showed that the diffusion disk method was

not suitable for PAA derivatives, L-Lactic acid nor sodium hypochlorite due to fast rates of diffusion. Resistance to a first generation QAC (BAC) was found to be prolific amongst the sample set, mixed results were seen for the fourth generation QAC that was tested. Mixed results were seen for the two QAC cocktails used which may highlight their potential in overcoming QAC resistance amongst *L. monocytogenes*, whilst still using QAC based sanitizers which have been proven to be effective against other bacteria found within an FPE, such as *Salmonella*. The QFS sanitizer produced by Byotrol was shown to be the most effective sanitizer in controlling *L. monocytogenes* within this study, however resistance was still encountered. This suggests that further tests, such as WGS, should be carried out against the QFS resistant isolates to ascertain where their resistance is coming from. The QFS sanitizer is increasing in popularity amongst South African food processors, due to concerns over QAC resistance coming from producers. Again, it is important to reiterate that the bacterial loads used were not what would be expected within a conventional FPE prior to sanitizer application. Preceding cleaning steps, including the application of a surfactant and mechanical action would drastically reduce the microbial load and thus the potential for sublethal exposure of the sanitizer onto the *L. monocytogenes* cells.

The responses of a single isolate of *L. monocytogenes* coming from a factory's drain was tested in the novel CEMS against BAC (a first generation QAC), PAA and QFS. The results showed that within 20 hours of exposure, the biofilm had begun to recover against the BAC treatment but not the PAA nor the QFS treatment. This shows that the isolate was expressing resistance against BAC but not PAA nor QFS. This shows the potential of the CEMS to study sanitizer resistance in real time and get a better indication of responses, especially in terms of PAA, versus conventional disk diffusion methods.

The implications of this study's findings shows that QAC resistance is widespread amongst the six FPEs and that the use of QACs should be decreased in favour of other alternatives. This does not mean that QAC usage should be drifted away from entirely: some isolates were still susceptible to varying generations of QACs and they may still prove to be a viable option should they be used responsibly (correct or increased concentrations and adequate contact time); as they are affordable and still prove effective when applied correctly for certain isolates. Not all bacteria that are food safety hazards within FPEs are sanitizer resistant *L. monocytogenes*, other pathogens are at play in FPEs as well, and a "blinkers on" approach to controlling contamination within an FPE must not be adopted. Instead, a holistic approach to FPE hygiene needs to be adopted and QACs may still have a role to play.

Without proper sanitizer application, the phenomenon of sanitizer resistance is bound to continue due to the issue of sublethal exposure. This issue of sublethal exposure is further snowballed through the continuous application of sanitizers each and every day, after operational shutdown of

an FPE, in accordance with GMPs. This repeated action of cleaning every day, further increases the chances of sublethal exposure occurring, with an increased chance of slip ups following.

To conclude, without the implementation of a thorough cleaning protocol, which removes soil and mechanically dislodges biofilms and other residues, sublethal exposure of *L. monocytogenes* towards sanitizers can occur. Not following chemical manufacturers' guidelines and using too low of a concentration may also contribute to the phenomenon of sublethal exposure which is a driving force for the development of antimicrobial resistance. As has been seen globally in terms of QAC resistance, this issue may very well begin once more with different classes of sanitizer. Emphasis needs to be put on industry to control the abuse of sanitizers by following cleaning procedures in a sequential manner and finally to test their in-house strains for resistance towards sanitizers that are in use. By doing so they will ultimately stop the spread of resistance genes, halting the propagation of resistant isolates which will ultimately result in the safe production of their respective food products. This monitoring of resistance towards cleaning agents must be included within a factory's GMP for cleaning and this should be an auditable criterion.

Further investigations should be carried out into the genetic makeup of resistant isolates, their resistance to antibiotics and virulence in order to get a better understanding of the potential impact that these isolates can have further downstream. Furthermore, resistance of biofilms (and particularly mixed biofilms) towards the QAC-cocktails on offer as well as further investigations into the efficacies of PAA and QFS which are emerging as popular market options, should be explored. The use of bacteriophages against these mixed species biofilms should also be conducted. Finally further investigations into the monitoring of sanitizer resistance within a factory should be conducted. This will enable a factory to successfully implement sanitizer resistance monitoring into their cleaning GMP and ultimately help prevent further incidences of listeriosis outbreaks from sanitizer resistant isolates occurring thus ensuring food safety for the end consumer.