Profiling and antibiotic resistance of lactic acid bacteria isolated from commercial *aMasi* samples

Submitted in fulfilment of the academic requirement for the degree of Master of Applied Sciences in Food Science and Technology at the Department of Biotechnology and Food Technology

Faculty of Applied Sciences

Durban University of Technology, Durban, South Africa

By

Yovani Pillay

Supervisor: Prof. O. A. Ijabadeniyi

Co-supervisor: Prof. F. M. Swalaha

March 2020

REFERENCE DECLARATION

I, Ms Yovani Pillay and Prof. Oluwatosin A. Ijabadeniyi do hereby declare that in respect of the following dissertation:

Title: **Profiling and antibiotic resistance of lactic acid bacteria isolated from commercial *aMasi* samples**

1. As far as we ascertain:
   (a) No other similar dissertation exists;
   (b) The only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

SIMATENDE, P. 2016. Microbial ecology and diversity of Swazi traditional fermented foods. PhD, University of KwaZulu-Natal.

2. All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

Signature of student Date

Signature of supervisor Date

Signature of co-supervisor Date

AUTHOR'S DECLARATION

I, Ms Yovani Pillay, hereby declare that the work reported in this dissertation and submitted to the Department of Biotechnology and Food Technology at the Durban University of Technology for the Degree of Master of Applied Sciences is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education and Learning Institution.

Yovani Pillay Date

Student

As the student's supervisors, we agree with the submission of this dissertation:

Prof. Oluwatosin A. Ijabadeniyi Date

Supervisor

Prof. Feroz M. Swalaha Date

Co-supervisor

ABSTRACT

aMasi is traditionally fermented milk that constitutes part of the South African heritage and is regarded as a supplementary staple food. Its inclusion into the South African Food Based Dietary Guidelines has led to the encouraged consumption of this product. Given the fact that *aMasi* is a rich source of lactic acid bacteria (LAB), such bacteria are of economic importance to the food, feed and pharmaceutical industries. The main concern regarding food safety is LAB's inherent ability to acquire and disseminate antibiotic-resistant genes. Although LAB have regulatory statuses such as 'generally recognised as safe' (GRAS) and 'qualified presumption of safety' (QPS), concerns are expressed over the possible mobility of resistance genes to human and animal opportunistic and pathogenic bacteria which could make treatment of bacterial infections more complex to treat in the future. Numerous reports globally, have documented antibiotic resistance among LAB isolated from commercial dairy and pharmaceutical products over the last decade. Therefore, the aim of this study was to determine if LAB isolated from commercial *aMasi* samples harbour antibiotic-resistant genes.

To achieve this aim, the total bacterial population and LAB population of 10 *aMasi* samples were surveyed using culture-dependent techniques and the proportional prevalence of LAB to the total bacterial population were determined by using a 100% stacked-column. In all 10 samples, LAB was the predominating population ranging from 87.44% to 99.77%. A total of 30 LAB isolates were characterised after isolation and sequencing of 16S rDNA of these isolates showed that LAB were *Leuconostoc pseudomesenteroides* and *Leuconostoc mesenteroides* with two isolates being identified as *Lactococcus lactis* CP028160.1.

The relationship between the growth of LAB and selected physicochemical properties (pH, titratable acidity, water activity (a_w), moisture content, fat content and estimation of reducing sugars (lactose)) were determined using principal component analysis (PCA) and classification and regression tree (CART) to illustrate the likelihood of LAB present in *aMasi* samples based on LAB count and pH. From the PCA results, approximately 75.25% of variances in the data were retained by the first three principal components (PCs). The first principal component (PC1) had accounted for the highest total variance of 33.16%. PC1 increased with an increase in lactic acid % and a_w, whilst it negatively correlated with LAB count, moisture % and lactose (mg/25ml lactose·H_2O). The results showed an increase in LAB count with an increase in moisture % and lactose (mg/25ml lactose·H_2O) whilst, LAB count had decreased with an increase in lactic acid % and a_w. Moreover, pH and fat % had no effect on PC1, high LAB counts were observed for samples 6 and 7 whist low LAB counts were observed for samples 9 and 10. On the other hand, PC2 had accounted for approximately 27.53% of the total variance. PC2 increased with an increase in fat % and lactose (mg/25ml lactose·H_2O), whilst it negatively correlated with LAB count and pH. It was

observed that the growth of LAB had increased with an increase in pH, whilst it decreased with an increase in fat % and lactose (mg/25ml lactose·H_2O). Moreover, lactic acid %, a_w and moisture % had no effect on PC2. High LAB counts were observed for samples 7 and 8 and low LAB counts were observed for samples 2 and 4.

Nine out of the 30 LAB isolates were selected due to these isolates having a different GenBank Accession number and were subjected to antibiotic susceptibility testing using the disc diffusion method against a total of 11 antibiotics. Most of the LAB isolates exhibited multiple resistance towards some of the most commonly used antibiotics as well as last-resort antibiotics. All the isolates showed high levels of resistance towards vancomycin, colistin sulphate, fosfomycin and pipemidic acid except for *Lactococcus lactis* CP028160.1 which was susceptible to vancomycin. All isolates were susceptible to tetracycline and erythromycin whilst eight out of nine isolates were susceptible to chloramphenicol with seven out of nine isolates being susceptible to ampicillin. Furthermore, the isolates had displayed intermediate resistance mainly towards kanamycin and streptomycin.

The present study showed that multiple antibiotic resistance is prevalent in different species of starter culture strains, which may pose a food safety concern. LAB that exhibit phenotypic resistance to antibiotics should also be evaluated on a molecular level to monitor their resistance. The presence of such a variety of expressed AR genes in probiotic isolates is a worrying trend. The impact of the interactions of these bacteria with pathogenic strains and their transfer of these AR genes is yet to be assessed. Furthermore, antibiotic sensitivity is an important criterion in the safety assessment for the evaluation of food-grade and potential food-grade LAB.

ACKNOWLEDGEMENTS

The author would like to express gratitude and thanks to the following people and/or institutes for making this dissertation a success:

- I would like to sincerely thank the Divine Omnipresent, for the gift of intuition, providing me with the will, strength, courage and wisdom that I so desperately needed during my trying times on this journey. I am truly grateful for the many life lessons that I have learnt during my years of research as a Masters student.
- To my supervisor, Prof. Oluwatosin Ijabadeniyi, I thank you sincerely for accepting me as your Masters student and for providing me with assistance, timeous feedback and positive criticism during our research meetings to make this dissertation a success.
- To my co-supervisor, Prof. Feroz Swalaha, I thank you sincerely for always being patient with me during my difficult times and for availing yourself to meet with me despite your busy schedule to help, guide and assist me throughout my Masters study. Thank you for teaching me about the importance of planning and for stretching beyond your limits to ensure that I had the resources available to complete my research and for providing positive criticism to me.
- To both my parents, I thank you both sincerely for allowing me the opportunity to pursue further studies and for putting up with me during my trying times. Thank you for being ever so patient, understanding, sacrificing and for helping and guiding me throughout this journey. I can never repay the debt.
- To the staff at Inqaba Biotec, I thank you all sincerely for making my visit a very pleasant one. Thank you for assisting me with all the information that I needed and for allowing me the privilege to observe the workflow and analysis of my samples. I thank you all sincerely for assisting me with finding accommodation and for gifting me with souvenirs.
- To the staff and students at the Institute for Water and Wastewater Technology, I thank you for your assistance and guidance during my project and for allowing me the privilege to conduct research in your laboratories.
- To the technicians in the Undergraduate Food Technology Lab, Mr Vee Dilraj and Ms Samantha Govender, I thank you both sincerely for allowing me the privilege to use your resources and lab facilities to complete certain aspects of my work. I also thank you both for your help, guidance and advice given to me throughout my project.
- To Dr Depika Dwarka, Dr Sabiu Saheed, Dr Mahmoud Nasr, Dr Al Govender, Prof. John Mellem, Ms Kameshnee Mellem, Ms Sarla Juglal, Ms Sohana Ranglal, and technicians in the Undergraduate Microbiology Lab, Ms Rena Brijlal and Ms Siphi Mbamali, I thank you all sincerely for the different roles that you have

significantly played in my research. Be it to providing me with encouragement, assistance, expertise, guidance, advice and support to consumables, resources and administrative assistance when I needed them the most.

- To Ms Mellisa Jula, I thank you sincerely for assisting me with information and advice to help me move forward from my challenges faced during the early stages of my project and for moral support.
- To my friends, lab colleagues, lab technicians and anyone to whom I may have missed out, thank you all sincerely for providing me with the help, guidance, support, advice and positive company that I needed to be successful in achieving my Masters. Most of all, thank you for the light-hearted moments, memories, fun and laughter that helped restore my equilibrium during challenging times.
- To the DUT Postgraduate Research and Support Division, I thank you sincerely for providing me with funding throughout my Masters programme.
- To the National Research Foundation, I thank you sincerely for partial funding towards my Masters programme.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

a_w	- Water activity
AACs	- N-acetyltransferases
AMEs	- Aminoglycoside-modifying enzymes
ANOVA	- Analysis of Variance
ANTs	- O-nucleotidyltransferases
AOAC	- American Association of Analytical Chemists
APHs	- O-phosphotransferases
API	- Analytical Profile Index
AR	- Antibiotic resistance
ATP	- Acid tolerance response
BMi	- Business Monitor International
CART	- Classification and Regression Trees
CIP	- Cleaning-in-place
CLSI	- Clinical Laboratory Standards Institute
CPE	- Carbapenemase-producing Enterobacteriaceae
DGGE	- Density Gradient Gel Electrophoresis
E.	- *Enterococcus*
EFSA	- European Food Safety Authority
EPI	- Expanded Programme for Immunisation
EPS	- Exopolysaccharide
ESBL	- Extended spectrum-beta-lactamase
EU	- European Union
FBDG	- Food-Based Dietary Guidelines
FDA	- United States Food and Drug Administration
FEEDAP	- EFSA's Panel of Additives and Products or Substances used in Animal Feed
GAD	- Glutamate decarboxylase
GARP-SA	- Global Antibiotic Resistance Partnership in South Africa
gDNA	- Genomic deoxyribose nucleic acid
GI	- Gastrointestinal
GRAS	- Generally recognised as safe
HACCP	- Hazard analysis and critical control points
HGT	- Horizontal gene transfer
HIV	- Human Immunodeficiency Virus
HRC	- High resource countries
HTST	- High-temperature, short-time
IPC	- Infection Prevention and Control
ISO	- International Organisation for Standardisation

KZN - KwaZulu-Natal
LAB - Lactic acid bacteria
Lb. - *Lactobacillus*
Lc. - *Lactococcus*
Leu. - *Leuconostoc*
LMIC - Low- and middle-income countries
LSM - Lactic acid bacteria Susceptibility Medium
MGE - Mobile genetic elements
MIC - Minimum inhibitory concentration
MID - Minimum infectious dose
MLST - Multilocus Sequencing Typing
MRS - de Man Rogosa and Sharpe
MRSA - Methicillin-resistant *Staphylococcus aureus*
NCD - Non-communicable diseases
NCS - National Core Standards
PC - Principal component
PCA - Principal component analysis
PCR - Polymerase chain reaction
PCSMA - Plate Count Skimmed Milk Agar
PEFR - Plasmid-encoded fosfocin resistance
QPS - Qualified presumption of safety
S. - *Staphylococcus*/*Streptococcus*
SAASP - South African Antibiotic Stewardship Programme
TB - Tuberculosis
US - United States
VGT - Vertical gene transfer
VRE - Vancomycin-resistant enterococci
WHO - World Health Organisation

CHAPTER ONE: INTRODUCTION

South Africa (SA) has a food framework that is predominated by foods that are highly energy-dense, palatable, cheap and nutrient-poor (Joubert, 2013, Steyn and Ochse, 2013). Given the fact that SA is a middle-income earning country, it is not surprising to note that increased consumption of these foods leads to higher occurrences of non-communicable diseases (NCDs) (Labadarios *et al*., 2011). The South African National Health and Nutritional Examination Survey in 2014 had estimated a higher incidence of obesity among women than men with 39.2% and 24.8% respectively (Shisana *et al*., 2014). In contrast to this, undernourishment among young children aged 0-3 years had the highest prevalence with 26.9% for boys and 25.9% for girls respectively showing stunting (Shisana *et al*., 2014). The first set of Food-Based Dietary Guidelines (FBDGs) in SA was published in 2001 consisting of 10 guidelines which excluded a specific dairy guideline where milk was previously included in the protein group (Vorster *et al*., 2001). Vorster *et al*. (2013b) pointed out that the incorporation of milk into the protein group was because of the FBDGs emphasis on affordability for most of the population. Moreover, the only inclusion of milk in the protein group without the inclusion of a separate dairy guideline was through the possible high prevalence of lactose intolerance amongst South Africans (Du Plooy *et al*., 2017).

In 2012, the FBDGs were amended and republished. These guidelines now feature a specific guideline for dairy products and currently states: “Have milk, *maas* or yoghurt every day.” This guideline was included after the low intake of calcium and potassium were consistently reported and the high incidence of hypertension and NCDs reported amongst the South African population (Vorster *et al*., 2013a). There are many reasons for the inclusion of *maas* (also known as *aMasi*) into the dairy guidelines. These reasons include: the recognition of *aMasi* as a traditional food that is widely consumed; beneficial health effects from the incorporation of probiotics in fermented milk; improvement of lipid profiles; the acidic nature of fermented milk and their ability to hinder gastric emptying which can result in appetite regulation and lastly, the low sodium-to-potassium ratio is contemplated to be advantageous for the prevention of cardiovascular disease and hypertension (Du Plooy *et al*., 2017).

aMasi production is founded on the principle of fermenting cows’ milk through the activity of indigenous occurring or added microflora. The final product is white in colour, thick in consistency and contains lumps once ample fermentation has concluded (Du Plooy *et al*., 2017). There are two commercial methods used to produce *aMasi* i.e. “in-container fermentation” and “tank fermentation.” Both methods are based on the incorporation of a permitted/defined starter culture to milk. The most common starter cultures used to produce commercial *aMasi* are mesophilic and include *Lactococcus lactis* subsp. *lactis*, *Lc*. *lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris* (Vorster *et al*., 2013b).

Given the fact that *aMasi* constitutes part of the South African heritage and is regarded as a supplementary staple food, its inclusion into the SA FBDGs has led to the encouraged consumption of this product (Du Plooy *et al*., 2017). Keeping in line with the United Nations' (UN) One Health Approach, it is vital to safeguard the consumer against foodborne and environmental contaminants that negatively affect human health (Bell *et al*., 2018). This is done through joint actions by preventing and controlling foodborne zoonoses, antibiotic resistance (AR) and emerging microbiological hazards (Bell *et al*., 2018). A crucial element of their assessments, management and communication is the identification of risks and health hazards at the human-animal-ecosystems interface (Rostal *et al*., 2018). The One Health Approach is crucial because of the development of AR and focuses on widespread health concerns, which accounts for emerging infections, parasitic and zoonotic diseases. It was estimated that 60% of human infectious diseases are of animal origin; nearly 75% of emerging human infectious diseases over the past 30 years had originated in animal-borne (even aquatic) diseases or pathogens (Gebreyes *et al*., 2014).

Through secure collaborations with civil society organisations, animal, environmental and human health experts, an ideal reality of a safe and secure world with fewer infectious disease threats to human security can be possible through simulating advances in these mentioned aspects (El Samra, 2017). Thus far, the UN One Health Approach has demonstrated to be successful from a developing and infectious disease perspective. However, its relevance still needs to be demonstrated with respect to the interactions and exchanges of other microbiomes and fundamentals of microbial communities' transfer among humans, animals and the environment (El Samra, 2017).

Lactic acid bacteria (LAB) are of economic importance to the food, feed and pharmaceutical industries which are also interchangeably referred to as probiotics (Chelule *et al*., 2015). These probiotics are intentionally incorporated into food products, mainly dairy products, and are commercially available on a large scale (Jose *et al*., 2015, Sharma *et al*., 2017). The main concern regarding food safety is their inherent ability to acquire and disseminate AR genes (Toomey *et al*., 2010). Such bacteria are also common commensals of the human and animal gastrointestinal (GI) tracts. LAB are ubiquitous in nature hence, these bacteria may serve as vectors for the transmission of AR genes via the food chain to the consumer (Erginkaya *et al*., 2018). This is particularly true for fermented milks that do not undergo any heat treatment or pasteurisation prior to consumption which provides a channel for which AR bacteria may associate with human intestinal microflora, forming a direct link between animal indigenous microflora and the human GI tract (Sharma *et al*., 2015, Carruth *et al*., 2017).

Additionally, LAB have been reported to harbour and disseminate AR to food-borne or enteric pathogens (Ouoba *et al*., 2008). Antibiotic resistance is a continuous menace globally. This phenomenon is mainly attributed to the misuse of these drugs in nosocomial environments,

agriculture and animal husbandry which had led to the selective pressure and emergence for point mutations and the acquisition of mobile genetic elements (MGE) encoding AR leading to the dissemination of multiple AR determinants (Ouoba *et al*., 2008, Cahill *et al*., 2017). This makes AR one of the greatest threats to food security, global health and development in recent times. This tendency implies that treatment for patients with bacterial infections in the years to come would be more difficult, time-consuming and expensive to treat, due to the inefficacy of current antibiotics that were once effective in these treatments (Abriouel *et al*., 2015a, Sabtu *et al*., 2015, Fymat, 2017). Bacteria use a variety of multiplex mechanisms to disseminate AR genes (Munita and Arias, 2016, Peterson and Kaur, 2018). The main mechanisms of horizontal gene transfer (HGT) in bacteria are contemplated to be conjugation and transduction via bacteriophages (Lerner *et al*., 2017). Conjugation, being the most prominent mechanism of transfer involves the presence of plasmids being MGE and play a vital role in the dissemination of AR (Peterson and Kaur, 2018).

Given the fact that AR has become a subject of debate presently, it has received limited scrutiny in SA with regards to LAB, which is globally the most freely consumed bacterial group (Sharma *et al*., 2017). A rise in the number of reports documented regarding AR among LAB strains has been observed over the last decade (Sharma *et al*., 2017). Despite their positive benefits, there is a need to guarantee their safety in terms of disseminating AR genes. Safety is not an issue if AR is intrinsic, but; it does become a significant problem if these bacteria are able to transfer AR genes to other possibly pathogenic bacteria (Gueimonde *et al*., 2013). Although LAB have regulatory statuses such as ‘generally recognised as safe’ (GRAS) and ‘qualified presumption of safety’ (QPS), concerns are expressed over the possible mobility of resistance genes to human and animal opportunistic and pathogenic bacteria (Nawaz *et al*., 2011).

Studies by D'Aimmo *et al*. (2007), Liu *et al*. (2009), Gad *et al*. (2014) and Sharma *et al*. (2015) have reported AR in LAB isolated from commercial dairy and pharmaceutical products. According to Moračanin *et al*. (2017), there are two atypical genes in LAB namely, *tet*(M) encoding for tetracycline resistance and *erm*(B) encoding for erythromycin resistance, followed by *cat* genes which encode for chloramphenicol resistance. This poses a serious problem to food safety and to the general public. It would, therefore, be interesting to investigate the possibility of AR in LAB from South African commercial *aMasi*. With SA having well-established probiotic markets and the most active surveillance for AR compared to the rest of Africa (Moyane *et al*., 2013), there is a paucity with regards to documented research on AR of LAB.

Therefore, the main aim of this research is to investigate if LAB isolated from commercial *aMasi* samples harbour AR genes. The general objectives are to:

1. Survey the total population of bacteria present in commercial *aMasi* samples and to determine the proportion of LAB present.

2. Identify the LAB isolates by 16S rDNA sequencing.

3. Characterise each of the *aMasi* samples by carrying out selected physicochemical tests (pH, titratable acidity, water activity (a_w), moisture content, fat content and estimation of reducing sugars (lactose)) and relating them to the growth of LAB using principal component analysis (PCA) and classification and regression tree (CART) modelling.

4. Determine if LAB isolated from the *aMasi* samples harbour antibiotic-resistant genes. This objective is mainly achieved through the objectives, 4a. and 4b. listed below:

    a. The first part of this objective was to phenotypically determine by the Kirby-Bauer disc diffusion method if the LAB isolates had resistance to any of the eleven antibiotics (ampicillin, chloramphenicol, colistin, erythromycin, fosfomycin, gentamicin, kanamycin, pipemidic acid, streptomycin, tetracycline and vancomycin).

    b. The second part of this objective was to select the isolates that exhibited phenotypic resistance to any of the eleven antibiotics and conduct a polymerase chain reaction (PCR) to determine if the isolates had harboured antibiotic-resistant genes in the genome or plasmid using gene-specific and degenerate primers.

CHAPTER TWO: LITERATURE REVIEW

This chapter aims to review the characteristics and functions of the different types of LAB utilised as lactic starter cultures, their applications in South African fermented milk as well as their microbial dynamics, biochemical activities, safety in terms of antibiotic resistance and potential risks associated with their application in fermented dairy products.

2.1 LACTIC STARTER CULTURES

LAB are considered to be the most significant and commonly used starter cultures in dairy fermentation to produce high-quality fermented milk products (Mullan, 2014, Hickey *et al*., 2015, Rakib *et al*., 2017). Products include cheese, yoghurt, cultured buttermilk and sour cream (Bandler and Singh, 2018) as well as indigenous products such as *aMasi* and *Kefir* amongst many others (Surono and Hosono, 2011, Behare *et al*., 2016). In the industrial process, starter cultures are defined as food-grade microorganisms that have been carefully selected to be intentionally inoculated into milk during the manufacturing process of fermented dairy products (Surono and Hosono, 2011, Hickey *et al*., 2015, Rakib *et al*., 2017).

LAB may originate from the microbial flora naturally present in raw milk (Quigley *et al*., 2013, Mullan, 2014). Often, the composition of microbial flora in raw milk is unpredictable, uncontrollable and futile. Furthermore, their natural microbial flora may altogether be destroyed by pasteurisation during milk processing and may not be available to use as secondary starter cultures. Adding a pre-grown starter culture to milk in a more controlled manner can aid in providing specific characteristics, leading to a more reliable and predictable fermentation (Goff, 2018c).

The pivotal role that LAB play in the fermentation process is to manage the pH of maturing milk and related products via the process of glycolysis, i.e. a process involving the conversion of lactose present in milk into lactic acid (Hickey *et al*., 2015). The reduction of pH to approximately 4.60, occurs quite rapidly within four to eight hours in fermented milk products. Since the milk environment becomes acidic, such conditions favour the growth of acid-tolerant bacteria. The purpose of reducing the pH is to serve as a control mechanism for non-starter microflora (Hickey *et al*., 2015). Apart from LAB producing lactic acid as the main end product, such bacteria also perform secondary functions (Goff, 2018c). These functions are to produce antimicrobial metabolites such as organic acids (acetic, folic and propionic acids), carbon dioxide (CO_2), hydrogen peroxide (H_2O_2), bacteriocins, ethanol, etc. (Reis *et al*., 2012) which will be discussed further.

2.1.1 Characteristics and Functions of Dairy Starter Cultures

LAB are ubiquitous in nature and are mainly located in habitats rich in carbohydrates such as plants, fermented foods and the mucosal and GI tracts of animals and humans (Teuber *et al*., 1999, Devirgiliis *et al*., 2011, Florou-Paneri *et al*., 2013, Quinto *et al*., 2014). Starter cultures are genetically diverse, the prevalent characteristics of dairy LAB are Gram-positive, non-sporulating, facultative aerobes or anaerobes and lack cytochromes and porphyrins which therefore make them oxidase and catalase-negative (Darsanaki *et al*., 2013, Liptáková *et al*., 2017). Furthermore, the DNA base composition of LAB ranges from 33 to 55 ymol% G+C (Stiles and Holzapfel, 1997).

Such bacteria are non-pigmented cells that are divided into rods and cocci (Liptáková *et al*., 2017) according to their cellular morphology and are further divided into two groups i.e. homofermentative and heterofermentative, based on how they ferment glucose (Gänzle, 2015). Homofermentative LAB convert carbohydrates to lactic acid as the main or sole end product whilst, heterofermentative LAB produces in addition to lactic acid, other secondary products such as CO_2, ethanol, succinate, acetate and formate (Florou-Paneri *et al*., 2013, Abriouel *et al*., 2015a). Having mentioned this, the main metabolic process of LAB is to primarily produce energy and lactic acid by metabolising different carbohydrates and related compounds (Florou-Paneri *et al*., 2013). The nutritional requirements of lactic starter cultures differ among species with many of these cultures being nutritionally fastidious, frequently needing vitamin B, certain amino acids and other growth factors (Behare *et al*., 2016).

Starter cultures have to provide a multifunctional role so as to ensure a high-quality product (Rakib *et al*., 2017). These functions are to: (i) produce lactic acid and other secondary metabolites (i.e. acetic acid, propionic acid, CO_2 and alcohol) (Surono and Hosono, 2011, Hati *et al*., 2013). This is to rapidly reduce the pH of the milk and to serve as a control measure to inhibit non-starter cultures, aforementioned; (ii) yield aromatic compounds such as acetoin, acetaldehyde and diacetyl (Rakib *et al*., 2017); (iii) provide an antimicrobial effect against spoilage microbial flora and foodborne pathogens (Hati *et al*., 2013, Rakib *et al*., 2017); (iv) initiate flavour development through glycolysis, lipolysis and proteolysis (Behare *et al*., 2016); (v) enhance texture and body of certain products through exopolysaccharide (EPS) production (Hati *et al*., 2013, Behare *et al*., 2016) and lastly, contribute to overall acceptability of the final product (Rakib *et al*., 2017).

2.1.2 Types of Lactic Acid Bacterial Starter Cultures

As mentioned previously, LAB are perceived to be the most significant and commonly used starter cultures in dairy fermentation (Rakib *et al*., 2017). Due to this, most of these cultures are manufactured and supplied on a commercial scale (Mullan, 2014). Several LAB starter

cultures used presently today, have emerged from the outset of LAB present in the contaminating microbial flora of milk (Kongo, 2013). Given the fact that these bacteria are ubiquitous in nature (Quinto *et al*., 2014), there is a high likelihood of these bacteria originating from various sources in the environment. For example, the most prevalent LAB used as dairy starters are lactobacilli, lactococci, leuconostocs, bifidobacteria and enterococci. Lactococci and leuconostocs originated from plant material (Campedelli *et al*., 2015, Song *et al*., 2017); bifidobacteria had originated from the faeces of healthy breast-fed infants (Lee and O'Sullivan, 2010) whilst other bacteria such as lactobacilli and enterococci have originated from the GI tract of humans and animals (Bull *et al*., 2013, Lebreton *et al*., 2014).

Modern-day LAB starter cultures have derived from a traditional process called back-slopping i.e. whereby a small amount of inoculum from a successful fermentation the preceding day would be used as an inoculum for the next fermentation of a product the following day (Mullan, 2014). Presently, however, starter cultures are industrially produced and have evolved significantly to meet the technological demands for their respective use in food fermentation processes (Terefe, 2016). Based on their technological use, safety is an integral criterion. All cultures produced must at least attain one regulatory status such as GRAS by the United States Food and Drug Administration (FDA approval) or QPS by the European Food Safety Authority (EFSA approval) (Laulund *et al*., 2017).

Based on the safety approval of these cultures aforementioned, a variety of these starter cultures can be divided into two groups viz. a) simple or defined, whereby one or more strains are used, provided the number is known and; b) mixed or compound, whereby more than a single strain is utilised with each strain imparting its own specified attributes (Goff, 2018c). These starter cultures provide a compilation of positive aspects from a variety of bacterial strains (Hickey *et al*., 2015).

In the past, LAB were assigned a genus primarily based on characteristics such as morphology and Gram-reaction; growth at different temperatures; catabolic reactions such as carbohydrate fermentation and mode of glucose fermentation (homo- or heterofermentation); ammonia production from arginine; tolerance towards bile, salt or alkaline conditions; and isomer(s) of lactic acid produced (von Wright and Axelsson, 2011). Two types of lactic acid are produced by LAB i.e. L-(+) lactic acid or D-(-) lactic acid, however, being species-dependent, racemic mixtures of both types of acids can be produced (Mullan, 2014).

The different characteristics of LAB outlined hitherto, are established on phenotypic attributes and have insubstantial validity in present-day microbial classification. However, they are still employed, on condition that the limitations are understood and have some service in applications. In modern-day applications, chemotaxonomic and molecular methods are progressively employed to allocate genus and species nomenclatures (Mullan, 2014). Certain molecular methods have been particularly useful in the creation of a genus. These methods

include nucleotide sequencing of the 16S and 32S rRNAs, similarity profiling produced by chromosomal mapping of chromosomal DNA and, the degree of DNA-DNA and DNA-rRNA hybridisation. Other methods include serology, which has provided further confirmation of the authenticity of nomenclature (e.g. antisera) against purified superoxide dismutase, which was used to indicate a similarity between lactococci but not enterococci or streptococci (Mullan, 2014).

After recent taxonomic studies, changes were made to the *Streptococcus* genus in particular with several new bacteria being included, inclusive of one motile species, *S. milleri*. In addition, species that were previously under the *Streptococcus* genus have now been allocated into two new genera namely, *Enterococcus* and *Lactococcus*. Currently, LAB have 16 genera viz.: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella* (Álvarez-Cisneros and Ponce-Alquicira, 2018). Out of these genera, species belonging to the *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* genera are important and most commonly used as starter cultures in dairy fermentation and will be discussed further.

2.1.2.1 Enterococcus

Enterococci are coccoid cells that form chains of different length. Lactic acid produced by these bacteria are from the L-isomer. Enterococci are known to be ostensibly similar to lactococci despite these bacteria being halophilic, alkaliphiles, thriving in high bile salt concentrations, ability to proliferate at 45°C, having general tolerance to heat and their resistance to a range of antibiotics (Byappanahalli *et al.*, 2012). Prior to modern-day taxonomic exploration, *Enterococcus* species employed as starter cultures were designated as Group D streptococci and faecal streptococci. Additionally, these bacteria comprise the normal commensal bacteria of the human intestinal tract as well as that of animals and are frequently applied as indicators of faecal contamination in microbiology. Species found and employed in specialist cheeses, cheesemaking and artisanal cultures include *E. faecalis*, *E. faecium* and *E. durans* which belong to the *E. faecium* group (Mullan, 2014).

The use of enterococci as starter bacteria is debatable. Concerns have been expressed with regards to the safety of enterococci in foods for a myriad of reasons. These include the ability of some strains to illicit nosocomial infections and produce bioamines which can cause headaches and other physiological effects (Mullan, 2014). However, other LAB can produce amino acid decarboxylases, recommending that screening new strains for bioamine production to be conducted. Additionally, the concern of these bacteria being able to disseminate AR genes, particularly for glycopeptide antibiotics (vancomycin and teicoplanin), raises critical concern (Eliopoulos and Gold, 2001, Depardieu and Courvalin, 2017). Vancomycin is currently considered as a last-resort drug that may be successful in the

treatment against methicillin-resistant *Staphylococcus aureus* (MRSA) (Mullan, 2014). Despite this, enterococci are commonly prevalent at high concentrations in fermented foods such as artisanal cheese starter cultures and have a long history of use as probiotics, particularly for the treatment of constant diarrhoea. Some enterococci are best suited for use as cheese starters due to their ability to rapidly produce acid in milk, produce acid at room temperatures used in scalding some cheeses and proliferate in the presence of 6.5% salt (Mullan, 2014). A QPS assessment performed by the EFSA in 2010, for *E. faecium* concluded that it was not suitable to screen for the species as a whole and that strain-specific evaluations should be attempted before employing *Enterococcus* stains for use in food production. This suggestion was accepted due to the uncertainty that exists about virulence determinants in enterococci (Herman *et al*., 2019).

2.1.2.2 Lactobacillus

The genus *Lactobacillus* are non-sporulating, Gram-positive rods or coccobacilli that are notably heterogeneous with chromosomal DNA constituting between 32% to 53% G+C content (Radulović *et al*., 2012, Kumar and Kaushik, 2016, Liptáková *et al*., 2017). Species of this genus are often arranged into various groups. This is because of the diverse manner in which they metabolise sugar and the presence or absence of phosphoketolase and fructose-1,6-bisphosphate aldolase (Gänzle, 2015, Kumar and Kaushik, 2016). Presently, more than 100 *Lactobacillus* species have been identified. In addition, several species of *Lactobacillus* are utilised as probiotics and these include *Lb. acidophilus* NCFM, *Lb. casei* strain Shirota, *Lb.* GG (*Lb. casei* subsp. *rhamnosus* or *Lb. rhamnosus*), *Lb. helveticus*, *Lb. jensenii*, *Lb. johnsonii* 100-100. *Lb. johnsonii* LA1, *Lb. plantarum* and *Lb. reuteri* (Kumar and Kaushik, 2016).

Lactobacilli are facultative anaerobes, aciduric, homo- or heterofermentative and have nutritional requirements that are complex because of their ability to adapt to diverse environments (mammalian GI tract, meat, plants and dairy) that are generally enriched in proteins and carbohydrates (Devirgiliis *et al*., 2011, Florou-Paneri *et al*., 2013, Quinto *et al*., 2014, Kumar and Kaushik, 2016). Several species of *Lactobacillus* are superior adjunct and starter cultures for fermenting milk into a diverse range of fermented milk products such as cheese, yoghurt, yakult, etc.

In addition, out of all the starter cultures, lactobacilli are the most acid-tolerant. Such bacteria prefer to initiate growth at acidic pH of 5.6 to 6.2 and lowering the pH of milk further to less than 4.0. Furthermore, some of these species mainly produce L-lactate from glucose whilst others produce D-lactate. Racemase activity is exhibited by certain strains and since a racemase is an isomerase, D/L lactic acid is also produced (Behare *et al*., 2016). The growth of lactobacilli in pure cultures are generally stagnant. It is due to this reason that these cultures are combined with other fastidious cultures to facilitate better growth. A few species of

lactobacilli are employed as probiotics for the treatment of various disorders (Behare *et al*., 2016).

2.1.2.3 Lactococcus

Species of the genus *Lactococcus* are Gram-positive LAB which are adenine-thymine rich, catalase-negative, non-motile, homofermentative and non-sporulating cocci. These bacteria inhabit plant matter and dairy environments (Kumar and Kaushik, 2016). Thus far, only *Lc*. *lactis* is utilised as a starter culture out of the five recognised species. This is because of *Lc*. *lactis* having more practical significance in dairy fermentations as opposed to the other four recognised species (Behare *et al*., 2016, Kumar and Kaushik, 2016). Presently, there are two subspecies namely, *Lc*. *lactis* subsp. *cremoris* and *Lc*. *lactis* subsp. *lactis* and a single variant viz. *Lc*. *lactis* subsp. *lactis* biovar. *diacetylactis*. These strains are generally employed as single strain or mixed strain starter cultures (Behare *et al*., 2016).

Lactococci are mesophilic and homofermentative i.e., greater than 95% of their end product, when grown in milk, is lactic acid (L(+) isomer). Furthermore, they have weak proteolytic properties; they can utilise milk proteins and survive at 10°C but cannot thrive at 45°C. *Lactococcus lactis* subsp. *lactis* is also more thermophilic and halotolerant as compared to the other subspecies (Behare *et al*., 2016). It can grow in pH 9.5 and at 40°C as well and it can also ferment maltose and produce ammonia from arginine. *Lactococcus lactis* subsp. *cremoris* do not exhibit these attributes. A close relationship exists between *Lc*. *lactis* subsp. *lactis* and *Lc*. *lactis* biovar. *diacetylactis* except, that the latter differs by displaying citrate positive ability and lactic acid production in milk is not as great as that produced by *Lc*. *lactis* subsp. *lactis* (Behare *et al*., 2016).

Well-known bacteriocins produced by *Lc*. *lactis* subsp. *lactis* and *Lc*. *lactis* subsp. *cremoris* are nisin and diplococin whilst, bacteriocins produced by *Lc*. *lactis* biovar. *diacetylactis* are unnamed. In addition, some lactococcal species can produce EPS to improve the overall texture of cultured dairy products (Behare *et al*., 2016).

2.1.2.4 Leuconostoc

Species of this genus have cocci shaped bacteria that are often ellipsoidal and appear in either pairs or chains. Having mentioned this, it is difficult to differentiate between species of the *Leuconostoc* and *Lactococcus* genera as both bacteria from these genera form chains of cocci to ovococci shaped cells and they both are catalase-negative. A handy method to aid in the differentiation of the two bacteria is to grow them in litmus milk. *Leuconostoc* spp. do not reduce litmus before coagulation whereas, *Lactococcus* spp. do (Behare *et al*., 2016).

Leuconostocs are heterofermentative producing D-lactate except for *Leu*. *lactis* which exhibit no change in milk. They also require several B vitamins for proliferation and they do not

hydrolyse arginine. *Leuconostoc* spp. can ferment a variety of carbohydrates such as fructose, galactose, lactose and ribose. They can also proliferate at 10°C but not at 40°C. End products from these species include acetoin from citrate, CO_2 and diacetyl. Species that are commonly utilised in the dairy industry are *Leu*. *mesenteroides* subsp. *cremoris*, *Leu*. *mesenteroides* subsp. *dextranicum*, *Leu*. *lactis*, *Leu*. *mesenteroides* subsp. *mesenteroides* and *Leu*. *pseudomesenteroides* (Behare *et al*., 2016). Like lactobacilli cultures, leuconostocs are also combined with other fastidious lactic cultures. They are employed as lactic starters to impart flavour to fermented dairy products like cheese, butter and flavoured milk.

2.1.2.5 Streptococcus

Streptococcus thermophilus is the only species of the *Streptococcus* genus which is ascribed the regulatory status, GRAS. This bacterium is the second most valued dairy starter following *Lc*. *lactis* and is currently used in the production of yoghurt and a variety of cheeses (Behare *et al*., 2016, Rakib *et al*., 2017).

Streptococcus thermophilus is closely related to *S*. *salivarius* which is common microflora of the mouth. According to Axelsson (2004), *S*. *thermophilus* was initially combined with *S*. *salivarius* however, DNA hybridisation data analysis had revealed these two bacteria as being different hence, *S*. *thermophilus* having a separate species status. Unlike species of the genera *Leuconostoc* and *Lactococcus*, *S*. *thermophilus* can be easily differentiated based on their growth temperature and sugar fermentation profile. In contrast to *Lactococcus* and *Leuconostoc* spp., *S*. *thermophilus* are more thermophilic and can grow at 40°C but will cease to grow at 10°C (Behare *et al*., 2016).

Furthermore, *S*. *thermophilus* can be distinguished from other species of LAB based on their ability to utilise galactose. The result of having high levels of galactose in the final product is attributed by using non-galactose fermenting strains (Anbukkarasi *et al*., 2014). It is common practice to solely select strains that utilise galactose to reduce the likelihood of undesirable colour changes in heated products due to the Maillard reaction considering that amino acids react with other reducing sugars and galactose. The majority of all *S*. *thermophilus* strains hydrolyse saccharose, aesculin and lactose (Behare *et al*., 2016).

In addition to this, *S*. *thermophilus* is one of the fastidious starter cultures that can coagulate milk in a short period of time. *Streptococcus thermophilus* and *Lactobacillus* spp. are used in combination and inoculated at temperatures greater than 40°C during the fermentation process to initiate acidification. Generally, streptococci are isolated from milk and milk products but can often be indistinguishable with *Enterococcus* species by exhibiting growth at 45°C and possessing similar morphological features. Most enterococci regardless can proliferate at 10°C in pH 9.6 and 6.5% NaCl and contain the Lancefield group ᴅ-antigen, which

distinguishes them from *S. thermophilus* as they do not exhibit these characteristics (Behare *et al*., 2016).

2.2 DYNAMIC STRESSES OF LACTIC ACID BACTERIA ASSOCIATED TO FERMENTATION PROCESSES

Food fermentation can be defined as the chemical degradation of major and minor food components by microflora such as bacteria and yeasts to produce fermented products (Lavefve *et al*., 2019). This process plays an extensive role in characterising fermented foods with respect to their chemical and sensorial attributes. Given the fact that fermentation is an ancient technique, it reserves a significant place in the history and evolution of human cuisine by modifying the taste profiles and extending the shelf life of these products (Serrazanetti *et al*., 2013, Mokoena *et al*., 2016). Such foods produced from lactic acid fermentations specifically have comprised an integral part of human diets since pre-historic times (Awaisheh, 2012, Papadimitriou *et al*., 2016). LAB have and still play a vital role in preserving agricultural resources and improving the nutritional and organoleptic characteristics of animal feed as well as a variety of human foods. Presently, these economically important bacteria are being considered as alternatives to antibiotics, vehicles for vaccine delivery, probiotic products, enzyme and metabolic factories (Serrazanetti *et al*., 2013).

Several factors must be considered when incorporating LAB into a food formulation. These factors may influence the capability of these microorganisms to survive, proliferate and adapt by becoming active in the new matrix. According to Heller (2001), these factors include a) the physiological state of the LAB to be used as starter cultures (whether cells are from the exponential or stationary growth phase); b) the physical conditions of product ripening and storage (e.g. temperature); c) the chemical composition of the matrix (e.g. acidity, a_w, carbohydrates content, mineral content, nitrogen source and oxygen concentration) and d) possible interactions of the starter cultures with probiotics and other microorganisms naturally added to the system. The key factors that influence the viability and the reactions of LAB from production to storage are illustrated in Figure 2.1.

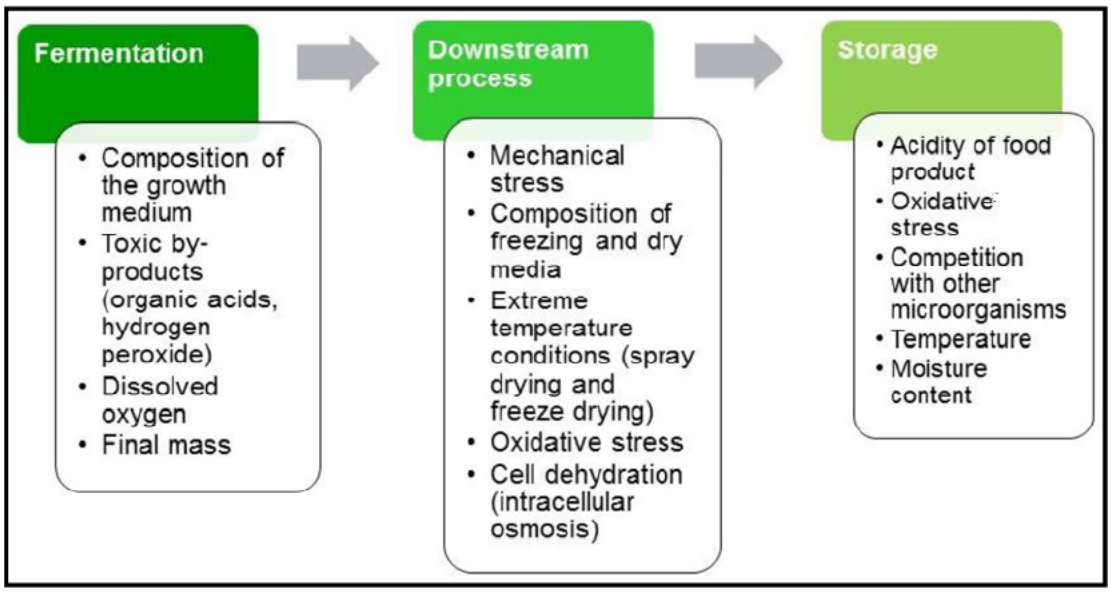

Figure 2.1: Key factors influencing the viability and reactions of LAB in the production of fermented foods. Adapted from Serrazanetti *et al*. (2013).

2.2.1 The Relationship between Lactic Acid Bacteria and Stress

Stress is defined as the interactions between subjects and their habitat that are perceived as being severely pressured and threatening to their well-being (Serrazanetti *et al*., 2013). Stress has administered evolutionary change in microorganisms through the process of natural selection. Therefore, microorganisms that were best adapted to stress (stressors) have evolved and currently survive in our natural environment (Brooks *et al*., 2011). Irrespective of their inherent environment, bacteria are vulnerable to perpetual shifts in their growth conditions. As a result, these bacteria have developed advanced responses, modified by the change in protein complexes and by the phosphorylation-dependent signal transduction systems, to acclimatise and endure an array of stressors (Serrazanetti *et al*., 2009). To ensure endurance to unfavourable environmental conditions, bacteria may habituate themselves to variations in their habitat by reacting to the imposed stress. These responses are dissimilar and vast and are dependent on the nature of the microorganism and environmental stress and are achieved by shifts in the patterns of gene expression for those genes whose products are required to combat adverse conditions. Cellular metabolic pathways, in particular, are closely related to stress responses and the flux of specific metabolites to apprehend the hypothetical changes and implications in food systems have been studied in LAB (Ndagijimana *et al*., 2006, Guerzoni *et al*., 2007, Lanciotti *et al*., 2007, Vannini *et al*., 2007, Vernocchi *et al*., 2008, Montanari *et al*., 2010, Serrazanetti *et al*., 2011).

LAB are used as functional ingredients in food (Chelule *et al*., 2015). They play a vital role in the development of organoleptic attributes and food safety of fermented products (Terefe, 2016). As mentioned previously, these bacteria are employed as starter cultures in an array

of fermented food products, therefore, the validity of starter cultures in terms of quality and functional attributes, growth kinetics and robustness, has become essential for favourable fermentations (van de Guchte *et al*., 2002). Some studies have described the physiological stress responses in LAB, specifically in *Lactobacillus* sp., which have a broad diversity (van de Guchte *et al*., 2002, Hörmann *et al*., 2006, Burns *et al*., 2008, Pavlovic *et al*., 2008).

LAB have developed specific mechanisms to react and endure stress and variation from their habitat (Papadimitriou *et al*., 2016). In a broader sense, microorganisms could have specific regulators customised for each of their regulated genes which enable them to acclimatise their expression congruent to their environmental conditions (Bleuven and Landry, 2016). These integrated regulatory systems serve as good examples of stress defences. Bacterial stress reactions are dependent on concerted expression of genes that modify different cellular processes such as DNA metabolism, maintenance of the cell, cell division, transport, membrane composition, etc. and act synergistically to optimise the bacterial stress tolerance (Serrazanetti *et al*., 2013). The coalition of these stress reactions is achieved by networks of regulators that allow the cells to react to a variety of complex environmental shifts. LAB react to stress in a very distinctive manner and are dependent on the species, strain and the type of stress encountered. The best-studied stresses are heat, acid, oxidative and cold-stresses, although, for the last-mentioned, most of the studies focused on specific family proteins rather than analysing the whole response (Serrazanetti *et al*., 2009). Other stresses include competition and communication and high-pressure stress response (Serrazanetti *et al*., 2013).

Most studies available have been done on the dynamic variations of fermented food mediums with imposed stress on *Lactobacillus* species but limited information exists on species from the genus *Leuconostoc*. Despite the extensive use of LAB in the food and beverage industry, literature is scarce regarding the stress-induced mechanisms studied *in vivo* for optimising the endurance of these bacteria during actual food processing. A better understanding of the dynamic reactions of LAB is vital because these bacteria are often exposed to adverse environmental conditions during the fermentation process (Serrazanetti *et al*., 2013). It should be mandatory for these bacteria to resist unfavourable conditions encountered during manufacturing processes, for instance, during starter administration and storage (lyophilising, freezing or pulverising) and during the dynamic variations that occur in the fermentation environment. These events emphasise the need for robust LAB since they may have to endure and proliferate in various adverse conditions expressing specific functions i.e. during the stationary phase or storage of the final product (van de Guchte *et al*., 2002).

2.3 TYPES OF SOUTH AFRICAN FERMENTED MILK, MARKET VALUE AND THEIR MICROBIAL DIVERSITY

Fermented milk is of great importance in Africa. The reason for this is because it possesses therapeutic and nutritional requirements that are essential for enriching our diet (Beukes, 1999, Shiby and Mishra, 2013). In addition, the African continent has an array of traditionally fermented foods embedded with socio-cultural values (Kayitesi *et al*., 2017). Having mentioned this, traditionally fermented milk may serve as a source of income for local ethnic entrepreneurs (Beukes, 1999).

In SA, there are only a few types of fermented milk. These products are yoghurt, cultured buttermilk, and the traditionally fermented *aMasi*. *aMasi* is the only traditionally fermented milk that has been successfully produced on an industrial scale and is also referred to as *Maas* or *Inkomasi* on the commercial market (Kayitesi *et al*., 2017) and will be reviewed in more detail in this review.

2.3.1 Market Value

Business Monitor International (BMi) Research had reported on the market trends for the category of buttermilk and *aMasi* products in 2017. This category had experienced constant growth year-on-year in both value and volume in 2017. Buttermilk and *aMasi* value growth were attributed to an escalation in both industry prices and volume. The mean industry selling price for these products had also increased in 2017. This was caused by the fluctuating price of raw milk after seasonal drought as well as an escalation in distribution costs (BMi, 2018).

Product distribution with a combined volume share of 81.3% had dominated in 2017 with forecourts and retail sectors (low- and high-end retailers) being in the forefront. Low-end retailers accounted for 20.9% of the total volume sold in 2017 as compared to the 20.6% share which was achieved in 2016 (BMi, 2018). This channel was predominated by regional players who sold larger pack sizes to their customers. This channel also encompassed farm stores which enabled customers to purchase directly from producers. Accounting for 60.4% of the total volume sold in 2017, forecourts and high-end retailers were the most predominant in this channel. This was mainly attributed to a variety of brands sold and price points that are typical with these channels (BMi, 2018).

The core region for the manufacturing and sales of *aMasi* and cultured buttermilk products was KwaZulu-Natal (KZN) as this province had accounted for 35.7% of the overall volume in 2017. This province has a considerable number of farmers that produce *aMasi* in comparison to the other provinces mainly since this product is considered to be a staple diet component to consumers in this region in comparison to the other regions. The mean recommended retail prices of these products were also marginally lower in KZN as compared to the other

provinces as this could be attributed to the increased competition in the region. The excess volume of *aMasi* produced in KZN is distributed throughout SA (BMi, 2018).

These figures only pertain to the established commercial sector and do not take into consideration home or traditional production which is still well practised in rural settings. Products in this category are contemplated to be an affordable source of protein that performs well when the economy is stagnant. Given this fact, products in this category are expected to expand in the short to medium term. Fermented milk, therefore, appears to have great economic prospective in the dairy industry (BMi, 2018). *aMasi* and cultured buttermilk are part of the staple diets in SA as these products are often used for culinary purposes or consumed with maize. However, despite their popularity among South Africans, there is a need for the development of novel or different types of fermented milk.

2.3.2 Production of *aMasi*

2.3.2.1 Traditional production

The production of *aMasi* has evolved over the decades with the first scientific evidence of traditional production being documented in 1939 (Du Plooy *et al*., 2017). Constituted as South African heritage, *aMasi* has been contemplated as a supplementary staple food. Traditionally, this fermented milk is produced at household level, to generate income to local entrepreneurs and form a nutritionally significant constituent to the diets of the rural population (Du Plooy *et al*., 2017).

In the past, fermentation was a common practice to preserve milk amongst disadvantaged communities where access to electricity was poor and facilities for cold storage were scarce. During the process of fermentation, LAB produces lactic acid which initiates a decline in the pH of the milk which causes the milk to coagulate and inhibits the growth of microorganisms responsible for deterioration and spoilage (Osvik *et al*., 2013). This fermented milk was termed "*aMasi*" which is the isiZulu word or "*Maas*" which is the Afrikaans word to describe this popular and diverse beverage in SA (Beukes, 1999).

Presently, it is still regularly produced and consumed by cattle-owning families in the indigenous rural areas of SA. Households commonly generate continuous supplies of *aMasi* using traditional milk fermentation techniques (Osvik *et al*., 2013). This is often achieved by modifying the temperature and duration of fermentation to achieve the desired thickness and taste of the product. Seasonal composition and the availability and volume of fresh milk in each household influences the quantity, bitterness and quality of *aMasi* (Osvik *et al*., 2013).

aMasi is traditionally prepared by collecting unpasteurised milk and pouring into a calabash (sealable container). The calabash may be pre-smoked to serve as a preventative measure

against mould growth. To prevent contamination, the calabash is then sealed and usually stored indoors in a warm environment with an ambient temperature of > 20°C (Dlamini and Buys, 2009). The fermentation process occurs within three to five days. The presence of naturally occurring bacteria in the milk together with the presence of residual bacteria within the container or contamination during the milking process accelerates the fermentation process. As time progresses, the milk separates and the whey is removed at regular intervals resulting in a thicker white coagulant referred to as *aMasi* – a product formed when it has acquired the desired qualities (Kayitesi *et al*., 2017).

Furthermore, it also results in the selection and adoption of LAB strains that dominate the fermentation. The stock is replenished by adding more unpasteurised raw milk which interacts with the internal surface of the calabash providing a suitable environment for bacterial growth to thrive. Pre- or post-fermentation has rarely been reported at household level together with heat treatment or filtering prior to consumption. This requires further investigation due to the potential microbial diversity and public health risks of traditional dairy products (Osvik *et al*., 2013).

2.3.2.2 Industrial production

In the commercial manufacturing of *aMasi*, raw milk is supplemented with additives (0.5% of gelatine and 3.0% of skim milk powder) to stabilise the product prior to pasteurisation. Gelatine is a stabiliser that serves the function of preventing syneresis and providing the final product with a smooth and uniform texture whilst, skim milk powder is used as a fortifying and stabilising agent to amplify the functional properties of *aMasi* by preventing syneresis and enhancing the nutritional profile (Kayitesi *et al*., 2017).

The milk then undergoes high-temperature, short-time (HTST) pasteurisation at 72°C for 15 seconds to eradicate foodborne pathogenic and spoilage microorganisms followed by instant cooling to approximately 30°C to prevent the growth of spore-forming microorganisms. Mesophilic lactic starter cultures such as *Lc*. *lactis* subsp. *lactis* and *Lc*. *lactis* subsp. *cremoris* are supplemented into the milk to provide an initial inoculum concentration of 10^6 CFU/ml. The product is then subjected to 24 hours of incubation at 30°C which is thereafter stored at refrigeration temperatures (4°C to 7°C) (Kayitesi *et al*., 2017).

2.3.3 Microbial Diversity of Fermented Milks

Literature with respect to the microbial composition of fermented milk was evident after the 19th century (Lekofa, 2009). Early investigations by researchers such as Metchnikoff and Grigoroff had reported on a diverse range of microorganisms present in fermented milk. In 1907, *Bacillus bulgaricus*, cocci and yeasts were identified by Metchnikoff in yoghurt whilst,

Grigoroff in 1905, had discovered *Bacillus* A, a rod-shaped bacterium isolated from Bulgarian milk (Lekofa, 2009).

With the advancement of food microbiology over the years and the existing quantity of knowledge available, it has become evident that the microflora of fermented milk is comprised of a variety of LAB strains belonging to species of the genera *Bifidobacterium*, *Lactobacillus*, *Lactococcus* and *Leuconostoc*. Yeasts and milk moulds in synergistic growth with LAB also exist in minor proportions (Lekofa, 2009).

South African fermented milk such as traditionally produced *aMasi*, is predominated with LAB species belonging to the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc*. Other groups that were identified had included pyogenic enterococci and streptococci (Beukes *et al*., 2001).

2.4 DETECTION AND ENUMERATION OF BACTERIA IN MILK

Research is presently ongoing in this field of research as new LAB need to be identified and characterised (Abriouel *et al*., 2015a). Having mentioned this, biochemical-based techniques for identifying LAB may be inconclusive in several cases due to LAB having similar growth and nutritional requirements. Conventional microbiological methods for bacterial identification was once regarded as the 'golden standard' and is still widely used today (Franco-Duarte *et al*., 2019). These techniques are based on physiological and morphological characteristics such as the fermentation of different carbohydrates, enzyme production, Gram staining, cell shape and spore formation (Moraes *et al*., 2013, Fguiri *et al*., 2015).

Biochemical tests and phenotypic identification have long been used for the conventional identification of LAB. Thus far the use of DeMan, Rogosa and Sharpe (MRS) Agar for LAB and M17 Agar for bifidobacteria are extensively used for the growth, identification and enumeration of LAB. However, in recent years, several methods have been established and several multi-test kits such as the Analytical Profile Index (API) Kits are widely used (Fguiri *et al*., 2015, Franco-Duarte *et al*., 2019).

Molecular techniques are presently being applied for the routine identification of microbes. This has led to an increase in the number of LAB identified. Many molecular methods that exist are based primarily on 16S rDNA sequences, partial or complete genomes. van Hijum *et al*. (2013) had reviewed the developments of molecular techniques for investigating the diversity of microbes in traditionally fermented foods particularly emphasising the application of novel sequencing technologies to generate metagenomes, metatranscriptomes and phylobiomes coupled with the related post-sequencing analyses, and in-depth insights in the responsible microbial genomes are described in Table 2.1.

As with any method, there are always pros and cons. With the conventional microbiological methods, the pros are that they are reliable, cost-effective, highly sensitive and provide both quantitative as well as qualitative results on the bacterial populations in a given sample. The cons are that the samples are often mixed cultures including pathogens as well as the normal microflora. In these cases, purifying the culture by isolation to obtain pure cultures would take several growth steps that are necessary to characterise the bacteria in-depth. As a result, these methods are often time-consuming (since results would only be available after one to three days) and labour intensive (Fguiri *et al*., 2015).

Molecular methods, on the other hand, have substantially revolutionised food microbiology. They have become more convenient than the conventional methods as they are faster, precise, more specific and preceding cultivation of microorganisms is not necessary. In contrast to conventional methods, the identification and characterisation of unculturable or slow-growing pathogens are easier to implement. Furthermore, these methods can be performed, and results can be analysed by someone who has no, to little expertise. These methods also provide more stable characteristics than conventional methods using phenotypic characteristics (Fguiri *et al*., 2015). These methods do have limitations. The limits of these advanced techniques have been questioned due to the extent of their database, the need for complex procedures, trained personnel, specialised and expensive equipment (Fguiri *et al*., 2015).

Table 2.1: Techniques suited for the identification of LAB from traditional fermentations

Technology/ technique	Description
16S rDNA sequencing	PCR amplicons are sequenced based on one or multiple variable (V) regions on the bacterial 16S gene. The amplicons can then be sequenced using Roche 454 for example or paired-end 100 bp Illumina. With the Illumina, the 16S amplicon is read from both directions where the choice of the parts V regions represented by both directions is critical.
Assembly	Sequence reads are aligned and integrated into larger contiguous sequences (contigs). Due to the complexity of metagenomic samples, factors such as the complexity of microbial population, sequencing depth and the sequencing platform dictates assembly success i.e. the error rate, the presence of chimeric sequences, length and coverage of contigs of the entire metagenome.
ARDRA	Amplified rDNA restriction analysis. An adaption of RFLP to create 'fingerprints' from the 16S rRNA gene that can be analysed on an agarose gel.
DGGE of 16S rRNA	Enables 'community fingerprinting' by PCR amplified DNA of 16S or 18S rRNA from mixed microbial communities to visualise variations in microbial diversity and give an estimate of richness/abundance of predominant microbial members.

Illumina	Sequencing method that generates millions of short reads from a single lane up to 100 bp.
Metagenomics	Application of sequencing methods to DNA obtained directly from a given environmental sample. Sequence reads can either be used or assembled into contigs for determining the prevalence of opening reading frames specifying molecular functions.
RNA-seq	Application of next-generation sequencing to cDNA reverse transcribed RNA.
Roche 454	Similar to the Illumina except that it can generate approximately one million longer reads from a single plate (450 - 700 bp).

Adapted from: van Hijum *et al*. (2013).

2.5 BIOCHEMICAL ACTIVITIES OF LACTIC ACID BACTERIA

2.5.1 Biochemical Activities

Microorganisms can deteriorate food and cause illness to humans due to their biochemical activities (Nychas and Panagou, 2011). However, some microbial biochemical activities are beneficial in the manufacturing of fermented milk products such as butter, cheese, yoghurt, etc. (Ghosh *et al*., 2019). The activities of specific microorganisms that are employed in the production of fermented milk products are regulated by the enzymes that they possess. These enzymes determine what the microbes can feed on and break down as well as result in the end products produced (Bylund, 2003).

Microorganisms possess several biochemical and enzymatic systems. The following systems covered in this review focuses on the main systems pertaining to milk and milk products which are sub-divided into which elements they break down and the effects that they have. The most significant enzymatic systems and biochemical activities of bacteria in milk products are those responsible for breaking down fats, lecithin, protein and carbohydrates; producing odours, slime or mucus as well as oxygen reduction and pathogenicity by foodborne pathogens. However, this review will cover the breakdown of carbohydrates, lipids, lecithin and pathogenicity in more detail.

2.5.1.1 Breakdown of carbohydrates

Most monosaccharides have the general empirical formula $(CH_2O)_n$. This is because they contain carbon, hydrogen and oxygen atoms (Solomon *et al*., 2010). Three types of saccharides exist i.e. mono-, di- and polysaccharides. Starch, chitin and cellulose are examples of polysaccharides which are composed of long chains of single or multiple sugars. The breakdown of carbohydrates and the extent to which it is broken down is determined by the enzymes the microorganism has. In milk, lactose which is a disaccharide is broken down into galactose and glucose via a process called hydrolysis (Fox *et al*., 2015a). These

constituents can further be degraded completely to CO_2 and water (which is oxidative metabolism), but fermentation occurs in the majority of cases (Bylund, 2003).

End products such as alcohols, gases and organic acids are produced as a result of fermentation (Ciani *et al*., 2008). There are many several types of fermentation that are significant due to the activity of microflora present in milk such as alcoholic fermentation, coliform fermentation, butyric acid fermentation and lactic acid hetero- and homofermentation (Bylund, 2003). Alcoholic fermentation involves the fermentation of carbohydrates to gas and alcohol. An example of this is the degradation of lactose to CO_2 and ethyl alcohol. Such fermentations generally take place in anaerobic environments (it can occur aerobically as well) and is primarily induced by yeasts (Dashko *et al*., 2014). Coliform fermentation (a mixture of butanediol and acids) with lactose produces an array of end products such as organic acids (acetic acid, formic acid, lactic acid and succinic acid), alcohols (butanediol and ethyl alcohol) and gases (CO_2 and hydrogen) (Rogers *et al*., 2013). Butyric acid fermentation occurs under an obligate anaerobic environment by a single strain of *Clostridium* bacteria which is spore-forming. In this type of fermentation, lactose is degraded to produce acid (butyric acid), gases (CO_2 and hydrogen) and in some cases alcohol (butyl alcohol) as end products (Ciani *et al*., 2008).

Lactic acid fermentation occurs in two ways viz. homofermentation and heterofermentation. Lactic acid fermentation by homofermentation of lactose results in lactic acid being the sole end product from the fermentation (Gänzle, 2015). This reaction is employed in the production of acidified products such as cheese, yoghurt, etc. Lactic acid heterofermentation of lactose, on the other hand, produces lactic acid with other organic acids (acetic acid), alcohol (ethyl alcohol) and gas (CO_2) (Gänzle, 2015). The main purpose of carbohydrate fermentation in milk is to consequently produce acidity in products (souring) and in some cases, gas (depending on the bacteria or yeasts used).

2.5.1.2 Breakdown of lipids

Milk fat is broken down by an enzymatic process called lipolysis. The key enzyme implicated in this process is called lipase. During this process, milk fat is hydrolysed to a glycerol molecule and either one, two or three separate fatty acids. Certain fatty acids are volatile and effuse strong odours. An example of this is butyric acid which imparts a rancid taste (Bylund, 2003). Microorganisms are incapable of degrading pure fat; however, they can break down fat when it exists in water or as a fat-in-water emulsion. Water plays an integral role in the enzymatic split. Milk fat in water emulsions such as cream and butter contains carbohydrates, proteins, minerals, etc. which occasionally makes it more prone to enzymatic breakdown. Proteins that are broken down by several bacteria and moulds can also break down fat oxidatively (Bylund, 2003).

2.5.1.3 Breakdown of lecithin

Membranes encompassing the fat globules constitutes of a phospholipid called lecithin. Lecithin is comprised of glycerol, two fatty acids, choline (organic alkali) and phosphoric acid. Enzymes produced by strains of *Bacillus cereus* are called lecithinases. These enzymes hydrolyse lecithin into phosphorylcholine and diglyceride. Sometimes clumps and flocs are present on the surface of the cream or milk, this is a result of the membranes of the fat globules splitting, which results in the formation of an unstable fat emulsion. This undesirable trait is often termed bitty or broken cream. Further degradation of choline into trimethyl would result in fishy odours and taste (Bylund, 2003).

2.5.1.4 Pathogens in milk and fermented milk

Food poisoning is caused by foodborne pathogens in two ways: a) by infection and/or b) by intoxication (WHO, 2019). Foodborne pathogens responsible for intoxication or infection are listed in Table 2.2. Infection implies the establishment, constant proliferation and manifolding of foodborne pathogens in the human host. In most cases, a significant number of foodborne pathogens are required to induce infection, however, sometimes pathogens like *Salmonella* Typhimurium, have a minimum infectious dose (MID) of one bacterium. In contrast to this, intoxication points to the yield of poisons in the food prior to its consumption (Bylund, 2003).

The incidence of foodborne pathogens and foodborne illnesses have been reported in fermented dairy products. In earlier studies by O'Mahony *et al*. (1990) and Dalu and Feresu (1996), foodborne pathogens such as *Listeria monocytogenes* and *Clostridium botulinum* were present in Zimbabwean industrially fermented milk samples and yoghurt samples, respectively. In the former study, *L*. *monocytogenes* in Zimbabwean industrially fermented milk samples was more prevalent at refrigerated temperatures whilst, *C*. *botulinum* found in yoghurt samples in the last-mentioned study, had resulted in 27 cases and 1 death. The cause behind this outbreak was due to insufficient processing of hazelnut conserve employed as a flavouring.

On the other hand, Cancino-Padilla *et al*. (2017), reviewed a high prevalence of foodborne pathogens particularly in cheese products in the North and South American regions and European region. The foodborne pathogens reviewed by the authors included *Salmonella*, *Salmonella enterica*, *L*. *monocytogenes*, *Escherichia coli*, *E*. *coli* O157:H7 and *S*. *aureus*. Furthermore, incidences across these regions had accounted for approximately 888 cases. More recently, in November 2019, cheese products had to be recalled due to potential *E*. *coli* contamination in Australia (Gerova, 2019).

Table 2.2: Foodborne pathogens in milk responsible for infection or intoxication

Infectious	Toxin producers
Campylobacter	*Bacillus cereus*
Corynebacterium diphteriae	*Clostridium perfringens*
Escherichia coli (selected strains)	*Staphylococcus aureus* (selected strains)
Listeria monocytogenes	
Mycobacterium bovis	
Mycobacterium tuberculosis	

Adapted from: Bylund (2003).

2.6 FOOD SAFETY OF LACTIC ACID BACTERIA

Lactic acid bacterial strains are used in several food manufacturing facilities to induce microbial fermentation in products such as dairy products, cheese, sausages and ham. Moreover, probiotics have become widely accessible on the market with products containing either a single strain or multiple strains. These bacteria must meet several criteria before they can be classified as probiotics which confer beneficial health properties to the host (Florou-Paneri *et al*., 2013).

The criteria to be met include: a) scientifically proven health benefits; b) have good technological attributes implying that their manufacturing and incorporation into food products must not lose viability; c) high survival through the upper GI tract and high viability at its site of action; d) antagonistic activity to pathogens; e) to be functional in the gut environment and lastly, f) antibiotic susceptibility which is the main focus in this project. Keeping in mind the impact and significance of AR in LAB in the food chain, the most vital criterion is that of antibiotic susceptibility when it comes to the selection of probiotic strains (Radulović *et al*., 2012, Florou-Paneri *et al*., 2013).

Given the fact that LAB naturally predominates the microflora of traditionally fermented foods and the GI tract, they are also intentionally incorporated as probiotics or starter cultures in the industrial production of fermented foods. Due to the long history of use in the food, feed and pharmaceutical industries, most LAB have acquired regulatory statuses such as GRAS and QPS (Radulović *et al*., 2012, Florou-Paneri *et al*., 2013).

Over the last five decades, food safety has been in the spotlight with respect to the spread of AR. The misuse of antibiotics in human medicine, agricultural and animal husbandry has led to extreme selective pressure for the emergence of resistant bacterial strains. The concern has been emphasised on pathogenic bacteria and their resistance to antibiotics, seeing that infections induced by these resistant bacteria have not only become more difficult to treat, but treatment and care has also become more intensive and time-consuming increasing the cost of treatment (Abriouel *et al*., 2015a, Founou *et al*., 2016).

Considering that LAB inhabits the GI tract in abundance and are also deliberately added to certain food products, concerns have been raised with respect to AR in these economically important bacterial species (Verraes *et al*., 2013). Although LAB having resistance to certain antibiotics could be beneficial to humans by helping to regulate the gut microflora in cases of diarrhoea induced by antibiotic treatment, there is, however, a risk. LAB have MGE such as plasmids which are contemplated to harbour AR genes (Sharma *et al*., 2015). The ability of these resistant LAB strains to transfer resistance genes to other, possibly pathogenic bacteria through bacterial conjugation, poses a risk that could make the treatment of a patient with an antibiotic-resistant bacterial infection or disease more complex (Abriouel *et al*., 2015a). Therefore, there is a need to evaluate the safety of potential probiotic and/or starter culture strains for acquired resistance and their dissemination. Furthermore, it is also essential to distinguish between intrinsic and acquired resistance genes.

2.7 CURRENT SITUATION OF ANTIBIOTIC RESISTANCE IN SOUTH AFRICA

Antibiotic resistance has been given a low priority in most developing and developed countries. With specific reference to SA in the present study, it was mentioned that very little attention was focused on AR (Moyane *et al*., 2013). This adversely impacts our society, as the misuse of antibiotics over the last seven decades have induced a 'post-antibiotic era' upon us (Mendelson, 2015). Internationally, only a few developed countries such as Denmark, The United Kingdom, Sweden and The Netherlands have managed to curb antibiotic consumption although, not always resulting in a substantial decrease in resistance (Moyane *et al*., 2013, Founou *et al*., 2016).

Schellack *et al*. (2017), had mentioned that a pre-requisite to understanding AR requires the availability of reputable data on antibiotic consumption. The misuse of antibiotics for prophylaxis, growth promotion and disease treatment in developing countries are significantly higher than that of developed countries (Founou *et al*., 2016). The World Health Organisation (WHO) had launched an initiative called the Global Action Plan on AR which contributes to a 'One Health' strategy for a national action plan development across animal, environmental and human health. South Africa has adopted this blueprint which aims to optimise the use of antibiotics in human and veterinary health as well as to amplify the knowledge and validation base through research and surveillance (Mendelson, 2015, Schellack *et al*., 2017).

Despite having this strategy in place, the paucity of antibiotic consumption data worldwide exists, with SA included. Several researchers have reported significant gaps in data sharing, standardised methodologies and in monitoring and surveillance with regards to (WHO, 2014, O'Neill, 2016, Tadesse *et al*., 2017, Tang *et al*., 2017). Furthermore, there is paucity in data regarding the volume of antibiotics used for animal husbandry and information about patterns of consumption of antibiotics among food animals also lacks. Having mentioned this, it isn't

surprising to note that data on the quantities used for specific purposes in agriculture and human medicine are also scarce (Moyane *et al*., 2013, Schellack *et al*., 2017).

2.7.1 Bacterial Resistance Rates to Antibiotics and Resistance Patterns in Humans

A *Klebsiella pneumoniae* strain which was identified in 2011, is currently resistant to all documented antibiotics available. This was noted as the most extreme case of a multi-drug resistant bacterial infection in SA. Surveillance data in SA has confirmed an increasing trend across infections caused by notable bacteria (South African Department of Health, 2015a).

There are two distinct classes of bacteria namely, Gram-positive and Gram-negative. However, innovative antibiotics created are not only expensive, but some have not yet been licensed for use in SA. Furthermore, these drugs only cater for infections induced by Gram-positive bacteria. On the other hand, infections caused by Gram-negative bacteria such as abdominal and urinary tract infections would be more challenging as there is no provision for novel antibiotics expected in the next 15 to 20 years, implying that strict conservation of these antibiotics used for treating infections induced by Gram-negative bacteria has to be adhered to (South African Department of Health, 2015a).

With respect to resistant patterns in humans, there are a few bacteria that have acquired resistance that make infections more difficult to treat (South African Department of Health, 2015a). It was shocking to note that limited information exists regarding diseases or infections caused by antibiotic-resistant bacteria. The main cause of this could be attributed to the fact that causes of illnesses and deaths are not well counted, as is often the case in developing countries where funding and technical resources are scarce (Moyane *et al*., 2013). Presently, these bacteria are carbapenemase-producing *Enterobacteriaceae* (CPE), extended-spectrum beta-lactamase-producing Gram-negative bacteria (ESBL producing Gram-negative bacteria), vancomycin-resistant enterococci (VRE) and MRSA (Mendelson, 2015, South African Department of Health, 2015a).

2.7.2 Resistance Patterns in Food-Producing Animals

There is a scarcity of documented research and surveillance on the resistance rates for bacteria in food-producing animals (Moyane *et al*., 2013, Schellack *et al*., 2017). In 2007, a collaborative surveillance activity between SA and Sweden had led to the surveillance and documenting of bacterial AR rates in food-producing animals from laboratories in all nine provinces. However, funding for the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs was discontinued after the completion of the grant from the National Research Foundation. The study did, however, highlight high rates of sulphonamide and tetracycline resistance in *Enterococcus* spp., *Escherichia coli* and *Salmonella enterica* (South African Department of Health, 2015a).

2.7.3 Present Efforts to Address Antibiotic Resistance in South Africa

Several initiatives have been taken by SA to manage AR. In 2011, as part of a collaboration between South African stakeholders and the Global Antibiotic Resistance Partnership in South Africa (GARP-SA), a situational analysis based on antibiotic use and resistance was published. The analysis was inclusive of AR in both veterinary and human health and had identified several limitations to implementing good practices for infection control and antibiotic stewardship initiatives (South African Department of Health, 2015a, Schellack *et al*., 2017). Representing the major burden of diseases in SA, are infections. This largely comprises of the human immunodeficiency virus (HIV) and tuberculosis (TB) as well as high rates of hospital and community-acquired infections (Moyane *et al*., 2013, Schellack *et al*., 2017).

Schellack *et al*. (2017), had reported that data from the Intercontinental Marketing Services, now known as IMS Health, in their 2011 analysis had fallen short of the true reflection of antibiotic consumption in SA. At the time, whilst data collection was still in its infancy, the collection of information regarding antibiotic consumption from the public sector required manual computation from accumulated data methods to explicate consumption in nosocomial settings. In addition to this, fluid catchment populations had also hindered the progress of acquiring accurate consumption data from communities. This, therefore, led to the paucity of trustworthy data which precluded antibiotic stewardship initiatives and the evaluation of these stewardship initiatives (Schellack *et al*., 2017).

Moyane *et al*. (2013) had mentioned that despite SA having the most active surveillance for AR in Africa, the country had not yet fully translated available AR surveillance data into policy and therefore, there was no evidence of any on-going AR surveillance for pathogens in SA. However, six years later, there has been much progress and a major shift was observed in policies towards combating AR in SA.

Several initiatives now exist to curb AR. The South African Antibiotic Stewardship Programme (SAASP) that was established in 2012, comprises a group of multidisciplinary experts from both veterinary and human health in the public and private health sectors. It was established to implement antibiotic stewardship programmes within nosocomial environments and advocates associations with increasing global drivers of change. Such drivers of change include strengthened partnerships for antimicrobial surveillance, efforts to ensure unceasing access to essential medication of high quality, enhanced control for the prevention of infection and efforts for the stimulation of new research and innovations (South African Department of Health, 2015a, Schellack *et al*., 2017).

After this initiative, much anticipation from the National Department of Health had culminated in a document entitled “South Africa’s Antimicrobial Resistance Strategy Framework 2014-2024”, with other initiatives such as Infection Prevention and Control and National Core

Standards (IPC and NCS) and Expanded Programme for Immunisation (EPI) which focused on antibiotic stewardship, infection control and pneumococcal conjugate vaccination in children and adults initiatives (South African Department of Health, 2015a, Schellack *et al*., 2017). However, there is a need to address AR by determining antibiotic utilisation across all sectors.

2.7.4 Limitations of These Initiatives

The initiatives taken to monitor AR in the country either emphasises only on resistance rates in the nosocomial setting or more recently on food-producing animals, however, despite SA having the most active surveillance for AR in Africa (Moyane *et al*., 2013, Mendelson, 2015, Schellack *et al*., 2017), there is paucity with regards to AR in the food chain and the possibility of LAB being resistant to antibiotics. The link between food-producing animals and human consumption via the food chain needs to be thoroughly investigated and researched to better understand resistance rates along the food chain.

Furthermore, there is a lack of published data regarding antibiotic consumption patterns for both human and animals. This could be due to a lack of resources, funding, electronic data capturing and linking facilities, access to laboratory facilities, inadequate legislation, trained personnel, etc. (Osvik *et al*., 2013, South African Department of Health, 2015a).

2.8 ANTIBIOTIC RESISTANCE IN THE FOOD CHAIN

2.8.1 The Development of Antibiotic Resistance

The introduction of antibiotics and drug innovation more than five decades ago has been a revolutionary discovery that has been implemented in both veterinary and human health to treat infectious diseases (Darsanaki *et al*., 2013, Verraes *et al*., 2013, Sharma *et al*., 2015). Furthermore, antibiotics have also been used to cure or serve as a preventative measure against bacterial infections in agriculture (Abriouel *et al*., 2015a). Their intensive and extensive use over the years has exerted pressure for the emergence and spread of AR bacteria (Nawaz *et al*., 2011, Abriouel *et al*., 2015a, Sharma *et al*., 2015).

According to Founou *et al*. (2016), antibiotics have become an “endangered species” that are currently facing extinction. This is due to the emergence of AR globally and the slow development of new therapeutic drugs to aid in the treatment of resistant bacteria. Several cases of treatment failure have been reported in patients suffering from infections caused by extensive-, pan- and multi-drug resistant bacteria.

According to Darsanaki *et al*. (2013), AR can be defined as the capacity of a microbe’s resistance to growth, killing or inhibitory action of an antimicrobial that is out of the specified susceptibility limit of the bacterial species of concern. Antimicrobials consist of any substance

that has a bacteriostatic or bactericidal influence on bacteria in a clinical setting for eliminating bacterial loads in surfaces and materials. These encompass antibiotics which are applied in treating bacterial infections in both animals and humans and, chemical biocides which are applied for the disinfection in food processing environments (Ullah and Ali, 2017). Resistance can be acquired by a microbe to an antimicrobial to which it was initially susceptible to, implying that the antimicrobial would be ineffective (in terms of bacteriostatic or bactericidal effect) to a microbe at the onset of treatment (Verraes *et al*., 2013). When this occurs, it becomes mandatory that other "last resort" alternatives be used which are more costly and/or more toxic (Founou *et al*., 2016).

The emergence and selection of resistant bacteria are caused by the misuse of antibiotics which is believed to be the main factor, with healthcare facilities and communities forming the primary ecological niches of occurrence in human health. The misuse of antibiotics in animal husbandry for growth promotion, therapeutic use or prophylactic use has however contributed to the dissemination of AR which is a global challenge (Zaman *et al*., 2017). The emergence of AR is a complex phenomenon. This phenomenon involves interactions between animals, bacteria, drugs, humans and the environment (Bester and Essack, 2010, Abriouel *et al*., 2015a) and is therefore contemplated to be a cross-sectoral problem due to three main reasons. The first reason is that antibiotics are widely used in animal husbandry and agriculture. The second reason is that AR bacteria and AR genes can be disseminated easily at any stage of the food production chain (Founou *et al*., 2016). Such AR bacteria have become resistant to ensure survival which poses a serious risk to not only veterinary and human medicine but, affecting food production as well, which is the third reason (Sharma *et al*., 2015).

The outcome of this poses a serious threat to public health and food safety (Nawaz *et al*., 2011). Several studies in literature have reported on food animals and products being colonised and/or infected and contaminated with antibiotic-resistant strains (Ewnetu and Mihret, 2010, Fischer *et al*., 2012, Price *et al*., 2012, Al Bayssari *et al*., 2015). The most infections being MRSA, AR *Campylobacter* spp. and ESBL producing-*Enterobacteriaceae* (namely *Klebsiella* spp., *Salmonella* spp. and *Escherichia coli*) (Ekwanzala *et al*., 2018). These mentioned strains have been recognised to have originated from animals and are currently associated with various hosts adaptability, high genetic exchanges and virulence mechanisms. It is due to these associations, exchanges and mechanisms that pose a serious threat globally as they could possibly lead to the development of new and more resistant, virulent mobile strains unidentified to the human immune system (Verraes *et al*., 2013, Founou *et al*., 2016).

The food chain is contemplated to be one of the primary routes for the transfer or AR from animal to human bacterial populations – a hypothesis that is supported by several studies in

literature (Sharma *et al*., 2015, Cahill *et al*., 2017, Zaman *et al*., 2017). Precisely, fermented foods that do not undergo pasteurisation or some sort of heat treatment before consumption serves as a vehicle for the transfer of AR genes from the microbiota inherent of animals to the commensal bacteria of the digestive system in humans (Sharma *et al*., 2015).

According to Founou *et al*. (2016), humans come into contact with AR bacteria in two ways. The first way is through indirect contact along the food chain by consuming contaminated food and the second way is through direct contact with infected livestock or biological substances such as blood, saliva and excreta among others. Seeing that humans have direct contact with animals, it is imperative to prevent the zoonotic transmission of AR bacteria and AR genes from food animals-associated reservoirs to humans.

It was observed in literature that several high resource countries (HRCs) such as Denmark, Netherlands, Japan, Sweden, the European Union (EU) and United States (US) have successfully implemented surveillance programs for monitoring antibiotic usage and AR in food animals, humans and food products (Moyane *et al*., 2013, Founou *et al*., 2016). Initiatives have also been taken in low- and middle-income countries (LMICs) to contain AR, however, the main focus in these countries is on human health and not the food chain.

In 2014, SA had implemented The National Framework of Antimicrobial Resistance for 2014-2024 to aid in combating AR (South African Department of Health, 2015b). India had also developed and implemented national guidelines for the use of antibiotics in 2013 (Kumar *et al*., 2013). A national strategy for emerging diseases inclusive of AR was implemented in Thailand together with a policy for rational drug use in 2011. Presently Thailand has an AR Containment Program for 2012-2016 that is in place (Sumpradit *et al*., 2012).

Despite the vast majority of LMICs having these surveillance programs in place, they still, however, lag far behind HRCs with respect to containing AR in general as well as specifically curbing AR via the food chain as significant risk factors for communicable diseases and considerable amounts of animal-occupational exposure exist in LMICs. AR in food animals in LMICs are particularly a true burden since they are rarely documented (Moyane *et al*., 2013). Furthermore, this burden is a threat to the food chain and is often under-estimated in LMICs. Seeing that there is an increasing trend in the human population, international travel, host migration and the globalisation of trade in food and animal products, AR is easily disseminated globally via the food chain. If surveillance programs employed are not effectively applied locally, regionally, nationally and internationally, the inadequacy of intervention in one country or continent for example, in developing countries, can endanger the efficacy of policies of curbing AR employed in other countries worldwide, inclusive of HRCs with the best surveillance for AR (Founou *et al*., 2016).

2.9 TYPES AND MECHANISMS OF ANTIBIOTIC RESISTANCE

2.9.1 Types of Antibiotic Resistance

According to Verraes *et al*. (2013), there are three types of AR – *in vitro* or microbiological resistance, pharmacological resistance and *in vivo* or clinical resistance. The first type of AR which is *in vitro* resistance is described as the bacterium's reduced susceptibility to antibiotics exceeding the specified limit of normal susceptibility of the bacterium in question. This is also referred to as epidemiological resistance (Verraes *et al*., 2013). Furthermore, it can be confirmed by molecular techniques such as genotypically, by establishing the presence of a specific AR gene or resistance mechanism (Bester and Essack, 2010, Verraes *et al*., 2013). Pharmacological resistance, the second type of AR, is based on normal susceptibility and pharmacokinetic parameters. A bacterium is susceptible to an antimicrobial if the minimum inhibitory concentration (MIC) for the specific bacterium meets the specified concentration range that can be acquired by that specific antimicrobial. A bacterium would be resistant when the MIC of an antibiotic significantly exceeds the concentration acquired from the site of infection (Bester and Essack, 2010, Verraes *et al*., 2013). The third type of AR, i.e. *in vivo* resistance is when the onset of a bacterial infection caused by a certain bacterium, would no longer be treated effectively hence, making treatment failure evident (Verraes *et al*., 2013).

2.9.2 Mechanisms of Antibiotic Resistance

There are two main biological pathways that are involved in the evolution of the emergence of AR. The first scenario of resistance describes natural bacterial populations that have pre-existing phenotypes. Throughout the evolutionary process, bacterial cells were able to accumulate genetic errors in the present genes (plasmid or chromosome). The transfer of resistant genes to the offspring cells was via vertical gene transfer (VGT) which lead to intrinsic resistance (Founou *et al*., 2016).

The second case of resistance is referred to as acquired resistance, which relates to genetic exchanges within and between bacterial species. This points towards HGT. According to Munita and Arias (2016), HGT encompasses three main mechanisms among bacteria i.e. conjugation, transduction and transformation. Such mechanisms may possibly occur in the GI tract of humans, the food that we consume as well as in the environment (soil and water).

Genetic structures like integrons, transposons and more especially plasmids significantly enhance HGT of AR genes, their sustenance in bacterial populations and generating multidrug resistance. These are known as mobile genetic elements since they embody a collection of mobile genetic material. HGT frequency is greatly dependent upon the characteristics of the donor and recipient populations, the properties of the MGE and the environment (Verraes *et al*., 2013).

The first type of mechanism is conjugation. This is referred to as the transmission of genetic material among living bacterial cells, furthermore, it needs absolute contact between the donor and recipient cells. Plasmids and transposons frequently have AR genes present on them (Bester and Essack, 2010). These can be additionally associated with genetic structures. Within bacterial cells, translocation of insertion and transposons sequences occurs. Complex assemblies, on the other hand, result from plasmids and other genetic elements.

As a result of several AR genes being identified on transposons and plasmids, conjugation is therefore contemplated to be the primary mode of AR gene transfer between bacteria (Álvarez-Cisneros and Ponce-Alquicira, 2018). There are five molecular and epidemiological factors that limit this mechanism. The first factor is that contact should be permitted between the strains within the ecosystem. The second factor is that the strains should have specific mobility, whether influenced by external factors or by themselves (Verraes *et al*., 2013). The third factor is that there is an inaptness between plasmids preventing the transmission of plasmids within the same inapt group entering the same cell. The fourth factor is that a cell's genetic background should permit the integration of integrated mobile elements. Lastly, is that some MGE have sustenance systems that when lysed from the cell, it would result in cell death. Bacteria have adapted systems for plasmid transfer (Verraes *et al*., 2013). Basic conjugative steps, however, can be identified in all of these systems.

Furthermore, conjugation in Gram-negative bacteria appears to follow a common process beginning with the development of conjugative pili to facilitate contact between donor and recipient cells. According to Grohmann *et al*. (2003), cell contact by Gram-positive bacteria is achieved by using alternative mechanisms such as aggregation facilitated plasmid transfer in *Bacillus thuringiensis* ssp. *israelensis*.

The second type of mechanism is transduction which involves a bacteriophage transfer process. The first process involves the attachment of the bacteriophage to the bacterium whereby DNA from the bacteriophage is injected into the bacterium, which could include host bacterial DNA (Bester and Essack, 2010). Once inside the bacterial cell, the DNA is stabilised by combing with the bacterial DNA or by forming an autonomously replicating element. The production of new phage particles is influenced by the foreign DNA being stabilised in the bacterial cell (Bester and Essack, 2010, Verraes *et al*., 2013). Depending on the phage involved, genomic DNA (gDNA) or bacterial plasmid of varying lengths can now be transmitted from one bacterium to another. However, host specificity of bacteriophages limits this mechanism, therefore, it is common for translocation to occur among bacterial strains that are closely related. The transducing capacity of a phage can be wider, however, is not necessarily limited to bacteria which can be infected. Presently, the transfer of AR genes by transduction is rarely reported (Verraes *et al*., 2013).

The third type of mechanism is transformation, whereby raw DNA from environmental origin is absorbed in bacterial cells. Four steps describe the transformation process. In the first step, death and lysis of the cell occur submissively when the bacterial genetic material is primarily released from the cell. For some bacteria in the growth cycle, this occurs vigorously at a specific point in the cycle. In the second step, competent bacteria in the environment absorb the genetic material, thirdly, the genetic material survives the harsh influence of nucleases in the bacterial cell and is stable fused in the acceptor cell. In the final step, the incorporated DNA is expressed Verraes *et al*. (2013), lists many studies done on transformation.

According to Verraes *et al*. (2013), the consequences of AR gene transmission in foodstuffs presently, by transformation has not been demonstrated. The difficulty and reduced frequency to detect the event compared to conjugation could possibly be reasons for the abovementioned phenomenon. This decreased frequency is the outcome of the different requirements that have to be met before the consequence is a successful transfer of AR genes. In the animal and human GI tract, the susceptibility of DNA is due to the effects of DNA nucleases, chemical and physical deterioration (Bester and Essack, 2010).

This was demonstrated in a variety of food products and fruit juice. The complexity of the food matrix and food processing facilities can protect the DNA. Individual food components in the food matrix such as maltitol and arginine can also protect DNA. There is an inverse function of the stability of DNA during food processing and the DNA length. For stabilisation of DNA to occur in the recipient cell, the transformed DNA recombination has to occur with homologous regions in the resident chromosome or be available on a mobile genetic element such as a plasmid. Transformation by means of HGT was identified as a major barrier because of the absence of homologous sequences or origins of replication (Verraes *et al*., 2013).

2.10 PREVALENCE AND DISSEMINATION OF ANTIBIOTIC-RESISTANT DETERMINANTS IN LAB

Many dairy food productions involve lactic acid fermentation, which is initiated by LAB (Hickey *et al*., 2015). Furthermore, probiotics have become available worldwide on the market available as a single strain or a combination of strains (Fukao and Yajima, 2012). In recent years, food safety has been in the spotlight regarding AR of LAB which is believed to have acquired resistance genes (Nawaz *et al*., 2011). Therefore, in the interest of safety, it is essential to distinguish between intrinsic and acquired resistance genes (Fukao and Yajima, 2012).

In literature, AR profiles of lactobacilli have been reported in European and Asian countries. A study by Adimpong *et al*. (2012), focused on the AR of LAB isolated from traditional African fermented foods but, the status regarding AR of LAB in SA is unknown. Lactobacilli have been found to be susceptible to ampicillin and penicillin which are inhibitors of cell wall synthesis,

in contrast to vancomycin and trimethoprim which they are intrinsically resistant to (Fukao and Yajima, 2012). Lactobacilli have also been found to be susceptible to protein synthesis inhibitors (chloramphenicol, clindamycin and erythromycin). Furthermore, resistance to tetracycline and antibiotics of the aminoglycosides and glycopeptides group has been more common in lactobacilli (Fukao and Yajima, 2012, Darsanaki *et al*., 2013).

LAB have an innate resistance to an array of antibiotics and may have the ability to acquire resistance to other antibiotics or transmit their resistance determinants to other pathogens that reside in the GI tract of humans and animals. An example demonstrating this was in a study done by Shao *et al*. (2015), who indicated that two isolates of *Lb*. *plantarum* carried two genes, *aaad*A and *ant*(6) encoding for streptomycin resistance, and that overexposure to this antibiotic had led to the marked increase of MIC and increased cross-resistance to other antibiotics of the aminoglycosides class. Alternatively, Gad *et al*. (2014), had isolated strains from dairy and pharmaceutical products of Egyptian origin with 6% of the stains having resistance genes encoding for tetracycline *tet*(M) and/or erythromycin *erm*(B). Similarly, Erginkaya *et al*. (2018), had reported a high incidence of *Lactobacillus* isolated from Turkish fermented dairy products to be resistant to vancomycin (58%), gentamicin (48%), tetracycline (43%), ciprofloxacin (26%) and erythromycin (10.8%). A study by Kastner *et al*. (2006), had shown that the *tet*(W) for tetracycline resistance and *lnu*(A) for lincosamide resistance genes were present in probiotic commercial *Lb*. *reuteri* ATCC 55730. Furthermore, a study by Ouoba *et al*. (2008), had demonstrated the comparison of probiotic lactobacilli of European and African origin for their susceptibility to antibiotics. The results had detected acquired resistance genes for aminoglycosides, tetracycline and erythromycin. In contrast to this, Flórez and Mayo (2017), have demonstrated no dissemination of the *tet*(M) and *erm*(B) genes encoding for tetracycline and erythromycin, respectively from *S*. *thermophilus* to *Lb*. *delbrueckii* during the manufacture and storage of yoghurt.

Moreover, the food chain can facilitate the spread of antibiotic-resistant bacteria between animals, foods and humans through the fermented milk and meat products that we consume. These foods have demonstrated to be common vehicles for antibiotic-resistant bacteria that, when ingested can interact with the indigenous microflora of the GI tract as most of these foods are consumed without pasteurisation or heat-treatment (Mathur and Singh, 2005). Although some studies confirm the transmittance of resistance genes, the two most typical resistant genes in LAB are *tet*(M) and *erm*(B) which encode for tetracycline and erythromycin respectively succeeded by *cat* genes which encode for chloramphenicol resistance (Moračanin *et al*., 2017). Taking into account the various potential industrial applications of LAB in both human and animal health, there is a need for their thorough investigation that involves the detection of acquirable AR genes.

2.11 CONTAMINATION AND INCIDENCE OF ANTIBIOTIC RESISTANCE IN FERMENTED FOODS

2.11.1 Contamination of Food with Antibiotic-Resistant Bacteria Harbouring Antibiotic-Resistant Genes

Food may be contaminated in several ways with AR genes and/or AR bacteria. Such bacteria may be located in the environment (soil and water) and in human and animal faecal matter (Teuber *et al*., 1999). Products of animal origin have the possibility of containing AR bacteria from the outcome of faecal contamination during slaughter. Products of plant origin may also have the possibility of being contaminated with AR bacteria during production with the continued use of irrigation water contaminated with faecal matter from both humans and animals or by sewage discharges (Verraes *et al*., 2013). The environment could also be a source of contamination. Contamination from such nature may occur after food processing which is referred to as post-contamination. Lastly, cross-contamination may occur where food is already contaminated with AR bacteria and/or AR genes originating from other food sources during the handling process of food by consumers (Verraes *et al*., 2013).

2.11.2 Deliberate Addition of Microbes with Antibiotic-Resistant Properties to Food for a Technological Purpose

Throughout the production process of certain food products, microbes which harbour AR genes are deliberately added to certain foods to provide a technological purpose. The intended effect to which these additional microorganisms are supposed to provide is grouped into four categories: starter cultures, probiotics, bacteriophages and bio-preservatives (Verraes *et al*., 2013).

Starter cultures are live cultures that are intentionally added to certain food products to induce the fermentation process (Holzapfel, 2002). The bacteria most commonly utilised for this purpose is LAB such as *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus*. Some starter cultures may also serve as bio-preservatives or possess probiotic properties (Hickey *et al*., 2015). Examples of fermented foods and beverages in which starter cultures are used to induce the fermentation process are sauerkraut, yoghurt and fermented sausages amongst many others.

Probiotics, like starter cultures, are also live cultures that are intentionally added to certain food products to provide beneficial effects to the host organism consuming the food product. The most common types of bacteria utilised as probiotics are bifidobacteria and LAB (Hickey *et al*., 2015). Certain bacilli and yeasts such as *Saccharomyces boulardii* are also used for this purpose. Probiotics are primarily added to yoghurts and supplementary foods and are used as feed supplements (Verraes *et al*., 2013).

Bacteriophages are phage types, used for the inactivation of foodborne pathogens and spoilage organisms. According to Colomer-Lluch *et al*. (2011), the prevalence of AR genes in bacteriophages is largely unknown. In a study done by the same author, 30 bacteriophage DNA samples were collected from river water and urban wastewater. Three genes were found to confer resistance to antibiotics – two β-lactamase-genes (bla_{TEM} and bla_{CTX-MA}) from β-lactam antibiotics and one gene coding for a modified penicillin-binding protein (*mec* A) was recovered from all of the 30 samples. These genes were also recovered from almost all of the bacteriophage DNA samples originating from the faecal waste of cattle, pigs and poultry. In conclusion, this study demonstrated that bacteriophages could be environmental reservoirs of AR genes, implying that bacteriophages could possibly act as environmental factors for HGT of AR genes.

Bio-preservation is a natural method to prolong the shelf-life of food by utilising natural or controlled microbiota function as inhibitors by inactivating spoilage pathogens and microorganisms because they compete for nutrients and/or produce antimicrobial agents (Verraes *et al*., 2013). Furthermore, they may also possess fermenting or probiotic properties. According to Verraes *et al*. (2013), LAB may function as bio-preservatives for a variety of food products such as fermented foods and cooked meat products. Because LAB produces lactic acid and bacteriocins, they provide antimicrobial actions against both pathogenic and spoilage microorganisms such as *Listeria monocytogenes* (Verraes *et al*., 2013).

Another bio-preservative is *Pichia anomala*, a yeast that may be added to products of plant origin due to its antifungal and inhibitory effect on Gram-negative bacteria. A prevalent finding on AR in starter cultures is that transferable genes were rare and that resistance against tetracycline is quite prevalent (Verraes *et al*., 2013). AR is sometimes found in probiotic strains and fermented foods. Resistance in *Enterococcus* was found to be most common amongst the LAB isolated from spontaneously fermented foodstuffs (Teuber *et al*., 1999). Vancomycin resistance is associated with this bacterium in the majority of cases although, resistances to erythromycin, chloramphenicol and tetracycline were also reported (Teuber *et al*., 1999). LAB isolated from dairy products were from the genera *Enterococcus, Lactobacillus* and *Lactococcus* which were found to possess multi-resistant plasmids. There have been high incidence reports of erythromycin and tetracycline resistance that was reported from *Lactobacillus* isolated from artisan cheese (Çataloluk and Gogebakan, 2004).

A German study by Klare *et al*. (2007), reported that 6 out of 473 examined probiotic LAB isolated from human and animal isolates, were found to be multi-resistant to tetracycline and erythromycin. *Streptococcus thermophilus* and *Lactococcus* that were isolated from dairy products were reported to have high incidences of resistance to erythromycin and tetracycline (Wang *et al*., 2006). Furthermore, resistance to tetracycline was also detected in probiotic

bifidobacteria which included seven *Bifidobacterium animalis* ssp. *lactis* and *Bifidobacterium bifidum* strains (Masco *et al*., 2006).

Forasmuch as probiotics, bio-preserving microorganisms and starter cultures are often encompassed under the same bacterial genera, whilst the mechanisms for the transmission of AR are the same (Verraes *et al*., 2013). After food has been ingested by the consumer, the added microbes accumulate in the human GI tract where digestion occurs, and the transmission of AR genes may occur. Transmission often takes place via conjugation although transduction and transformation cannot be discredited (Verraes *et al*., 2013). Bacteriophages aforementioned, are host specific and it is therefore assumed that the transduction by phages occurs only between strains that are closely related (Verraes *et al*., 2013). Transduction of a pathogenicity island by phages between *S. aureus* and *L. monocytogenes* has already been shown (Chen and Novick, 2009). Presently, thus far, the transmission of AR by means of transduction has rarely been reported *in vitro* studies. Studies in literature describe the transmission of genes for the multidrug efflux proteins *gac A* and *gec B* in MRSA strains by means of transduction (Verraes *et al*., 2013).

2.12 SAFETY ASSESSMENT OF ANTIBIOTIC-RESISTANT LACTIC ACID BACTERIA

In recent years, testing for AR in probiotic or starter culture LAB intended for use in dairy products has become more standardised. The QPS concept launched in Europe by the EFSA is similar to the GRAS system implemented in the United States by the FDA where both systems are aimed to permit strains with a well-known history and safety status to enter the market without stringent testing requirements (Sharma *et al*., 2015). The QPS system provides a better framework for decision making and in the safety assessments of AR because of the recommendations of The Panel of Additives and Products or Substances used in Animal Feed (FEEDAP) of EFSA (Fukao and Yajima, 2012).

According to Jorgensen and Ferraro (2009), the main aims of testing AR is to detect possible drug resistance in common pathogens and to assure the sensitivity to drug choice when treating infections. Automated instrumentation that provides rapid results with the use of commercially available devices and materials or broth microdilution methods are the most commonly used methods to test for antimicrobial susceptibility (Álvarez-Cisneros and Ponce-Alquicira, 2018). Other methods that provide cost-saving solutions and greater flexibility are the gradient diffusion and disc diffusion methods. Each method, however, has its own pros and cons including the accuracy of the test to determine an organism's susceptibility (Álvarez-Cisneros and Ponce-Alquicira, 2018).

All methods provide qualitative results by categorising the results as resistant, susceptible and intermediate. Quantitative results are also achieved from some methods by MICs. The

current methods provide accurate detection of AR mechanisms. New and emerging mechanisms of resistance, however, need constant monitoring regarding the ability of each test method to accurately detect resistance (Jorgensen and Ferraro, 2009).

According to Fukao and Yajima (2012), MIC is a phenotypic method and a FEEDAP requirement for the determination of the most relevant antibiotics for each bacterial strain intended for use as a feed additive to eradicate the possibility of acquired resistance. A microbiological breakpoint is a specified concentration (mg/L) of an antibiotic which determines whether a species of bacteria is susceptible or resistant to the antibiotic (Verraes *et al*., 2013). If the MIC exceeds the breakpoint, more extensive analyses are done to express the genetic basis of the observed resistance and to assess the risk for transfer of this resistance to other bacteria. The latest literature highlights the use of genotypic methods to identify acquired resistance genes using PCR-based techniques and/or micro-assays (Nawaz *et al*., 2011, Gad *et al*., 2014, Flórez *et al*., 2016, Guo *et al*., 2017). Both techniques serve as a powerful tool to identify resistant LAB strains.

Transferability tests are optional in the case of suspected acquired or intrinsic resistance. By conducting bacterial mating experiments, conjugation can be detected. The transfer of resistance genes is checked when the suspected donor with an AR phenotype is mixed with a recipient strain that is sensitive to the respective antibiotic. If resistance genes were transferred, then the strain would be prohibited for use in food products and further analyses would not be necessary (Fukao and Yajima, 2012). Guo *et al*. (2017), had conducted a conjugative experiment by bacterial filter mating. PCR analysis had identified *van*(X), *van*(E), *gyr*(A) and *tet*(M) resistance genes from lactobacilli present in Chinese fermented milk, however, none of these genes were transferred to *Lc*. *lactis* MG1614 and *E. faecalis* 181.

2.13 THE NEED FOR PROBIOTICS IN AFRICA

According to Franz *et al*. (2014), LAB producing lactic acid resulting from carbohydrate metabolism was suggested to be beneficial to health. While many studies have drawn attention to the health benefits of probiotics (Anukam and Reid, 2009), the mechanisms of probiotics possessing health beneficial properties are still incompletely understood. According to Franz *et al*. (2014), probiotics provide beneficial effects to humans in the following ways: anticarcinogenic properties, antimutagenic activities, reducing blood pressure and cholesterol levels, improvement in symptoms of lactose intolerance, immune modulation and reducing the symptoms of diarrhoea amongst many others.

In European countries, the biggest food segment in the functional food market comprises of foodstuffs made with synbiotics, probiotics and prebiotics. Recently over the years, rapid growth was experienced in the probiotic market (Bhadoria and Mahapatra, 2011). The growth

in this market was specifically directed towards products like fermented milk and yoghurt. The Scandinavian and Northern European regions have the most well-developed markets for these products since consumers in those regions have a well-known generation of fermented dairy products (Bhadoria and Mahapatra, 2011). Poor decision making in the European legislature has led to a decline in certain markets in recent years (Franz *et al*., 2014). With North American markets being one of the top five markets in the world, probiotics have been gaining increasing importance. Several companies in the USA market have products that claim to be probiotic in supplement form (Bhadoria and Mahapatra, 2011, Franz *et al*., 2014).

Data are limited regarding the probiotic market in Africa which is an extremely small market compared to global north. The interest and need regarding probiotics are expressed in SA where there are well-established markets providing probiotics in the form of fermented dairy products, supplements and fortified food items. An interest and need have been expressed for the addition of probiotics in traditional fermented foods (Anukam and Reid, 2009).

Food safety in Africa has always been a priority for the Food and Agricultural Organisation (FAO) agenda. In accordance with the FAO's priorities for food safety in Africa, researchers in the field have suggested that studies regarding probiotic properties in LAB strains found in traditional fermented foods be ongoing and to include developing *in vitro* selection procedures for such strains followed by research on the technical performance of these strains in traditional substrates (Holzapfel, 2002).

According to Franz *et al*. (2014), reputable probiotic strains and products have the capability of being sold in Africa, but they are subjected to a number of pivotal factors that have to be considered. An example of this is that local populations residing in third world countries may prefer an indigenous solution to nourishment, health care and restoration to the population. An indigenous solution may reduce the alternative of purchasing foreign products on a regular basis, particularly if these products do not feature in the normal diet or are unaffordable to the third world communities (Reid *et al*., 2005). In the African cultural society, an assortment of fermented foods are derived from cereals, vegetable proteins or starchy root fermentations. Some foods have to meet dietary requirements, for example, less on dairy which is more common in North America and northern European countries. Fermented dairy production in Africa does, however, occur primarily in the northern and northern west areas, the Savannah Hills, Sahara and eastern parts of Africa (Karenzi *et al*., 2013, Franz *et al*., 2014).

Traditional fermented foods are significant to Africans because such foods provide enhanced flavour to existing staple foods. Furthermore, it provides a cost-effective method to preserve foods, and boost nutritional quality and digestibility of raw products (Osvik *et al*., 2013, Franz *et al*., 2014). These foods with improved properties may be acceptable to populations with a traditional societal structure. A challenge that Africa and other third world countries experience is poor access to electricity and a scarcity of cold storage facilities (Osvik *et al*., 2013). This

implies that what would usually work in first world countries would not necessarily work in third world countries. Typical flavourings and delivery agents for probiotic products used in the developed countries may not be used in the developing countries, specifically in African rural communities (Franz *et al*., 2014). Products with yoghurt origin that are consumed at a later stage would require cold storage facilities to ensure the stability of both the probiotics and the product itself. It is for the aforementioned reason that such products would not be suitable for rural African communities to consume.

According to Reid *et al*. (2005), although *Lb*. *rhamnosus* GG or *Lb*. *casei* Shirota strains are reputable strains capable of being utilised in probiotic products, the reason is still presently unclear as they are not yet permitted to be sold in sub-Saharan African countries. This may be due to the reasons aforementioned or because manufacturing companies in Africa specialising in these types of products may not be gaining the expected profits (Reid *et al*., 2005). Furthermore, it could also be possible that the appropriate distributors may not have been available to justify sales. It is mainly the sub-Saharan region that is in dire need for probiotics since this is the region in Africa that is hit the hardest when it comes to food insecurity and poor nutritional profiles of these populations (Reid *et al*., 2005).

According to a 2018 FAO report, Africa still remains the continent with the highest prevalence of undernourishment, affecting more than 256 million people (FAO, 2018). Agronomic constraints and scarcity of locally suitable processing techniques are the main reasons for Africa's inability to feed the continent both qualitatively and quantitatively. It was shocking to note that 14 out of the 20 world's poorest countries come from Africa. Most of these countries continuously face the challenge of malnourishment amongst the population. Chronic malnutrition has been in the spotlight affecting young children in sub-Saharan Africa (Franz *et al*., 2014).

The causal pathway that leads to undernourishment is the malnutrition-infection cycle. Because undernourished children have characteristic symptoms of weak immune systems and poor physical stamina; such children are susceptible to infectious diseases such as diarrhoea and thus are at an increased mortality risk (Franz *et al*., 2014). This is significant since 37% of the cases are diarrhoea related and is the main cause of childhood mortality in sub-Saharan Africa. Africa is, therefore, a continent with a high incidence of diarrhoeal incidences affecting mainly young children where the mortality rate for children under the age of five is tremendously high (Franz *et al*., 2014).

A solution to this problem could be to utilise traditional fermented foods containing probiotics to help alleviate the problem by improving the health of children who consume such foods. Fermentation not only contributes to the preservation of food but it also minimises postharvest losses. Furthermore, it also detoxifies raw materials and increases the intake of both micro- and macro-nutrients reducing the symptoms of malnutrition (Holzapfel, 2002).

A question that often arises is why should fermented foods be vehicles for probiotics and which strains should be used? Fermented foods originating from animal sources harbouring animal proteins, inclusive of milk fermentations are not widely practised in Africa. Only a few regions in Africa such as Kenya, Ethiopia and the southern region of Africa practice milk fermentation where such products are consumed on a regular basis (Franz *et al*., 2014). It would make sense for such products to be indigenous to those specific areas and hence distribution of such products would not be very wide across Africa. Ideally, the development of probiotic food products in Africa should be specific to the traditional fermented foods of a specific region or form part of the local dietary requirements (Franz *et al*., 2014). Furthermore, dried probiotic products could be developed for additional purposes to a diversity of local foods with the ability to survive the heat in certain cases (Franz *et al*., 2014).

Strains used in the fermentation of foods should be reputable and well suited to the product exhibiting health beneficial properties. This implies for multifunctional starter cultures to be derived and developed from new bacterial strains or the evaluation and applicability of Western strains to an African environment (Holzapfel, 2002). The development of emerging probiotics would require research into the functional property of the strain, inclusive of the survival at the body target site, anti-disease effects, enhancement of immune response, to be beneficial to the indigenous microbiota, be cost-effective, to be adaptable to African food substrates and maintain good survival/viability during the storage of the product. Furthermore, their influence on taste and texture also plays a vital role in implementation into a food product (Franz *et al*., 2014).

Whilst most research in literature have focused on benefits of probiotics very little research has focused on AR genes being present in LAB which are primarily used as starter cultures in a variety of foods (Franz *et al*., 2014). Not only does this pose a serious risk to public health, but for food safety in general. Research should focus on screening existing and emerging probiotics for AR genes which according to the EFSA should not be allowed to be used in foodstuffs (Franz *et al*., 2014, Sharma *et al*., 2015). Furthermore, improving the quality of these strains could help to solve the serious crisis that Africa faces with food security and increase employment opportunities among the rural third world countries.

2.14 POTENTIAL RISKS FOR ANTIBIOTIC-RESISTANT GENE TRANSFER AND CLINICAL CASES INVOLVING LACTIC ACID BACTERIA

To gain a full understanding of the extent to which LAB strains transfer-resistant genes in nature, it is important to investigate genetic exchange in this context. The ability of wild-type AR genes (*erm*(B) and *tet*(M)), present in LAB strains that were isolated from food sources to be transferred to recipient strains were reported by Toomey *et al*. (2009). *In vitro* mating demonstrated that all four LAB mating pairs transmitted their resistance determinants at high

frequencies. *In vivo* transfer between LAB has only been demonstrated in the GI tracts of gnotobiotic mice and rats (Jacobsen *et al*., 2007).

An increase in transfer frequencies was observed when antibiotics were administered at subtherapeutic levels in the feed and drinking water of animals. This implies that increasing antibiotic pressure (by misuse, prophylaxis, disease treatment and growth promotion) could intensify the AR transfer between bacterial species. The aforementioned investigations imply that AR factors may be transferred from economically viable bacteria like LAB to other possibly pathogenic bacteria. The risks associated need to be well-thought-out, especially with the increasing concern and emphasis placed on foods acting as a potential reservoir for AR determinants.

Aforementioned, gut microflora could act as reservoirs for resistant genes that can be acquired from ingested bacteria. Although gene transfer between bacteria occurs in the GI tract, intrinsic resistance is believed to pose minimal potential for horizontal dissemination, whilst acquired resistance is believed to have a greater potential for lateral spread. In nosocomial environments, AR pathogens that have caused a myriad of treatment failures leading to both hospital illnesses and fatalities. Ashraf and Shah (2011), had reviewed clinical cases involving LAB. Correlations between systemic infections and probiotic consumptions were documented and found to be few. However, all of these correlations had occurred in patients with an existing medical condition. Despite several probiotic LAB having a safe history in patients receiving nutritional support, some probiotics, however, have exhibited an increase in the risk of complications in specific patient groups.

Clinical cases reviewed by Aguirre and Collins (1993) and Ashraf and Shah (2011) involving LAB and bifidobacteria between 1938 and 1993 and a summary of their outcomes are displayed in Table 2.3. In 155 cases of infections involving LAB or bifidobacteria, 95 cases involved *Lactobacillus* spp., furthermore, endocarditis was found to be the most frequent infection in which species of *Lactobacillus* have been mostly implicated.

Of approximately 180 cases of septicaemia involving LAB in humans, only a single case had identified LAB which was found to be identical to a commercially available dairy strain. Cannon *et al*. (2005), had observed a minuscule percentage (1.7%) of cases that were associated with high dairy consumption. Three cases were related to endocarditis, whilst only a single case was related to a liver abscess. Clinically visible symptoms had appeared after a patient's dental extraction with the patient having consumed several tubs of yoghurt a day. Presterl *et al*. (2001), had reported on the diet of a young man which comprised of large amounts of probiotic yoghurt who had developed endocarditis and septic arthritis caused by *Lb*. *rhamnosus*. Contradictory to this finding was a case of endocarditis found in a 73-year-old man caused by *Lb*. *casei* subsp. *rhamnosus*. The man was reported to have had no previous history of dental manipulation or daily yoghurt intake (Wallet *et al*., 2002).

Table 2.3: Lactic acid bacteria and bifidobacteria that were implicated in clinical cases

LAB identified	Bacteraemia	Endocarditis	Other infection	Total
Lactobacillus spp.	7	8	19	34
Lb. acidophilus	3	3	2	8
Lb. casei	12	-	-	12
Lb. plantarum	11	2	1	14
Lb. rhamnosus	19	5	3	27
Bifidobacterium	-	9	-	9
Leuconostoc	2	23	8	33
Pediococcus	-	11	7	18
Total cases	54	61	40	155

Adapted from: Ashraf and Shah (2011).

2.15 CONCLUSION

There are limited studies done on the AR profiles of commercial LAB. Generally, the majority of research done in this area of food safety, have been done in Eurasian countries which investigated the AR profiles of LAB that were spontaneous or naturally occurring in traditionally fermented foods. Therefore, this presents a research gap for investigating AR in commercial LAB in SA. *aMasi* is traditionally fermented milk sold on a commercial scale that is widely consumed by South Africans due to these products having a high LAB content. Although LAB may be perceived as beneficial bacteria, a need has been expressed to investigate the possibility of these bacteria harbouring AR genes, which, if transferrable to other bacteria, could pose a food safety risk to consumers consuming this product.

Despite SA having the most active surveillance for AR in Africa, little has been done to eradicate AR in the food chain. Current initiatives to combat AR in SA focuses mainly on the dissemination of AR in nosocomial settings, placing very little emphasis on the dissemination of AR in the food chain. Limited data regarding AR in SA exists, therefore, expressing the need to investigate the AR of LAB in commercial *aMasi* samples, since this product comprises a supplementary staple food to the lives of South Africans.

CHAPTER THREE: METHODOLOGY

3.1 INTRODUCTION

The overview of the methodology used in this study is illustrated in Figure 3.1. In summary, the numbers (1 to 4) illustrated in Figure 3.1 indicate the objectives set out to achieve the aim. Firstly, commercially produced *aMasi* samples were surveyed to determine the presumptive total bacterial and lactic acid bacterial counts. From these, the proportional prevalence of presumptive lactic acid bacteria was determined from the presumptive total bacteria. This step was done to confirm if LAB was indeed the predominating bacterial population among the samples. Secondly, LAB were then isolated after being surveyed and was identified by 16S rDNA sequencing. The third objective was to then establish if the LAB present in these commercial *aMasi* samples had exhibited any relationship to the physicochemical properties of the fermented milk medium. Finally, AR of the LAB was determined by screening the isolates phenotypically by the Kirby-Bauer Disc Diffusion Method and thereafter confirming the resistant LAB genotypically by PCR.

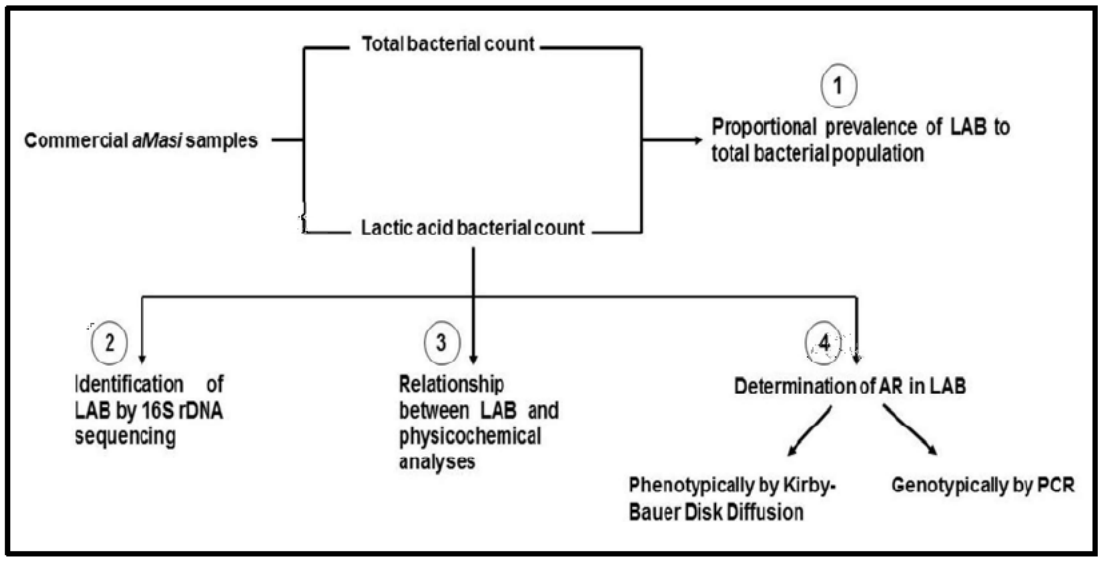

Figure 3.1: Brief overview of the methodology used to profile and determine AR of LAB isolated from commercial *aMasi* samples. Numbers 1 to 4 indicates the respective objectives undertaken to achieve the aim.

3.2 COLLECTION AND STORAGE OF SAMPLES

A total of 10 *aMasi* samples were collected from both well-established retail supermarkets as well as smaller supermarkets in Durban. It was observed prior to sample collection that well-established supermarkets had stocked the well-established brands of *aMasi* from well-known dairy companies whilst, the smaller retailers had stocked brands that were cheaper and produced by smaller dairy companies. Prices and sample servings sizes had also varied depending on the popularity of the brand. Therefore, samples were collected based on the

establishment of the retail supermarket, the price of the product and the establishment of the brand. All samples purchased were maintained at 4°C until further use, to prevent sample deterioration.

3.3 SURVEYING AND ENUMERATION OF BACTERIA

3.3.1 Total Mesophilic Bacterial Count

The Standard by ISO (2013) with modifications was followed. Serial dilutions were prepared by homogenising the sample with Buffered Peptone Water (Merck, Germany) in the ratio of 1:9. Thereafter, 100 μl of the homogenised solution was spread plated onto Plate Count Skimmed Milk Agar (PCSMA) (Merck, Germany) and incubated at 30°C for 72 hours aerobically to obtain an aerobic mesophilic bacterial count.

3.3.2 Isolation and Enumeration of Lactic Acid Bacteria

The standard by ISO (1998) with modifications was adhered to. Serial dilutions were prepared by homogenising the sample with Buffered Peptone Water (Merck, Germany) in the ratio of 1:9. Thereafter, 100 μl of the homogenised solution was spread plated onto MRS Agar (Merck, Germany) and incubated at 30°C for 72 hours anaerobically to obtain a LAB count. Thereafter, isolates with similar colony morphology were randomly picked and a single colony from the random selections were transferred to 100 μl of MRS Broth (Merck, Germany), vortexed and thereafter streaked onto MRS Agar (Merck, Germany) and incubated anaerobically using anaerobic jars with anaerobic gas packs (Oxoid AnaeroGen) for 72 hours at 30°C. This step was repeated at least three times to obtain pure cultures of the isolates.

3.4 IDENTIFICATION BY 16S rDNA SEQUENCING

This aspect of bacterial identification was completed at the laboratory of Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). Genomic DNA (gDNA) was extracted from LAB isolates using a commercial DNA extraction kit (Quick-DNA™ Fungal/Bacterial Miniprep Kit, Zymo Research) according to the manufacturer's instructions. The 16S target region was then amplified using a master mix (One-Taq® Quick-Load® 2X Master Mix, NEB) and universal primers: 16S-27F as the forward primer, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 16S-1492R as the reverse primer, 5′-CGGTTACCTTGTTACGACTT-3′. The PCR was performed in a 25 μl reaction according to the manufacturer's recommendation as described in Table 3.1 and thermocycling conditions for the PCR reactions carried out are described in Table 3.2.

Table 3.1: Constituents for 16S rDNA PCR reactions

Component	Volume for 1 reaction
One-Taq Quick-Load 2X Master Mix	12.5 µl
Forward primer: 16S-27F	0.5 µl
Reverse primer: 16S-1492R	0.5 µl
Sample DNA	1.0 µl
Sterile nuclease-free water	10.5 µl
Total volume	25.0 µl

Table 3.2: Thermocycling conditions for amplifying the 16S rDNA region in LAB

Step	Temperature	Time
Initial denaturation	94°C	30 seconds
	94°C	15-30 seconds
30 cycles	45-68°C	15-60 seconds
	68°C	1 minute
Final extension	68°C	5 minutes
Hold	4°C	∞

3.5 CHARACTERISATION OF THE *aMasi* MEDIUM BY SELECTED PHYSICOCHEMICAL TESTS

3.5.1 Fat Content

The fat content of *aMasi* samples was ascertained by using the Gerber method (AOAC, 2002) as described by James (1995) with modifications. The sample pack (*aMasi*) was placed into a water bath set at 40°C, gently mixed, and the temperature of the sample was adjusted to approximately 20°C. Following this, 10 ml of 91% sulphuric acid was added to the butyrometers. To this, 11.01 g of the sample and 1 ml of amyl alcohol (specific for milk testing) was added to the butyrometers, stoppered and shaken vigorously until the curd had dissolved and the contents within the butyrometers turned to a uniform dark brown colour. Thereafter, the butyrometers were placed into a water bath at 65°C for approximately 5 minutes. The stoppers were adjusted so as to bring the fat column into the scale section. The contents in the butyrometers were then centrifuged in a Gerber's centrifuge (Funke Gerber Nova Safety Centrifuge, art no. 3670) at 1 100 rpm for 3 minutes (a total of 350 ± 50 *g* within the butyrometer). Once centrifuged, the butyrometers were again immersed into a water bath at 65°C for approximately 5 minutes. The reading was thereafter taken to determine the percentage of fat in the samples.

3.5.2 pH

The pH of the samples were detected using the procedure outlined by Moyane and Jideani (2013). Before use, the pH meter (Thermo Fisher™ Orion™ 2-Star Benchtop pH Meter) was calibrated using the standard buffer solutions at pH 4 and 7 thereafter, immersing the pH probe into 50 ml of sample to obtain a reading.

3.5.3 Titratable Acidity

Titratable acidity was done on the samples using the method set out in AOAC (2000c) with modifications. The concentration of lactic acid was determined by diluting 20 g of sample with 30 ml of distilled water into an Erlenmeyer flask by swirling. One ml of phenolphthalein indicator was added to the diluted sample and 0.1 M sodium hydroxide (NaOH) from a filled burette was used to titrate the solution whilst swirling until an endpoint was reached (light pink colour retained). The volume of NaOH was recorded and the percentage of lactic acid was calculated using the equation:

$$\text{Lactic acid (\%)} = \frac{(\text{ml of NaOH used})(\text{concentration of NaOH})(0.090\ \text{milli-equivalent weight of lactic acid})(100)}{\text{Weight of sample}}$$

3.5.4 Water Activity

Water activity (a_w) was determined using a water activity meter (Novasina LabSwift a_w) according to the manufacturer's instructions (Novasina, 2010). Before using the a_w meter, the meter was allowed to warm up for 30 minutes and was standardised using the SAL-T verification standards provided by the manufacturer. One gram of sample was transferred into the sample dish and placed into the meter. After closing the hood, the 'actual' reading was taken after 20 minutes.

3.5.5 Moisture Content

Moisture content was investigated using the conventional oven drying method outlined by AOAC (2000a) with modifications. Three grams of the sample was massed out into an aluminium dish and was placed into a steam bath for 10 to 15 minutes to pre-dry the sample as described by Bradley (2010). Thereafter, the samples were placed into a conventional drying oven for 3 hours at 100°C. The loss in mass was determined and recorded as moisture content expressed as:

$$\text{Moisture (\%)} = \frac{\text{Loss in mass (g)} \times 100}{\text{Mass of sample (g)}}$$

3.5.6 Determination of Reducing Sugar – Lactose

The Munson and Walker method for the determination of lactose (AOAC, 2000b) was used in this study described by Aurand *et al*. (1987). Precipitation of proteins: 25 g of the homogenised sample was transferred into a 500 ml volumetric flask whereby, 400 ml of distilled water, 10 ml of Fehling's A solution (Fehling's A solution was made by dissolving 34.64 g of copper sulphate crystals ($CuSO_4 \cdot 5H_2O$) in distilled water up to 500 ml) and 44 ml of 0.1 N sodium hydroxide solution was added. This solution was shaken thoroughly and the precipitate was allowed to settle. The solution was then filtered through a dry filter (a Büchner funnel lined with Whatman no. 1 filter paper). The filtrate must have filtered through rapidly to obtain a clear filtrate. Lactose was determined from an aliquot of this filtrate.

For the reduction of copper, 25 ml of Fehling's A and 25 ml of Fehling's B solution (Fehling's B solution was made by dissolving 173 g of potassium sodium tartrate tetrahydrate and 50 g of sodium hydroxide and diluted in distilled water up to 500 ml) together with 50 ml of the filtrate obtained was transferred into a 400 ml beaker. The beaker was then covered with a watch glass and the solution was heated until it came to a boil in exactly 4 minutes. The solution was boiled for a further 2 minutes and was filtered immediately through a Büchner funnel and flask. The copper precipitate obtained from boiling was washed with 300 ml of hot distilled water (60°C) using suction through the flask. The hot water was emptied out of the flask and the Büchner funnel was first washed with 10 ml of 95% ethanol and thereafter with 10 ml of ethyl ether (Merck, EMSURE) using suction.

The Büchner funnel and precipitate were dried for 45 minutes at 100°C. This was then cooled and dried in a desiccator for 20 minutes and weighed. The Büchner funnel and precipitate were thereafter, re-heated for a 30 minute period, cooled and re-weighed to obtain a constant weight. The weight of lactose was obtained from the corresponding weight of Cu_2O (the amount of reduced copper) obtained from the Munson and Walker Table listed in AOAC (2000b).

3.6 STATISTICAL ANALYSES

3.6.1 Enumeration of Total Bacterial Count, Lactic Acid Bacterial Count and Determination of Proportional Prevalence of LAB to Total Bacterial Count

All analyses were performed in triplicate. A one-way analysis of variance (one-way ANOVA) with a Tukey's posthoc test was used to analyse results for the microbiological counts using GraphPad Prism v.7.00. A stacked-column was used to project the presumptive proportional prevalence of LAB to the presumptive total population using Microsoft Excel 365.

3.6.2 Determining the Relationship between LAB and the Physicochemical Attributes of the *aMasi* Medium

3.6.2.1 Principal component analysis

Principal component analysis (PCA) is a multivariate statistical method for representing core relationships in multifactorial data (Abdi and Williams, 2010). This is done by simplifying a data matrix *X* into two smaller matrices i.e. the score matrix *T* and the projection matrix *P*′. The score vector is represented by the columns *T* whilst, loading vectors are represented by the rows in *P*′. These two vectors are equal to the highest eigenvalues that encompass the most significant information relating to the system studied. The residuals matrix, *E*, includes variations between the projections and the initial coordinates. This can be expressed by Equation 1 (Awolusi *et al*., 2017):

$$X = 1 \cdot \bar{x} + T \cdot P' + E \tag{1}$$

where *X* represents the matrix of the data, $\bar{x}$ is the mean vector, *T* represents the score matrix, *P*′ is the projection matrix and *E* represent the residuals matrix.

Principal component analysis was achieved by following the steps by Awolusi *et al*. (2017): (1) the correlation matrix was calculated from Equation 2 listed below; (2) the function "pcacov" in MATLAB software R2015a was used to estimate the eigenvalues and eigenvectors of the covariance matrix and, (3) the eigenvalues were scored in descending order. The eigenvector complementary to the highest eigenvalue is the most dominant principal component (PC) of the data set which is referred to as (PC1). The second PC (PC2), computed under the constraint of being orthogonal to PC1, has the second-largest variance. In a specific PC, all variables having a loading value close to -1 or 1 are chosen for grouping. The PCA and calculations of the PC loadings were done using the function "pca" on MATLAB software R2015a.

$$r_{x,y} = \frac{\sum_{i=1}^{n} (x_i - \mu_x)(y_i - \mu_y)}{(n-1)\sigma_x \sigma_y} \tag{2}$$

3.6.2.2 Regression tree classification of Lactic Acid Bacterial relationship with physicochemical attributes

Decision trees are decision support tools that employ the use of models that resembles a tree-like structure illustrating a model of decisions and possible consequences. This process is illustrated by a root node which channels through the internal nodes to the leaf nodes. In this method, the root node represents the predictor that gives the optimum split of the target

values (Deepnarain *et al*., 2019). Internal or parent nodes symbolise a “test” or an attribute, each branch symbolises the implication of the test whilst, each leaf node portrays a class label. Splitting is continuously applied to the internal nodes until leaf nodes are obtained. The channels from the root node to the leaf nodes imply classification rules (Song and Lu, 2015). Overfitting problems are solved by pruning, which removes inessential nodes and optimises the tree size. Pruning can be done prior, to prevent the generation of inessential branches or afterwards, to remove branches from the established tree (Song and Lu, 2015). The construction of a decision tree is finished when (a) the leaf node has the same class label corresponding to the same target class value, (b) every determiner has already been used to divide a partition and (c) no more records for a specific value of a determiner variable.

Decision trees involve a learning process which is achieved by the training and validation steps. The training step is employed to construct and evaluate the decision tree model by reducing the difference between the measured and predicted outputs. On the other hand, the validation step is used to finalise the optimal tree size that overcomes the issues of overfitting and loss of generalisation ability. Both approaches are utilised to asses to the accuracy of the decision tree by comparing predicted outcomes with actual data. When the accuracy becomes adequate, the decision tree can be employed for classification and prediction purposes using a new dataset (Song and Lu, 2015).

The method by Deepnarain *et al*. (2019), was used in this study. The decision tree model was used to (a) elucidate the relationships between physicochemical properties of *aMasi* (as inputs) and LAB count (as an output), and (b) define the physicochemical factors that mainly influenced the LAB species. The function, “fitctree” in MATLAB software R2015a was used to estimate the decision tree based on the input variables. The function utilises the Classification and Regression Trees (CART) algorithm for constructing the decision tree by the binary split at each node (Song and Lu, 2015). This algorithm was chosen because of its application and flexibility towards complex data sets. The Gini index method for classification trees and mean squared error for regression trees were employed to select input factors having a great degree of purity of the child nodes (Song and Lu, 2015). This function illustrates the best sequence of pruned subtrees i.e. pre-pruning.

3.7 ANTIBIOTIC RESISTANCE DETERMINATION OF LACTIC ACID BACTERIAL ISOLATES

3.7.1 Phenotypic Antibiotic Resistance Determination of Lactic Acid Bacterial Isolates by Culture-Based Screening

Screening of the antibiotic susceptibility profiles of LAB was done by using the disc diffusion method employed by Sharma *et al*. (2015) with modifications. Due to LAB exhibiting poor growth on well-defined antibiotic susceptibility media such as Mueller-Hinton and Iso-

Sensitest (Egervärn *et al*., 2007), LSM developed by Klare *et al*. (2005) was used in this study. The bacterial cultures were grown overnight for 16 to 18 hours and, were adjusted spectrophotometrically at 600 nm to give an approximate cell density of 10^8 CFU/ ml. A volume of 100 µl of bacterial culture was swabbed onto each plate followed by the dispensing of the antibiotic discs which included: ampicillin (10 µg), chloramphenicol (30 µg), colistin sulphate (10 µg), erythromycin (30 µg), fosfomycin (50 µg), gentamicin (10 µg), kanamycin (30 µg), pipemidic acid (20 µg), streptomycin (25 µg), tetracycline (30 µg) and vancomycin (30 µg). The plates were kept at 25°C for 1 hour to allow for diffusion to occur and thereafter incubated at 30°C for 24 hours, anaerobically. The antibiotic susceptibility profiles of isolated LAB were assessed by measuring the zone of inhibition after 24 hours of incubation. Isolates that had exhibited phenotypic resistance were then selected for PCR analysis to investigate the prevalence of resistant genes encoding for resistance towards the different classes of antibiotics using gene-specific and degenerate primers.

3.7.2 Molecular Identification of Antibiotic Resistance Genes Using PCR

Polymerase chain reaction (PCR) was carried out to investigate the prevalence of resistant genes in LAB towards the different classes of antibiotics using gene-specific primers. Due to time constraints, optimisation was only completed for eight out of sixteen resistant genes' primer sets for three *Leu*. *mesenteroides* isolates. Genomic DNA was extracted using a modified heat lysis ('colony boil') method by Englen and Kelley (2000). Briefly, 5 to 7 colonies were picked from a 48-hour pure culture using a sterile loop and dissolved in 70 µl of sterile MiliQ water. This suspension was then placed onto a 100°C heating block for 10 minutes and thereafter, centrifuged at 13 000 rpm using an Eppendorf Mini Spin centrifuge for 5 minutes. The supernatant was transferred into a sterile 2.0 ml Eppendorf tube and stored at -20°C for future use.

A modified method by Morandi *et al*. (2013), was used as a standard optimisation protocol for PCR thermocycling that was done for the eight resistant genes' primer sets. Briefly, a 25 µl PCR reaction consisted of 12.5 µl of 2X PCR Master Mix (Thermo Fisher), 1 µl each of the forward and reverse primers, 2 µl of template DNA (concentration at 1 µg/µl) and 8.5 µl of sterile nuclease-free water. This PCR reaction was subjected to the thermocycling conditions listed in Table 3.3 with gene-specific primer sets and, expected amplicon size listed in Table 3.4. The PCR products ran on a 1% agarose gel at 60 V for 1 hour 15 minutes using 1X Tris-acetate-EDTA buffer. A GeneRuler 1kb Ladder (Thermo Fisher) or GeneRuler 1kb Plus Ladder (Thermo Fisher) was used as a DNA marker.

Table 3.3 Thermocycling conditions for antibiotic-resistant PCR reactions

Step	Temperature	Time
Initial denaturation	95°C	30 seconds
30 cycles	95°C (Denaturation)	1 minute
	55 – 61°C (Annealing)	30 seconds
	72°C (Extension)	1 minute
Final extension	72°C	4 minutes
Hold	4°C	10 minutes

Table 3.4 Primers used for the identification of antibiotic-resistant genes in LAB

Antibiotic and class	Primer	Sequence	Expected amplicon size (bp)	References
Vancomycin (glycopeptides)	*van*A	F: 5'-TCTGCAATAGAGATAGCCGC-3' R: 5'-GGAGTAGCTATCCCAGCATT-3'	377	Lemcke and Bülte (2000); Morandi *et al*. (2013)
	*van*B	F: 5'-GCTCCGCAGCCTGCATGGACA-3' R: 5'-ACGATGCCGCCATCCTCCTGC-3'	529	Fraimow *et al*. (1994); Lemcke and Bülte (2000); Morandi *et al*. (2013)
	*van*D	F: 5'-TGTGGGATGCGATATTCAA-3' R: 5'-TGCAGCCAAGTATCCGGTAA-3'	500	Depardieu *et al*. (2004); Morandi *et al*. (2013)
	*van*E	F: 5'-TGTGGTATCGGAGCTGCAG-3' R: 5'-GTCGATTCTCGCTAATCC-3'	513	Depardieu *et al*. (2004); Guo *et al*. (2017)
	*van*X	F: 5'-TCGCGGTAGTCCCACCATTCGTT-3' R: 5'-AAATCATCGTTGACCTGCGTTAT-3'	454	Liu *et al*. (2009); Guo *et al*. (2017)

Gentamicin (aminoglycosides)	*aac*(6')-*aph*(2'')	F: 5'-CCA AGA GCA ATA AGG GCATA-3' R: 5'-CACTATCATAACCACTACCG-3'	220	Rojo-Bezares *et al.* (2006); Guo *et al.* (2017)
	aph(2'')-*Ib*	F: 5'-CTTGGACGCTGAGATATATGAGCAC-3' R: 5'-GTTTGTAGCAATTCAGAAACACCCTT-3'	867	Vakulenko *et al.* (2003); Guo *et al.* (2017)
Kanamycin (aminoglycosides)	*aph*(2'')-*Ic*	F: 5'-CCACAATGATAATGACTCAGTTCCC-3' R: 5'-CCACAGCTTCCGATAGCAAGAG-3'	444	Vakulenko *et al.* (2003); Guo *et al.* (2017)
	aph(2'')-*Id*	F: 5'-GTGGTTTTTACAGGAATGCCATC-3' R: 5'-CCCTCTTCATACCAATCCATATAACC-3'	641	Vakulenko *et al.* (2003); Guo *et al.* (2017)
	aph(3")-IIIa	F: 5'-GCCGATGTGGATTGCGAAAA-3' R: 5'-GCTTGATCCCCAGTAAGTCA-3'	292	Ouoba *et al.* (2008); Guo *et al.* (2017)
	ant(2")-I	F: 5'-GGGCGCGTCATGGAGGAGTT-3' R: 5'-TATCGCGACCTGAAAGCGGC-3'	329	Ouoba *et al.* (2008); Toomey *et al.* (2010)
Streptomycin (aminoglycosides)	*str*A	F: 5'-CTTGGTGATAACGGCAATTC-3' R: 5'-CCAATCGCAGATAGAAGGC-3'	548	Ouoba *et al.* (2008); Toomey *et al.* (2010)
	*str*B	F: 5'-ATCGTCAAGGGATTGAAACC-3' R: 5'-GGATCGTAGAACATATTGGC-3'	509	Ouoba *et al.* (2008); Toomey *et al.* (2010)
	*aad*A	F: 5'-ATCCTTCGGCGCGATTTTG-3' R: 5'-GCAGCGCAATGACATTCTTG-3'	283	Ouoba *et al.* (2008); Toomey *et al.* (2010)
	*aad*E	F: 5'-ATGGAATTATTCCCACCTGA-3' R: 5'-TCAAAACCCCTATTAAAGCC-3'	386	Ouoba *et al.* (2008); Toomey *et al.* (2010)
Tetracycline (tetracyclines)	DI DII DIII	F: 5'-GAYACICCIGGICAYRTIGAYTT-3' R: 5'-GCCCARWAIGGRTTIGGIGGIACYTC-3' R: 5'-CKRAARTIGCIGGIGTISWIRCIGG-3'	1 500	Cleremont *et al.* (1997); Flórez *et al.* (2006)

CHAPTER FOUR: RESULTS

4.1 ENUMERATION OF PRESUMPTIVE TOTAL BACTERIA AND LAB

4.1.1 Enumeration of Total Bacteria

The presumptive total bacterial counts obtained for all 10 commercial *aMasi* samples are depicted in Figure 4.1. The samples had ranged from 6.42 Log CFU/ml to 8.47 Log CFU/ml. In addition to this, the presumptive mean total bacterial count for these samples was 7.36 Log CFU/ml. One-way ANOVA and the Tukey's posthoc comparison test had revealed extremely significant differences ($P \leq 0.0001$) among the *aMasi* samples except for samples 2 and 7, 2 and 3 and 3 and 7 which shared the same superscript letter/s implying that there were no significant differences ($P \geq 0.05$) between these samples.

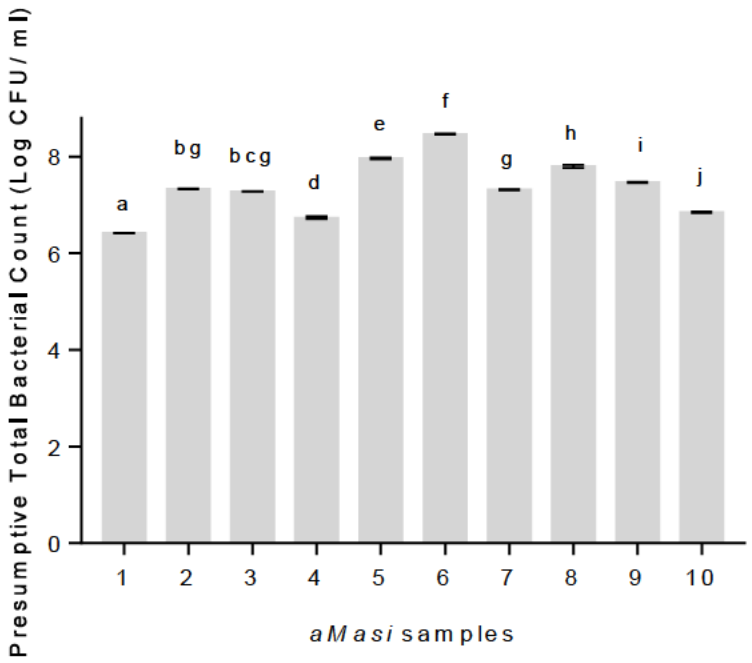

Figure 4.1: Presumptive TBC for the commercial *aMasi* samples. Bars with whiskers are represented as mean ± standard error of the mean; n=3 and bars with whiskers sharing similar letter/s indicate no significant differences ($P \geq 0.05$).

4.1.2 Enumeration of Presumptive Lactic Acid Bacteria

A similar trend was observed for the LAB counts presented in Figure 4.2. The presumptive LAB counts for commercial *aMasi* samples in this study had ranged from 6.39 Log CFU/ml to 7.86 Log CFU/ml. Additionally, the mean presumptive LAB count in this study for these samples was 7.15 Log CFU/ml. One-way ANOVA and the Tukey's posthoc comparison test evinced extremely significant differences ($P \leq 0.0001$) for these samples except for samples

2 and 3, 3 and 7, 4 and 10, 6 and 8 and, 7 and 9 which shared the same superscript letter/s indicating no significant differences (P ≥ 0.05) between these samples.

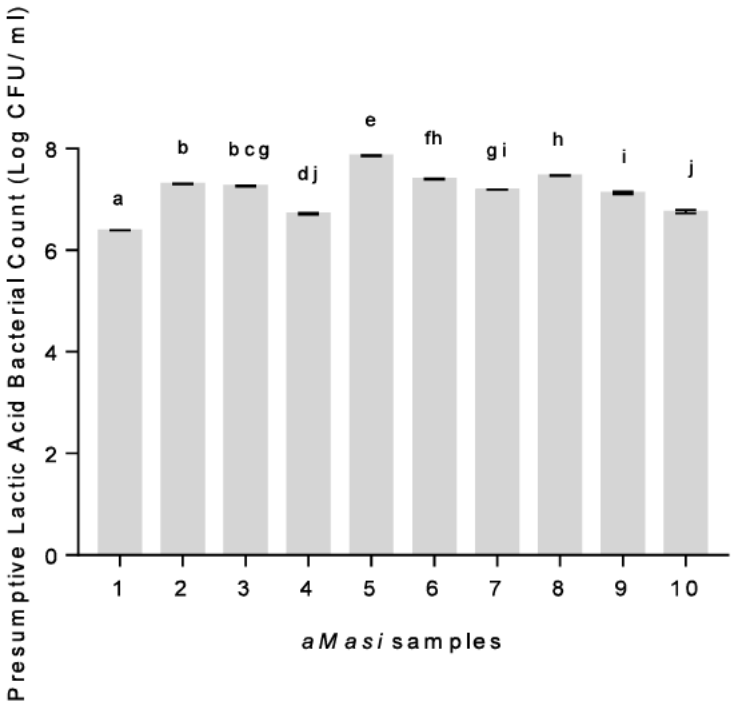

Figure 4.2: Presumptive LAB counts for commercial *aMasi* samples. Bars with whiskers are represented as mean ± standard error of the mean; n=3 and bars with whiskers sharing similar letter/s indicate no significant differences (P ≥ 0.05).

4.2 PROPORTIONAL PREVALENCE OF PRESUMPTIVE LACTIC ACID BACTERIA TO THE PRESUMPTIVE TOTAL BACTERIAL POPULATION

The proportional prevalence of presumptive LAB to the presumptive total bacterial population is illustrated in Figure 4.3. The presumptive LAB population in relation to the presumptive total bacterial population for the *aMasi* samples except for sample 6, had ranged from 95.47% to 99.77%. These results implied that LAB was the presumptive predominating population among these samples. Sample 6 had a lower percentage of LAB present (87.44%) in contrast to the other nine samples. Although the presumptive LAB population had predominated this sample, the percentage of other bacteria present was 12.56% which was significantly higher (P ≥ 0.05) in contrast to the other samples.

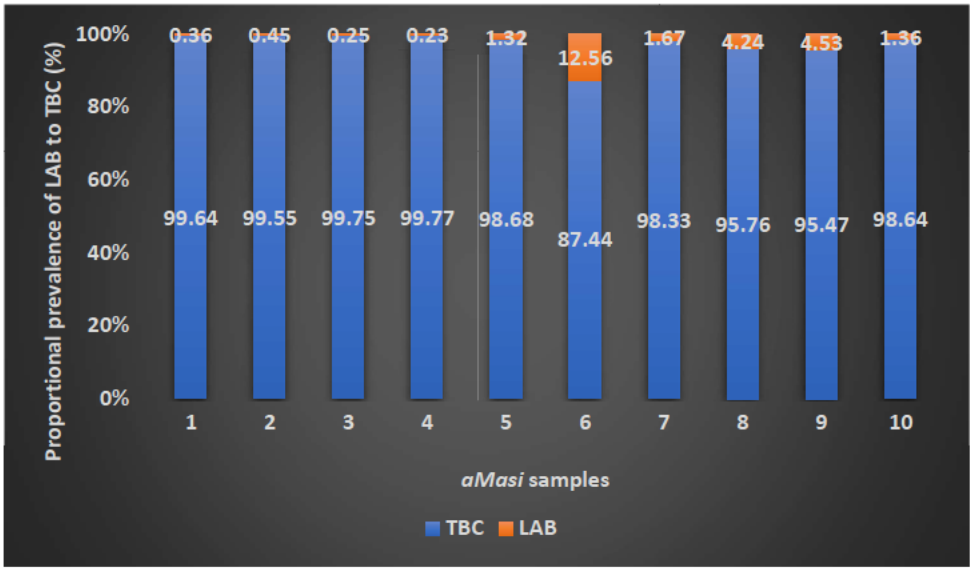

Figure 4.3: The proportional prevalence of presumptive LAB to the presumptive total bacterial population.

4.3 IDENTIFICATION OF *Bacillus* sp. IN COMMERCIAL *aMasi* SAMPLES

During the enumeration of total bacteria and presumptive LAB, colonies with radial swarming motility were observed and are depicted in Figure 4.4. These colonies were identified by 16S rDNA sequencing as either *Bacillus subtilis*, *Bacillus amyloliquefaciens* or *Bacillus velezensis* listed in Table 4.1. These bacteria are ubiquitous in nature as *B. subtilis* is predominantly found in soil, GI tract of humans and ruminants and hay, hence, is also sometimes commonly referred to as hay or grass bacillus (Bhunia, 2018). For swarming motility to occur, it normally requires a solid medium that is energy-rich, for example, PCSMA used in this present study. Specific conditions that influence swarming are dependent on the organism being considered. Certain bacteria like *B. subtilis* can swarm on an array of media that are energy-rich while other bacteria like *Salmonella enterica* and *Yersinia enterocolitica* require specific supplementation such as glucose to swarm. Swarming is encouraged by an elevated rate of proliferation which justifies the requirement for conditions to be energy-rich (Kearns, 2010, Kumari and Sarkar, 2016).

Figure 4.4: Swarming motility of *Bacillus* sp.

4.4 IDENTIFICATION OF LACTIC ACID BACTERIA BY 16S rDNA SEQUENCING

A total of 30 bacterial isolates were isolated from commercial *aMasi* samples. These isolates were initially screened by using macroscopic or colony morphology on MRS agar, Gram staining and microscopic morphology. Out of the 30 isolates, only 29 were considered as presumptive LAB, as these isolates had exhibited growth on MRS agar with round colonies that were white in colour and had an entire margin with a smooth and shiny surface and had a Gram-positive reaction. All isolates were then identified to species level by 16S rDNA sequencing. The predominating bacteria in commercial *aMasi* samples were LAB in which 29 out of the 30 isolates had comprised of *Leu*. *pseudomesenteroides* (16), *Leu*. *mesenteroides* (11) and *Lc*. *lactis* (2). The swarming bacterium found in all 10 samples of *aMasi* was identified as either *Bacillus subtilis*, *B*. *amyloliquefaciens* or *B*. *velezensis* (1). The identity of these isolates is displayed in Table 4.1. A total of nine isolates had different GenBank Accession numbers which were selected for antibiotic susceptibility testing.

Table 4.1: Identity of isolated lactic acid bacteria by 16S rDNA sequencing

Sample number	Isolate code	Bacteria identified	GenBank Accession Number
1	1.1	*Leu. mesenteroides**	MF098088.1
	1.2	*Leu. mesenteroides**	MG953247.1
	1.3	*Leu. mesenteroides**	KY660395.1
2	2.1	*Leu. pseudomesenteroides**	KY649503.1
	2.2	*Leu. pseudomesenteroides*	KY649503.1
	2.3	*Leu. pseudomesenteroides*	KY649503.1
	2.4	*Leu. pseudomesenteroides*	KY649503.1
3	3.1	*Leu. pseudomesenteroides**	KY649503.1/KT633928.1
	3.2	*Leu. mesenteroides*	MG953247.1

	3.3	*Leu. pseudomesenteroides*	KY649503.1/KT633928.1
4	4.1	*Leu. mesenteroides*	KY660395.1
	4.2	*Leu. mesenteroides*	KY660395.1
5	5.1	*Leu. pseudomesenteroides**	MG550992.1
	5.2	*Leu. mesenteroides*	KY649503.1
	5.3	*Lc. lactis**	CP028160.1
	5.4	*Lc. lactis*	CP028160.1
6	6.1	*Leu. pseudomesenteoides**	MF103729.1
	6.2	*Leu. pseudomesenteroides*	MF103729.1
7	7.1	*Leu. pseudomesenteoides*	MG550992.1
	7.2	*Leu. pseudomesenteroides*	KY649503.1
	7.3	*Leu. pseudomesenteroides**	MG694682.1
8	8.1	*Leu. pseudomesenteroides*	MF103729.1
	8.2	*Leu. pseudomesenteroides*	MG694682.1
	8.3	*Leu. pseudomesenteroides*	MF103729.1
9	9.1	*Leu. mesenteroides*	MG953247.1
	9.2	*Leu. mesenteroides*	MG953247.1
10	10.1	*Leu. mesenteroides*	MF098088.1
	10.2	*Leu. mesenteroides*	MF098088.1
	10.3	*Leu. mesenteroides*	MF098088.1
	10.4	*Bacillus subtilis/ Bacillus amyloliquefaciens* or *Bacillus velezensis*	MG733923.1/MG733918.1/CP033054.1

NB: All strains marked with an asterisk (*) were the strains selected for antibiotic susceptibility testing.

4.5 DETERMINING THE EFFECT OF PHYSICOCHEMICAL PROPERTIES ON THE GROWTH OF LACTIC ACID BACTERIA IN *aMasi* SAMPLES

4.5.1 Principal Component Analysis of Physicochemical Properties on the Growth of LAB

The aim was to represent the integration, grouping and interaction of seven variables using PCA: LAB count, pH, titratable acidity (lactic acid %), a_w, moisture (%), fat (%) and lactose (mg/25ml lactose·H_2O). Loadings that had an absolute value of >0.30 were taken into consideration for evaluation. As listed in Table 4.2, approximately 75.25% of variances in the data were retained by the first three principal components (PCs) i.e. PC1, PC2 and PC3. In addition, each of these PCs had an eigenvalue >1; however, only the first two PCs were graphically illustrated in Figure 4.5 a) and b) due to them accounting for the largest variances in the data set.

With respect to the first principal component (PC1); this PC accounted for the highest total variance of 33.16%, and its parameters were effectively used to describe the data illustrated in Table 4.2 and Figure 4.5a) and b). PC1 had high loadings for LAB count (-0.32), lactic acid % (0.53), a_w (0.54), moisture % (-0.33) and lactose (-0.36). PC1 increased with an increase in lactic acid % and a_w, whilst it negatively correlated with LAB count, moisture % and lactose (mg/25ml lactose·H_2O). The results showed an increase in LAB count with an increase in moisture % and lactose (mg/25ml lactose·H_2O) whilst, LAB count had decreased with an increase in lactic acid % and a_w. Moreover, pH and fat % had no effect on PC1, high LAB counts were observed for samples 6 and 7 whist low LAB counts were observed for samples 9 and 10.

PC2 on the other hand, had accounted for approximately 27.53% of the total variance, accounting for the second-highest PC and is displayed in Table 4.2 and Figure 4.5 a) and b). This PC had retained high loadings for LAB count (-0.38), pH (-0.50), fat % (0.57) and lactose (mg/25ml lactose·H_2O) (0.40). PC2 increased with an increase in fat % and lactose (mg/25ml lactose·H_2O), whilst it negatively correlated with LAB count and pH. It was observed that the growth of LAB had increased with an increase in pH, whilst it decreased with an increase in fat % and lactose (mg/25ml lactose·H_2O). Moreover, lactic acid %, a_w and moisture % had no effect on PC2. High LAB counts were observed for samples 7 and 8 and low LAB counts were observed for samples 2 and 4.

PC3 had accounted for approximately 14.56 % of the total variance. This PC had retained high loadings for LAB count (0.48), lactic acid % (0.46) and moisture % (0.63). This PC however, was not graphically represented due to this PC accounting for only 14.56% of the total variance which is relatively small compared to PC1 and PC2 which had accounted for 33.16% and 27.53% respectively, constituting 60.69% which was sufficient for graphical representation.

Table 4.2: Factor loadings of principal component analysis for the seven variables

	PC1	PC2	PC3
LAB count	**-0.32**	**-0.38**	**0.48**
pH	0.27	**-0.50**	-0.14
Titratable acidity (lactic acid %)	**0.53**	0.09	**0.46**
a_w	**0.54**	0.23	0.29
Moisture (%)	**-0.33**	0.25	**0.63**
Fat (%)	0.11	**0.57**	-0.21
Lactose (mg/25ml lactose·H_2O)	**-0.36**	**0.40**	-0.07
Variance (%)	33.16	27.53	14.56
Cumulative variance	33.16	60.69	75.25
Eigenvalue	2.32	1.93	1.02

Note: Values in bold font indicate absolute values >0.30.

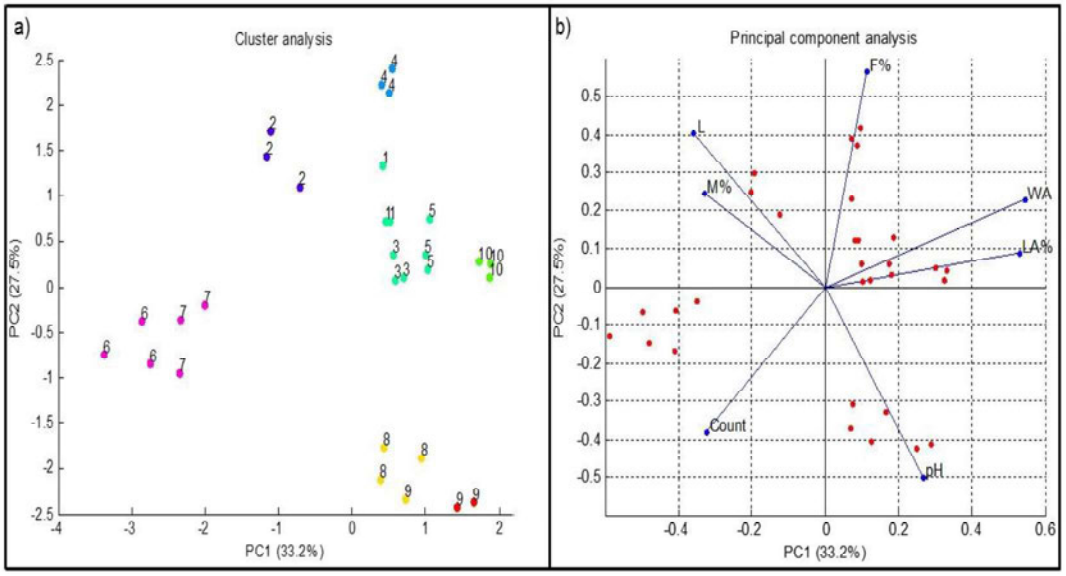

Figure 4.5: a) Score plot of data on PC1 and PC2, numbers 1 to 10 indicate *aMasi* samples and **b)** PCA biplot of data on PC1 and PC2. L - lactose, F% - fat %, WA - water activity, LA% - lactic acid %, Count - LAB count, pH - pH and M% - moisture %.

4.5.2 Regression Tree Model of the Physicochemical Properties of *aMasi* and Lactic Acid Bacterial Growth

A CART was used to illustrate LAB count and pH to determine if a certain LAB would be present in *aMasi* samples, depicted in Figure 4.6. If the LAB count was < Log 7.18, < Log 6.99 and with a pH < 4.45, then *Leu*. *mesenteroides* KY660395.1 was likely to be present in the *aMasi* samples, alternatively, if the pH was ≥ 4.45 then *Leu*. *mesenteroides* MF098088.1 was present in the samples. On the other hand, if LAB count was < Log 7.18 but ≥ Log 6.99, then *Leu*. *mesenteroides* MG953247.1 was present in the samples. Moreover, if the LAB

count was ≥ 7.18 with a pH of < 4.195 then *Leu*. *pseudomesenteroides* KY649503.1/KT633928.1 were likely to be in the samples. However, if the LAB count was ≥ 7.18 with a pH of ≥ 4.195 but with LAB count being < Log 7.35 then, *Leu*. *pseudomesenteroides* KY649503.1 would be present in the *aMasi* samples. Alternatively, if the LAB count was ≥ 7.18 with a pH of ≥ 4.195 but with LAB count being ≥ Log 7.35 then, *Leu*. *pseudomesenteroides* MF103729.1 would be present in the *aMasi* samples.

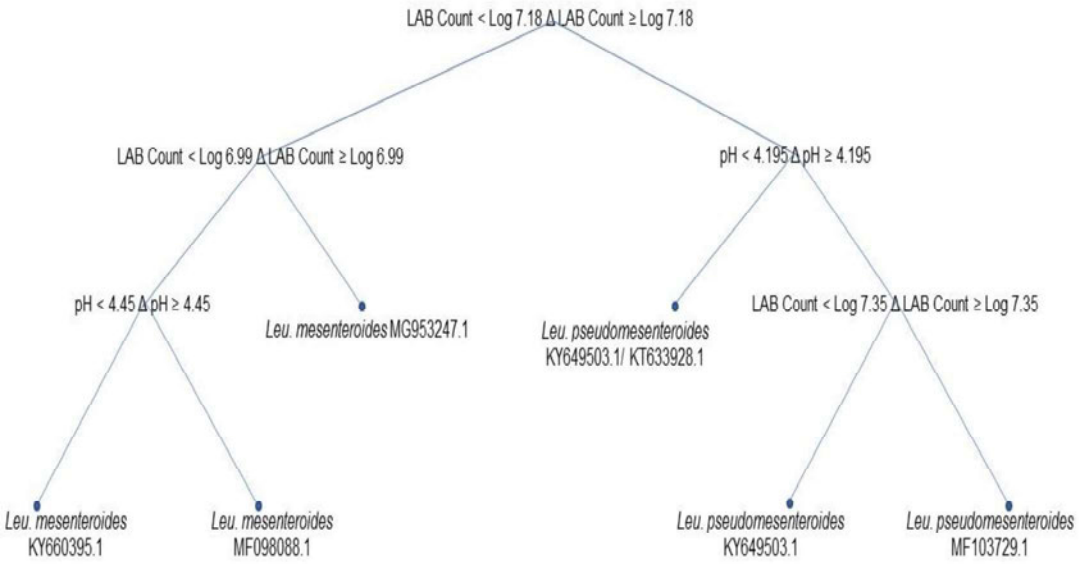

Figure 4.6: CART illustrating the LAB count and pH to determine which LAB would be present in *aMasi* samples.

4.6 PHENOTYPIC ANTIBIOTIC SUSCEPTIBILITY TESTING OF LACTIC ACID BACTERIA

The isolated LAB were screened for AR using the disc diffusion method. All the isolates showed a high level of resistance towards vancomycin, colistin sulphate, fosfomycin and pipemidic acid except for *Lc*. *lactis* CP028160.1 which was found to be susceptible to vancomycin. All isolates were susceptible to tetracycline and erythromycin whilst eight out of nine strains were susceptible to chloramphenicol with seven out of nine isolates being susceptible to ampicillin. Furthermore, the isolates had displayed intermediate resistance mainly towards kanamycin and streptomycin although four isolates had shown resistance towards the aminoglycosides i.e. either gentamicin, kanamycin or streptomycin. These results were compared to the guidelines outlined by CLSI (2012). The AR profiles of these LAB isolates are illustrated in Table 4.3. Resistance towards selected antibiotics such as vancomycin, pipemidic acid, colistin sulphate and fosfomycin are shown in Figures 4.7 to 4.9.

Table 4.3 Antibiotic susceptibility testing of LAB strains isolated from commercial *aMasi* samples

	LAB susceptibility to antibiotics										
	Van (30 μg)	**Col (10 μg)**	**Fos (50 μg)**	**PA (20 μg)**	**Gen (10 μg)**	**Kan (30 μg)**	**Strep (25 μg)**	**Tet (30 μg)**	**Ery (30 μg)**	**Amp (10 μg)**	**Chl (30 μg)**
LAB strains from commercial *aMasi* samples	**Average zone of inhibition diameter (mm)**										
***Leu. mesenteroides* MF098088.1**	0	9 ± 0.00	0	0	14 ± 0 58	12 ± 0.67	16 ± 0.88	22 ± 0 33	24 ± 0.33	20 ± 0.67	18 ± 0.88
***Leu. mesenteroides* MG953247.1**	0	9 ± 0.33	0	0	21 ± 0 00	18 ± 0.88	17 ± 0.33	27 ± 0 00	29 ± 1.00	29 ± 0.00	25 ± 1.86
***Leu. mesenteroides* KY660395.1**	0	10 ± 0.00	0	0	20 ± 0 00	17 ± 0.00	17 ± 0.58	28 ± 0 00	29 ± 1.00	16 ± 0.58	25 ± 1.16
***Leu. pseudomesenteroides* KY649503.1/ KT633928.1**	0	9 ± 0.00	0	0	16 ± 0 33	16 ± 0.58	17 ± 0.33	25 ± 0 00	30 ± 0.00	17 ± 0.33	22 ± 1.00
***Leu. pseudomesenteroides* KY649503.1**	0	9 ± 0.00	0	0	15 ± 0 33	16 ± 0.00	19 ± 0.00	23 ± 0 00	30 ± 0.00	26 ± 0.00	21 ± 0.33
***Leu. pseudomesenteroides* MG550992.1**	0	9 ± 0.00	0	0	20 ± 0 33	18 ± 0.58	20 ± 0.88	29 ± 0 33	33 ± 0.33	28 ± 0.00	22 ± 0.58
***Lc. lactis* CP028160.1**	20 ± 0 33	0	12 ± 0.67	0	14 ± 0 00	16 ± 0.00	13 ± 0.67	27 ± 0 33	24 ± 0.00	24 ± 0.33	25 ± 0.00
***Leu. pseudomesenteroides* MF103729.1**	0	11 ± 0.00	0	0	10 ± 0 33	15 ± 0.67	18 ± 0.33	30 ± 0 33	30 ± 0.33	24 ± 0.33	23 ± 0.88
***Leu. pseudomesenteroides* MG694682.1**	0	8 ± 0.67	0	0	16 ± 0 33	13 ± 0.67	16 ± 0.00	26 ± 0 00	26 ± 0.67	25 ± 0.00	26 ± 0.33
***Staphylococcus aureus* ATCC 25923 (control strain)**	17 ± 0 88	0	22 ± 2.40	10 ± 0.33	17 ± 1 20	18 ± 1.16	15 ± 0.00	29 ± 0 67	23 ± 0.58	33 ± 0.00	25 ± 1.00

NB: Van – Vancomycin, Col – Colistin Sulphate, Fos – Fosfomycin, PA – Pipemidic Acid, Gen – Gentamicin, Kan – Kanamycin, Strep – Streptomycin, Tet – Tetracycline, Ery – Erythromycin, Amp – Ampicillin and Chl – Chloramphenicol. Values are represented as average zone of inhibition diameter (rounded to nearest whole (mm)) ± standard error of the mean, n=3 using the guidelines determined by CLSI (2012) whereby, LAB strains were regarded as either: resistant (R) ≤ 14 mm, intermediate (I) 15 – 19 mm and susceptible (S) ≥ 20 mm.

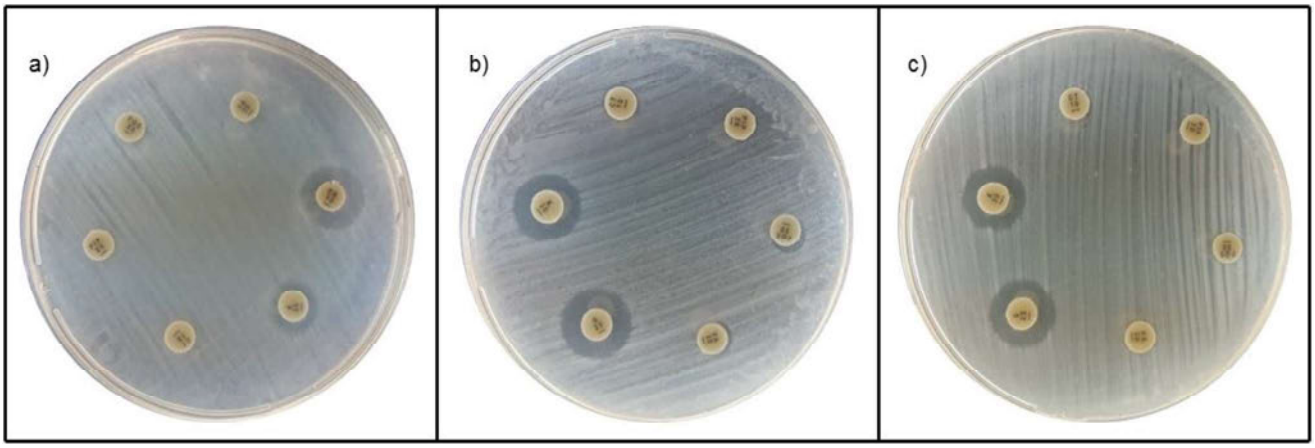

Figure 4.7 *Leu. mesenteroides* isolates showing resistance towards antibiotics: vancomycin, pipemidic acid, fosfomycin and colistin sulphate; a) *Leu*. *mesenteroides* MF098088.1, b) *Leu. mesenteroides* MG953247.1 and c) *Leu. mesenteroides* KY660395.1.

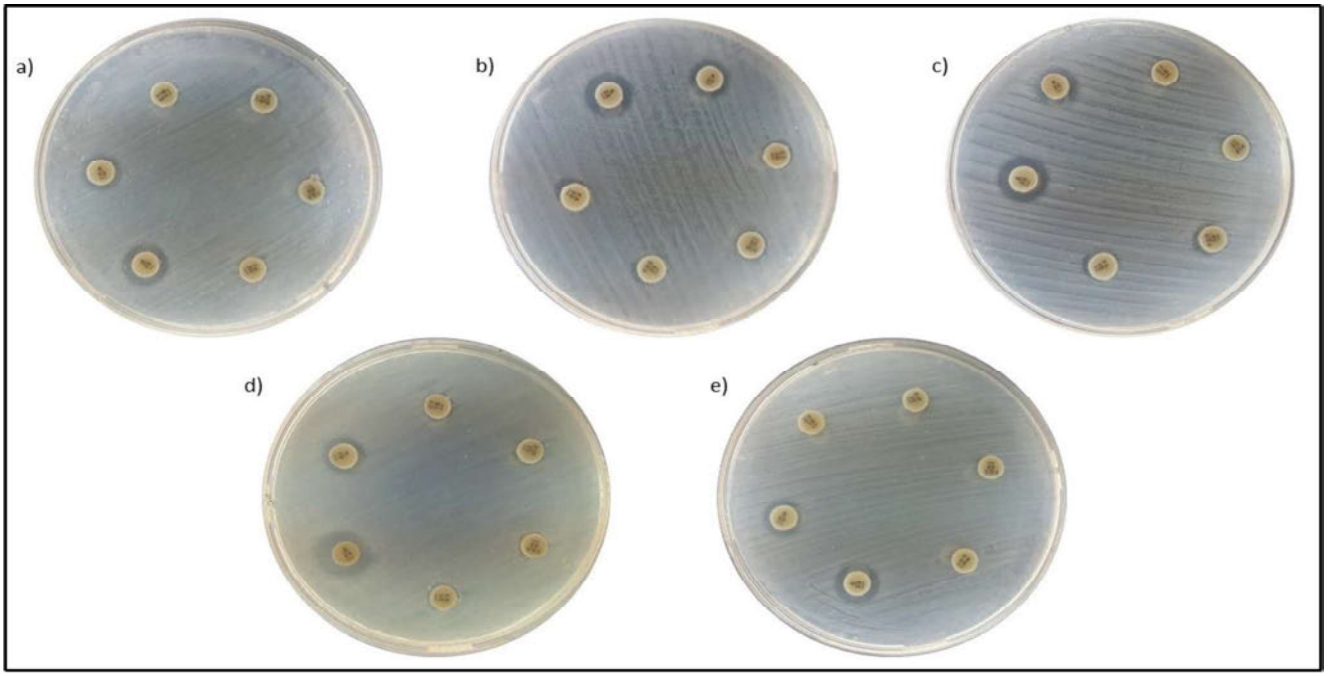

Figure 4.8 *Leu. pseudomesenteroides* isolates resistant to antibiotics: vancomycin, pipemidic acid, colistin sulphate and fosfomycin; a) *Leu. pseudomesenteroides* KY649503.1/ KT633928.1, b) *Leu. pseudomesenteroides* KY649503.1, c) *Leu. pseudomesenteroides* MG550992.1, d) *Leu. pseudomesenteroides* MF103729.1 and e) *Leu. pseudomesenteroides* MG694682.1.

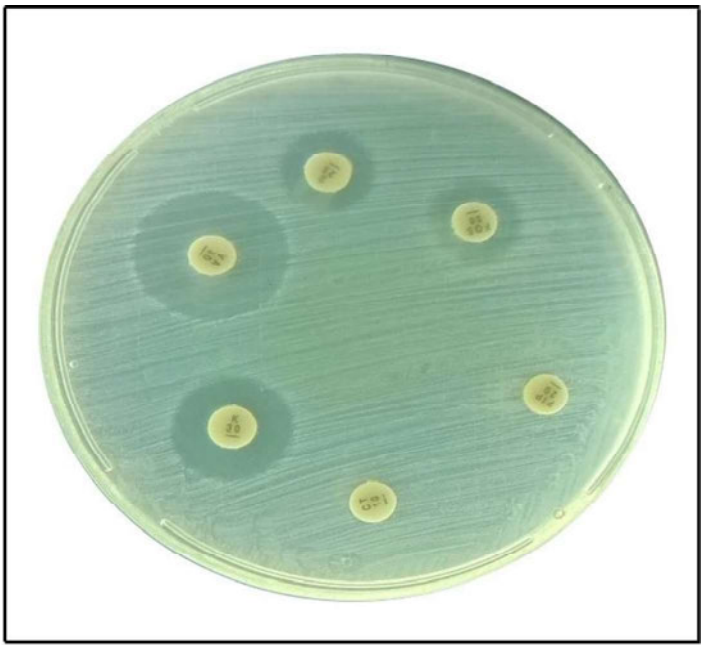

Figure 4.9 *Lc. lactis* CP028160.1 resistance to antibiotics: colistin sulphate, fosfomycin, pipemidic acid and streptomycin.

4.7 MOLECULAR SCREENING OF ANTIBIOTIC-RESISTANT GENES IN LACTIC ACID BACTERIA USING PCR

The optimisation screening process was only done on 3 *Leu*. *mesenteroides* isolates i.e. *Leu. mesenteroides* MF098088.1, MG953247.1 and KY660395.1 and these LAB were screened for eight out of sixteen resistance genes (*van* A, *van* B, *van* D, *van* E, *van* X, *aac*(6')-*aph*(2''), *aph*(2'')-Ib and *aph*(3'')-III). None of the three bacteria yielded amplicons for the presence of these eight resistant genes (figures are not shown).

CHAPTER FIVE: GENERAL DISCUSSION

5.1 TOTAL BACTERIAL AND LACTIC ACID BACTERIAL COUNTS IN *aMasi* SAMPLES

Milk is a highly nutritious substrate hence being the perfect substrate to support the growth of a wide variety of microorganisms. Hence, the purpose of surveying the total and LAB populations depicted in Figures 4.1 and 4.2 was to strengthen the hypothesis that LAB would be the predominating population in the *aMasi* samples. The Tukey's posthoc comparison test was chosen due to it being regarded as the most useful test for multiple comparisons. The test compares all potential pairs of means; however, the limitation of this test is that it is not statistically strict or robust as the means are sensitive to the requirement of following a normal distribution (Granato *et al*., 2014). Moreover, when significant differences exist between a pair of means, the Tukey test would be unable to single out the differences. Despite this limitation, the Tukey posthoc test is the procedure of choice favoured by several researchers in the field as it avoids Type II errors (rejecting equality when samples are not different) (Granato *et al*., 2014).

The presumptive mean total bacterial count in this study depicted in Figure 4.1 was slightly lower than the mean total plate count of 5.5×10^{8} CFU/ml (8.74 Log CFU/ml) for South African fermented milk reported by Beukes *et al*. (2001). The milk used in the study by these authors were spontaneously fermented, hence it would be acceptable for the total plate counts to be higher due to the fermentation being carried out in a non-controlled environment. Moreover, the presumptive mean total bacterial count in this study was slightly higher than the total mesophilic counts reported for Botswana *Madila* (Matsheka *et al*., 2013). The mean total mesophilic count reported by the authors was 6.5 Log CFU/ml which was approximately 0.86 Log CFU/ml lower in comparison to the results achieved in this study.

In contrast to the results achieved in this study, the range for total bacterial counts reported in this study was significantly higher than the range reported for total plate counts obtained by Moyane and Jideani (2013) for commercial South African *aMasi* samples. The authors had reported a range from 4.2×10^{3} CFU/ml to 9.1×10^{4} CFU/ml which equated to 3.62 Log CFU/ml to 4.96 Log CFU/ml. A possible reason for these results could be due to the fact that the study by these authors had only reported on the total plate counts from five *aMasi* samples. A better comparison of these results could have been deduced if a bigger sample size was used by the authors.

The presumptive LAB range achieved in this study, presented in Figure 4.2 ranged from 6.39 Log CFU/ml to 7.86 Log CFU/ml which was lower to those reported in literature. A study by Simatende (2016), had reported LAB counts from Swazi *Emasi* to be in the range of 7.30 –

8.82 Log CFU/ml. LAB counts from Zimbabwean *aMasi* had ranged from 8.29 – 9.88 Log CFU/g cited by Simatende (2016). LAB counts in this study were in accordance with LAB counts of 9.8×10^6 CFU/ml (6.99 Log CFU/ml) in Nigerian *Nono* and was cited by Simatende (2016). The mean presumptive LAB count in this study was also lower than the mean presumptive LAB count of 7.7×10^8 CFU/ml (8.89 Log CFU/ml) for South African fermented milk reported by Beukes *et al*. (2001). Furthermore, LAB counts from the Ghanaian fermented milk, *Nunu* was reported to have had up to 8.82 Log CFU/ml (Akabanda *et al*., 2010). However, Matsheka *et al*. (2013) had reported contrasting results in *Madila*, which had a LAB count of 5.3 Log CFU/ml which was approximately 1.85 Log CFU/ml lower than the mean reported in this study.

5.2 LACTIC ACID BACTERIA AS THE PREDOMINATING BACTERIAL POPULATION

The proportional prevalence of presumptive LAB to the presumptive total bacterial population is illustrated in Figure 4.3. It was observed in literature that various types of fermented milk such as Swazi *Emasi*, Zimbabwean *aMasi*, Nigerian *Nono*, traditionally prepared South African fermented milk and Ghanaian *Nunu* were a rich source of LAB since they had predominated the microflora of these fermented milks (Beukes *et al*., 2001, Akabanda *et al*., 2010, Matsheka *et al*., 2013, Simatende, 2016). It was also an indication that since most of these types of milk form staple diets in third world settings (Mokoena *et al*., 2016), the high amounts of LAB present constitutes one of the requirements for probiotic food (Simatende, 2016).

The presumptive LAB population in relation to the presumptive total bacterial population for the *aMasi* samples except for sample 6, had ranged from 95.47% to 99.77%. These results implied that LAB was the presumptive predominating population among these samples. Sample 6 had a lower percentage of LAB present (87.44%) in contrast to the other nine samples. Although the presumptive LAB population had predominated this sample, the percentage of other bacteria present was 12.56% which was significantly higher in contrast to the other samples.

Samples 1, 2, 6, 7 and 8 were purchased from well-established supermarkets and are well-established brands whilst, samples 3, 4, 5, 9 and 10 were purchased from smaller supermarkets and are less popular brands. The samples purchased irrespective of the establishment of the supermarket or brand showed that sample 6 was an outlier and would have no effect on the remaining nine samples in this regard, hence, indicating that this specific manufacturer may not have adhered to proper food safety practices. This could indicate poor hygiene, poor implementation of hazard analysis and critical control points (HACCP) for microbial hazards or that pasteurisation (Sandrou and Arvanitoyannis, 2000) employed for

this sample was not effective during the manufacture of this product. Furthermore, it could also be an indication that cleaning-in-place (CIP) procedures need to be implemented more frequently to prevent microbial contamination and biofilm formation within the milk processing equipment, milk handling devices, gaskets, pipes and floors (Cherif-Antar *et al*., 2016, Ostrov *et al*., 2016, Friedlander *et al*., 2019, Weber *et al*., 2019).

A possible reason for the contrasting lower microbial counts achieved in this study is the fact that commercial *aMasi* is manufactured from pasteurised milk (Kayitesi *et al*., 2017). In addition to this, defined adjunct cultures in the form of single and/or mixed cultures of LAB are used and the manufacture often occurs under controlled, hygienic conditions and fermentation occurs in a controlled manner according to specifications within the dairy manufacturing plant (Goff, 2018c). Therefore, the microbial diversity of these samples is limited as compared to the traditionally produced samples which occur spontaneously, and do not undergo any form of heat treatment or pasteurisation to kill vegetative cells, spore-formers and foodborne pathogens that are usually implicated in milk products (Sharma *et al*., 2015, Kayitesi *et al*., 2017).

5.3 THE USE OF PLATE COUNT SKIMMED MILK AGAR IN PROVIDING A TOTAL BACTERIAL COUNT

Plate Count Agar is a broad-spectrum medium for the enumeration of viable microorganisms from animal feed, food, dairy and water samples (Merck, 2019). However, it was observed initially during the preliminary investigation of this study that the use of Plate Count Agar (Biolab, Merck) had not been suitable to quantify or enumerate the 'total' presumptive population of bacteria from dairy samples. The main reason for this was that the plate counts obtained were much lower than the presumptive LAB population (at least 2 to 3 Log CFU/ml lower) and some of these plate counts were not suitable to report due to them falling below the counting specification range of 30 to 300 colonies in the 10^{-3} to 10^{-5} dilutions which would have affected the statistical outcome of these results. The results achieved initially were similar to those reported by Beukes *et al*. (2001) and Moyane and Jideani (2013), where the presumptive LAB counts reported by these authors were higher than the total plate counts. This was also a possible shortfall in literature, given the fact that the authors may have most likely used Plate Count Agar instead of PCSMA which will be discussed further.

The typical composition per litre of Plate Count Agar consists of four main constituents namely; 5 g/L of tryptone or enzymatic digest of casein (equivalent to tryptone), 2.5 g/L of yeast extract and 1 g/L of glucose and 9-18 g/L of agar (ISO, 1998). However, this medium was not suitable for enumerating bacteria from fermented dairy products as recommended by the supplier (Biolab, Merck). These constituents are essential for the growth of most bacteria, including LAB since tryptone serves as a source of nitrogen which contains significant levels

of amino acids, yeast extract which mainly provides microorganisms with B-complex vitamins and glucose serves as a carbon source which provides energy to facilitate the growth of bacteria whilst the purpose of agar is to solidify the medium (Merck, 2019).

Despite these constituents being present they did not facilitate or take into the account the growth of LAB. Succeeding these findings, the standard set out by ISO (1998) was adhered to whereby, the recommendation given when examining dairy products was to add 1 g of skimmed milk powder (free from inhibitory substances) to the original Plate Count Agar composition.

Milk is typically regarded as an excellent source of all primary nutrients inclusive of macro-minerals, carbon and nitrogen. Several micronutrients such as vitamins and micro-minerals are also acquirable which makes milk nutritious and an excellent substrate for the growth of several microorganisms. In addition to this, lactose is characteristic in milk, since it is intrinsically present (Quigley *et al*., 2013, Goff, 2018a, IFIC, 2019).

Most microorganisms, however, utilise glucose as a carbon source, therefore, acquirable carbohydrates that are present in the medium must be converted by the microorganisms to glucose in order for metabolic processes to prevail (Goff, 2018a). Since there is an absence of the enzyme lactase in most microorganisms to break lactose into its monomers i.e. glucose and galactose, this provides LAB with the advantage of possessing lactase which readily breakdown lactose into its monomers and can utilise glucose as a source of energy (Goff, 2018a, IFIC, 2019). It is due to this advantage that LAB have a competitive advantage in milk because of their ability to predominate the medium by out-competing the other bacteria which are unable to acquire glucose from lactose (Goff, 2018a). PCSMA (Merck) was therefore used to enumerate viable microorganisms to determine the presumptive total population from each of the *aMasi* samples which accounted for the growth of LAB.

5.3.1 The Role of Hazard Analysis and Critical Control Points in Controlling Microbiological Hazards

Hazard Analysis and Critical Control Points play an integral role in the dairy industry especially when it comes to microbiological hazards. Milk and milk products as with other types of food can cause foodborne illness. The reason behind this is because the quality of milk can be affected by factors such as pathogen contamination and proliferation, environmental pollution, nutrient degradation and chemical additives (FAO, 2019).

Given the fact that milk is an excellent medium for the proliferation of bacteria and other microflora, it has become a major food safety concern in the dairy industry. Microbiological hazards can be introduced into the milk from the environment or from the dairy animals themselves. Such products may contain undesirable bacteria such as *Brucella abortus*,

Bacillus cereus, *Brucella melitensis*, *Clostridium botulinum*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Mycobacterium bovis*, *Salmonella*, *Staphylococcus aureus* and *Y. enterocolitica* (FAO, 2019).

However, critical control points (CCP) to control microbiological hazards such as pasteurisation at 72°C for 15 seconds to kill pathogenic and spoilage microorganisms followed by rapid cooling to approximately 30°C is employed to deter the outgrowth of spores (Sandrou and Arvanitoyannis, 2000, Kayitesi *et al*., 2017). Despite this, spore-forming bacteria can survive industrial pasteurisation because of their ability to form spores when exposed to unfavourable or stressed environments (Gopal *et al*., 2015), hence it was not surprising to find *Bacillus* sp. depicted in Figure 4.4 in all these pasteurised *aMasi* samples. This was identified by 16S rDNA sequencing and makes reference to what was identified in Table 4.1 under Chapter 4.4. In addition to this, such bacteria are also able to form biofilms within the pipes and stainless-steel equipment located in dairy manufacturing plants (Gopal *et al*., 2015).

5.4 IDENTIFICATION OF LACTIC ACID BACTERIA BY 16S rDNA SEQUENCING

A study by Beukes *et al*. (2001), had reported on the microbial composition of South African traditionally fermented milk whereby, the dominant genera of LAB were found to be *Lactobacillus*, *Lactococcus* and *Leuconostoc*. From these South African fermented milk samples, *Lc*. *lactis* subsp. *lactis* was found to be a dominant species among the lactococci whilst, the majority of *Leuconostoc* isolated were identified as *Leu*. *mesenteroides* subsp. *dextranicum*. Other species that were identified from the study had included *Lb*. *delbrueckii*, subsp. *lactis*, *Lb*. *plantarum*, *Leu*. *citreum* and *Leu*. *lactis*. The results observed in this present study, listed in Table 4.1 were similar to the results reported by Beukes *et al*. (2001), whereby, *Leu*. *mesenteroides* was one of the predominating bacteria from the *Leuconostoc* genus whilst, *Lc*. *lactis* was also present in the commercial *aMasi* samples from this study, but it did not predominate the population. The results from this study were also similar to the results reported by Simatende (2016) whereby, *Leu*. *pseudomesenteroides* was identified as a predominating species in Swazi *emasi* after 16S rDNA sequencing.

The current observations in this study are also in similar accordance with the results reported by Osvik *et al*. (2013), who used a more modern approach in molecular techniques to identify LAB using 16S rDNA sequencing and Denaturing Gradient Gel Electrophoresis (DGGE) from *aMasi* samples originating from KZN. From this author's study, most of the strains identified belonged to the genus *Lactococcus*, whilst, several other species from *Leuconostoc*, *Lactobacillus* and *Enterococcus* were also identified. A greater consortium of microorganisms is expected because these *aMasi* samples were traditionally produced, hence having a greater variety of other LAB present as opposed to the commercially produced varieties.

In a study on the isolation and identification of the microbial community present in Sub-Saharan fermented milk by Schutte (2013), the predominating LAB that was present in fermented milk such as *omaere* was lactococci (94%), *chambiko* and *chekapmkaika* was lactobacilli (30% and 45%, respectively), in *omashikwa* it was enterococci (43%) and in *masse,* it was leuconostocs (68%). All these fermented milk are produced in a similar manner and share a similar microbial consortium to traditionally produced *aMasi*.

The predominating population of fermented milk will vary according to the origin of the product, the substrate used in the fermentation, utensils used, maintenance of the cultures and the method of production (traditionally or commercially produced) (Prado *et al*., 2015). In this study a reason for *Leu*. *mesenteroides* and *Leu*. *pseudomesenteroides* predominating the microbial population is because they were commercially produced whereby, well-defined starter culture strains were used. In traditional production, however, fermentation occurs spontaneously in a natural environment. The natural niche of *Leuconostoc* species originates from green vegetation and roots. It is from these natural niches where they can propagate to the raw materials and/ or substrates that are used in the production of fermented milk. Therefore, they have a high prevalence as part of the natural LAB community involved in the manufacture of fermented dairy products (Flórez *et al*., 2016).

Given the fact that the milk samples from previous studies were naturally fermented, it would be common for these samples to have a greater microbial consortium since the fermentation process of these products are spontaneous and often occur in uncontrolled environments that lack good hygiene practices and do not undergo any heat treatment before consumption. In contrast to this, commercially fermented milk like the samples used in this present study occurs under controlled parameters whereby, the milk is pasteurised before starter cultures are added. Starter cultures added are well-defined strains that are either added solely or as a mixed culture at a defined concentration and the manufacture of these products occur in facilities that practice good manufacturing and good laboratory practices and have proper specifications in place for temperature, time and storage.

5.5 IDENTIFICATION OF *Bacillus* sp. IN COMMERCIAL *aMasi* SAMPLES

The *Bacillus* species identified by 16S rDNA sequencing, listed in Table 4.1 were identified as either *Bacillus subtilis*/*Bacillus amyloliquefaciens* or *Bacillus velezensis* and is depicted in Figure 4.4. These bacteria are ubiquitous in nature as *B*. *subtilis* is predominantly found in soil, GI tract of humans and ruminants and hay, hence, is also sometimes commonly referred to as hay or grass bacillus (Bhunia, 2018). Similarly, *B*. *amyloliquefaciens* and *B*. *velezensis* are ubiquitous in the environment whereby these species are also predominantly found in soil and water (Bhunia, 2018). *B*. *cereus* is the most significant spore-forming spoilage bacterium in the dairy industry, due to its ability to produce enterotoxins which poses a safety concern

in the dairy industry since they are associated with incidences of food poisoning (Kumari and Sarkar, 2016).

Limited information exists on the prevalence of *B. subtilis*/*B. amyloliquefaciens* or *B. velezensis* in fermented milk products, however, there are studies that highlight the prevalence of *B. cereus* in fermented milk products. In a study by Tirloni *et al*. (2017), two *B. cereus* strains were absent in yoghurt and this was attributed to the synergistic effect of the natural microflora present in the milk and low pH (<5). However, in the same study by the authors, the growth of these *B. cereus* strains occurred rapidly in pasteurised milk and mascarpone cheese and was present up to approximately 7 Log CFU/ml indicating that pH was not restrictive, and no significant innate microflora was present in this scenario. In another study by Owusu-Kwarteng *et al*. (2017), *B. cereus sensu lato* was present at 1.8 Log CFU/ml in 35% of Ghanaian *Nunu* samples and at 2.6 Log CFU/ml in 39% of *Woagashie* (West African soft cheese) samples, respectively. However, according to the authors, these counts obtained were at levels that were considered safe for consumption.

In general, *Bacillus* sp. can enter the dairy environment through various channels during handling, processing and production. This primarily occurs from equipment that has not been improperly cleaned and sanitised. Resistance towards disinfectants, desiccation, heat and the hydrophobic properties of endospores allow these bacteria to anchor themselves to processing equipment and survive cleaning procedures (Kumari and Sarkar, 2016). This can result in biofilm formation which could possibly serve as a significant reservoir for recurrent contamination of dairy products. Aforementioned, spore-forming bacteria are ubiquitous in nature and contamination has been shown to transpire along the entire processing line inclusive of equipment such as filling machines, pasteurisers, packaging boards and blanks can be a source of contamination (Kumari and Sarkar, 2016).

In this present study, the *Bacillus* colonies appeared to have had a radial swarming motility as depicted in Figure 4.4. For swarming motility to occur, it normally requires a solid medium that is energy-rich, for example, PCSMA used in this present study. Specific conditions that influence swarming are dependent on the organism being considered. Certain bacteria like *B. subtilis* can swarm on an array of media that are energy-rich while other bacteria like *Salmonella enterica* and *Y. enterocolitica* require specific supplementation such as glucose to swarm. Swarming is encouraged by an elevated rate of proliferation which justifies the requirement for conditions to be energy-rich (Kearns, 2010, Kumari and Sarkar, 2016).

Even though certain bacteria can swarm over almost any agar surface, the requirement of soft agar in a limited range of concentrations is a necessity for most bacteria. Agar concentrations of 0.3% to solidify media usually excludes swimming motility and forces bacteria to move, most probably over the agar surface whilst agar concentrations greater than 1% forbids the swarming motility of several bacterial species (Kearns, 2010). Thus far, an

agar concentration at 1.5% is contemplated to be the standard that is used to solidify media in the laboratory that has been specifically chosen for swarming inhibition (Kearns, 2010). This proved to be partially true in the present study as the final agar concentration was adjusted to a final concentration of 2.5% by adding Agar Bacteriological (Merck, Germany) to the SMPCA to suppress the swarming motility of *Bacillus* sp. and permit the growth and enumeration of other viable microorganisms.

5.6 THE EFFECT OF SELECTED PHYSICOCHEMICAL PROPERTIES OF *aMasi* ON THE GROWTH OF LACTIC ACID BACTERIA

5.6.1 Principal Component Analysis on the Effect of Selected Physicochemical Properties and the Growth of Lactic Acid Bacteria

The principal component analysis (PCA) presented in Table 4.2 and Figure 4.5 a) and b) revealed a directly proportional relationship between the LAB count and the physicochemical properties, moisture % and lactose (mg/25ml lactose·H_2O). With regards to moisture, milk, in general, is a high-moisture substrate (Fox *et al*., 2015b). Having mentioned this, it was expected for the *aMasi* samples to have a high-moisture content ranging from 87.15% to 90.57%. This finding was comparable to the findings reported for yoghurt by authors Hassan and Amjad (2010) ranging from 84.58% to 86.14% although, this was slightly lower than the results achieved in this present study. High-moisture content makes fermented milk an excellent growth medium which could be the reason for the directly proportional relationship between LAB and moisture %, however, it is not definitive in defining microbial growth as the critical parameter for determining this is a_w and not moisture content (Goff, 2018a).

As previously mentioned, a directly proportional relationship was also observed for LAB count and lactose (mg/25ml lactose·H_2O). In this regard, lactose is the main sugar or carbohydrate naturally present in milk (Shendurse and Khedkar, 2016). It is a disaccharide that is comprised of the monomers, glucose and galactose. During fermentation, LAB utilises the lactose present in the milk substrate as an energy source which is cleaved by microbial beta-galactosidase during hydrolysis into monosaccharides, D-glucose and D-galactose. Pyruvic acid is then produced from the fermentation of the glucose monosaccharide, which is then processed to lactic acid (McKevith and Shortt, 2003, Shendurse and Khedkar, 2016). It would be acceptable for these two variables (LAB count and lactose) to have a directly proportional relationship between them at the outset of fermentation. This is because, at the beginning, LAB starter culture/s are added at a specific concentration to a vat whereby, the substrate (pasteurised milk) would have a higher concentration of lactose (McKevith and Shortt, 2003) which would decrease during the fermentation process whilst LAB growth would increase and try to reach a stable equilibrium. After fermentation, the expected result would be a decrease

in lactose (McKevith and Shortt, 2003) and LAB count due to the final product being rapidly cooled or refrigerated which could impose stress on the final LAB population.

The PCA had also revealed an inverse relationship between LAB count and physicochemical properties, lactic acid % and a_w. The inverse relationship between LAB count and lactic acid % is attributed to the action of LAB metabolising lactose to produce lactic acid as an end-product. This results in a decrease in lactose during the fermentation, due to the metabolism of LAB requiring lactose as a source of carbon and energy, therefore, increasing the production of lactic acid (Shendurse and Khedkar, 2016). The production of lactic acid acts as a preservative, inhibiting other undesirable microflora. Given the nature of LAB, they inherently acidify their food environment in a short period of time. This, in turn, self-imposed stress for themselves whilst concurrently being a necessity to respond to acidification to fulfil the criterion for probiotic selection (ability to function in the gut environment) as well as providing genetic and physical integrity (Kajfasz and Quivey, 2011, Radulović *et al*., 2012). However, such bacteria are equipped with an array of acid resistance mechanisms such as glutamate decarboxylase (GAD) and acid tolerance response (ATR) to survive these harsh conditions (Kajfasz and Quivey, 2011).

It would be clear and advantageous, for any microorganism to have the ability to withstand the adverse effects of environmental acidification. Under these circumstances, LAB proliferate in highly antagonistic environments among several antagonists and being able to tolerate acidic environments could be the primary reason by which these bacteria dominate their niche (Kajfasz and Quivey, 2011). Metagenomic data has been able to provide meaningful evidence about bacteria being intrinsically tolerant to acidic environments and how it contributes chiefly to fitness and survival traits of the bacteria (Kajfasz and Quivey, 2011). Moreover, such data also tells us how these traits allow these organisms to subsist periods of starvation and acidification to outcompete other microorganisms during periods of relative abundance of carbohydrate availability. A similar process is also true for food-related LAB that, by acidifying the food matrix, they predominate the population of the food matrix by inhibiting the growth of undesirable food spoilage and pathogenic bacteria (Kajfasz and Quivey, 2011). The results achieved in this study, however, seemed contradictory on this note, as other conditions such as cold shock treatment to stop the fermentation could explain a decrease in LAB growth.

The inverse relationship between LAB count and a_w was an expected result. The a_w for *aMasi* samples in this study had ranged from 0.82 to 0.95 averaging 0.90. There was limited literature available to compare these results to, however, it was reported in an earlier study that inconsistencies exist with respect to moisture requirements for the majority of LAB of commercial significance (Troller and Stinson, 1981). Practical knowledge by fermenting foods with LAB have been greatly accountable for empirically establishing minimal water activity (a_w) limits for growth in the 0.91 to 0.94 a_w range (Troller and Stinson, 1981). In addition to

this, since LAB are Gram-positive, most of these bacteria exist with an a_w limit of 0.90 to 0.95 (Erkmen and Bozoglu, 2016). The mean a_w for this study can be compared to this limit.

During the fermentation process, LAB can be subjected to osmotic stress when significant amounts of solutes (sugar and/or salt) are incorporated into the product (Serrazanetti *et al*., 2013). It was reported that lactobacilli inhabiting most food environments are challenged with salt (Piuri *et al*., 2003) and sugar stress (Piuri *et al*., 2005). A study on the disparities between sugar and salt osmotic stress have indicated that the hyperosmotic conditions imposed by sugar stress are much less inimical and are only temporary (transient osmotic stress). This is because of the ability of the cells to balance the excess and intracellular concentrations of sugars (lactose and sucrose) (van de Guchte *et al*., 2002). To survive and adapt to environmental changes, bacteria often accumulate compatible organic solutes (by synthesis or uptake) under hyperosmotic conditions. The compatible solutes are referred to as osmoprotectants. The main strategy of non-halophilic bacteria to adapt to high osmolarity is associated with the intensification of osmotolerance. Furthermore, osmoprotectants offer stability to enzymes and provide protection against osmotic stress but also against other types of stresses such as freezing, drying and high temperatures. Water loss is prevented by intracellular accumulation of compatible solutes caused by high external osmolarity and allows the regulation of turgor (Serrazanetti *et al*., 2013).

pH and fat % had no effect on PC1. It was a surprising observation that pH had no effect on PC1. The pH results obtained for *aMasi* samples in this study had ranged from 4.14 to 4.62 with an average of 4.34. This pH range was comparable to studies in literature by Moyane and Jideani (2013), who had reported pH values for commercial *aMasi* samples from South Africa, ranging from 4.22 to 4.34 with an average of 4.29. pH values for Swazi *emasi* samples in a study by Simatende (2016), had ranged from 4.31 to 5.03 averaging 4.68 and Beukes *et al*. (2001), had reported a pH range of 4.00 to 5.40 averaging 4.60 for South African fermented milk.

Whilst the pH of *aMasi* samples in this present study was in accordance to various studies aforementioned in literature, it was characteristic of the final *aMasi* product to have a pH within this range and was compliant to the South African Agricultural Product Standards Act, 1990 with all *aMasi* samples having a pH of less than 4.6 (South African Department of Agriculture and Fisheries, 2015). This is primarily due to fermentation by LAB which utilises lactose in the medium to produce lactic acid as the main product (Gänzle, 2015).

Acidity is benchmarked by pH and titratable acidity, however; pH is considered as a more purposeful measurement for quality and process control purposes since it is regarded as a judicious indicator of the preserving and safety effects of acidity (Goff, 2018b). However, in certain processes such as cheese making, titratable acidity is regarded as the standard

procedure to measure initial acid development. This is done to verify starter culture activity and due to this reason, titratable acidity is considered a more reliable indicator as opposed to pH because of its sensitivity to negligible changes in milk acidity during such processes (Goff, 2018d). pH, on the other hand, is a better process and quality control tool, because it measures undissociated free hydrogen ions (H^+) in solution with salts and proteins. This plays a significant role because protein functionality is modified by the presence of free H^+ ions and is responsible for imparting the sour taste profile (Goff, 2018b).

Fat content in the present study had ranged from 3.00% to 3.87% averaging 3.45%. These results were in accordance to the South African Agricultural Product Standards Act, 1990 as 9 out of 10 samples analysed were within the stipulated range of 3.3% to 4.5% (South African Department of Agriculture and Fisheries, 2015). It was observed from the nutritional information on the labels of 8 out of 10 products that the fat content had remained constant and was in accordance with their full-cream milk counterparts since these products are manufactured from full-cream pasteurised milk as the base substrate. The remaining 2 samples had no nutritional information on the label for any observations to be made.

From these observations, it can be deduced that non-lipolytic strains of LAB could have been used in the manufacture of these products as LAB possessing the enzyme lipase would have broken down or metabolised the fat globules into free fatty acids and glycerol within the milk matrix. This could explain why fat % had no effect on PC1. A lack of research exists on LAB lipases (Akabanda *et al*., 2014). In a study by the same authors on LAB isolated from *Nunu*, predominating LAB such as *Lb*. *fermentum* (32.18%), *Lb*. *plantarum* (9.09%), *Leu*. *mesenteroides* (21.95%), *E*. *faecium* (22.50%), *E*. *italicus* (17.95%) and *Lb*. *helveticus* (8.57%) had exhibited no lipolytic activity when grown on tributyrin agar plates (Akabanda *et al*., 2014).

PC2 on the other hand, had accounted for approximately 27.53% of the total variance, accounting for the second-highest PC and is displayed in Table 3.1 and Figure 3.1 a) and b). This PC had retained high loadings for LAB count (-0.38), pH (-0.50), fat % (0.57) and lactose (0.40). PC2 increased with an increase in fat % and lactose, whilst it negatively correlated with LAB count and pH. It was observed that the growth of LAB had increased with an increase in pH, whilst it decreased with an increase in fat % and lactose. Moreover, lactic acid %, a_w and moisture % had no effect on PC2. High LAB counts were observed for samples 7 and 8 and low LAB counts were observed for samples 2 and 4.

The directly proportional relationship between LAB count and pH can be attributed to the fact that at the onset of fermentation, milk is at a near-neutral pH (6.5-6.7). When the milk in vats are inoculated with LAB starter culture, being neutrophilic in nature (Hutkins and Nannen, 1993), they proliferate in the neutrophilic environment as they first catabolise lactose in the

medium into its monosaccharides, D-glucose and D-galactose. It is from these monomers, D-glucose to be specific, that is further metabolised into lactic acid as an end product, which rapidly reduces the pH of the milk from a near-neutral pH to a more acidic pH of ≤ 4.5. Additionally, LAB have stress-adaption mechanisms to assist them in being acid-tolerant. These mechanisms were previously discussed when discussing the inverse relationship between LAB count and lactic acid %.

5.7 PHENOTYPIC ANTIBIOTIC SUSCEPTIBILITY TESTING OF LACTIC ACID BACTERIA

Probiotic consumption by consumers is growing immensely, globally, as awareness of their therapeutic properties in establishing optimal gut and general health is increasing (Grover *et al*., 2012). Since there is an increasing trend in the consumption of these products, there is a need to exercise precaution to ensure the safety of consumers about the presence of AR in LAB. This present study was carried out to evaluate the prevalence of AR among LAB in commercially available *aMasi*. The highest frequency of resistance (%) was recorded against colistin sulphate, fosfomycin and pipemidic acid (n= 9), vancomycin (n=8), gentamicin (n=3), kanamycin (n=2) and streptomycin (n=1) (n, represents the number of isolates having resistance and is not inclusive of intermediate resistance). Most of the LAB isolates (7 out of 9), depicted in Table 4.3 and Figures 4.7 to 4.9 had intermediate resistance towards kanamycin and streptomycin. Apart from having resistance towards kanamycin and streptomycin, *Leu*. *pseudomesenteroides* KY649503.1/KT633928.1, KY649503.1 and MG694682.1 had also displayed intermediate resistance towards gentamicin, *Leu*. *mesenteroides* KY660395.1 and *Leu*. *pseudomesenteroides* KY649503.1/KT633928.1 had displayed towards ampicillin whilst, *Leu*. *mesenteroides* MF098088.1 had exhibited intermediate resistance to chloramphenicol. All LAB isolates were susceptible to tetracycline, erythromycin, ampicillin and chloramphenicol except *Leu*. *mesenteroides* MF098088.1 which had intermediate resistance towards chloramphenicol and *Leu*. *mesenteroides* KY660395.1 and *Leu*. *pseudomesenteroides* KY649503.1/KT633928.1 which had intermediate resistance towards ampicillin. *Leu. mesenteroides* MG953247.1, KY660395.1 and *Leu. pseudomesenteroides* MG550992.1 had also rendered susceptible to gentamicin whilst *Lc*. *lactis* CP028160.1 was the only isolate that was susceptible to vancomycin.

LAB have been reported to have resistance towards several classes of antibiotics due to their possession of AR genes. Their resistance towards antibiotics can either be intrinsic or extrinsic. Intrinsic mechanisms often involve the absence of the target, low cell permeability, antibiotic inactivation and the presence of efflux mechanisms (Radulović *et al*., 2012). Other resistant mechanisms include inhibition of cell wall synthesis (most common resistance mechanism), inhibition of protein synthesis (translation, which is the second most common mechanism), alteration of cell membranes, inhibition of nucleic acid synthesis and

antimetabolite activity. Thus far, LAB have intrinsic resistance towards antibiotics belonging to the classes of aminoglycosides (kanamycin, neomycin and streptomycin), glycopeptides (vancomycin and teicoplanin), tetracyclines, mupirocin and quinolones (ciprofloxacin, nalidixic acid and norfloxacin) (Álvarez-Cisneros and Ponce-Alquicira, 2018).

In the present study, most of the *Leuconostoc* species (6 out of 8) and *Lc*. *lactis* CP028160.1 were found to be susceptible towards ampicillin (penicillin-β-lactam) whilst concurrently, all the *Leuconostoc* species with the exception for the latter bacterium exhibited a high percentage of resistance to vancomycin (glycopeptide) and fosfomycin (phosphoric acid derivatives). Although *Lc*. *lactis* was resistant to fosfomycin, a slightly lowered susceptibility was observed in this regard compared to the *Leuconostoc* species. All three of these antibiotics are inhibitors of cell wall synthesis. With regards to resistance towards inhibitors of protein synthesis, all strains were susceptible to tetracycline and erythromycin (macrolide) whilst, susceptibility patterns had varied for all the strains towards gentamicin (aminoglycoside). Intermediate resistance was more prominent for most of the strains towards kanamycin (aminoglycoside) except for *Leu*. *mesenteroides* MF098088.1 and *Leu*. *pseudomesenteroides* MG694682.1 which was resistant. A similar trend was noted for most of the strains exhibiting intermediate resistance to streptomycin (aminoglycoside) except for *Lc*. *lactis* CP028160.1 which was resistant and *Leu*. *pseudomesenteroides* MG550992.1. Resistance towards colistin sulphate (polymyxins) and pipemidic acid (pyridopyrimidines) were observed for all strains. These antibiotics are responsible for altering the cell wall membrane and inhibition of DNA synthesis respectively (Mathur and Singh, 2005). Susceptibility towards chloramphenicol (amphenicol) which is an inhibitor of protein synthesis was also observed for all the strains except *Leu*. *mesenteroides* MF098088.1 which displayed intermediate resistance. Among these resistance profiles, the resistance of aminoglycosides and β-lactams are of specific interest as their corresponding AR determinants have been described in leuconostocs and lactococci (Mathur and Singh, 2005, Ammor *et al*., 2007, Radulović *et al*., 2012).

In studies done by previous authors, tetracycline, an inhibitor of protein synthesis, was reported to be the most frequently acquired resistance in food isolates of *Lactobacillus* and *Streptococcus* (Huys *et al*., 2004, Zago *et al*., 2011). However, all isolates in the study were found to be susceptible to tetracycline. The results achieved in this study are in agreement with those achieved by Ammor *et al*. (2007), D'Aimmo *et al*. (2007), Klare *et al*. (2007), van Hoek *et al*. (2008) and Hoque *et al*. (2010). Among the antibiotics used in therapy, vancomycin is justified for use as a last resort drug in the treatment of multidrug-resistant organisms; therefore, its resistance in commercial LAB is of critical interest (Ogier *et al*., 2008). Vancomycin belongs to a class of antibiotics called glycopeptides and is active against most Gram-positive bacteria. Intrinsic resistance towards glycopeptides has been reported in several studies (Patel *et al*., 2012, Erginkaya *et al*., 2018). In many species of LAB, the

terminal D-alanine residue is substituted by D-lactate or D-serine in the muramyl pentapeptide, preventing the binding of vancomycin (Delcour *et al*., 1999). All of the isolates in this study exhibited resistance to vancomycin except for *Lc*. *lactis* CP028160.1. It has been reported that LAB of the genera *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weisella* have resistance mechanisms to vancomycin (Handwerger *et al*., 1994, Nelson, 1999, Eliopoulos and Gold, 2001, Courvalin, 2006, Abriouel *et al*., 2015b). These studies also pointed out that species of these genera have intrinsic resistance towards vancomycin. Although several LAB have intrinsic resistance towards antibiotics, it is not considered a food safety issue. It only becomes a risk to food safety when the risk of gene transfer is present (Gueimonde *et al*., 2013). Having mentioned this, LAB having intrinsic resistance towards several antibiotics might be favourable as such resistance could assist in the sustainable utilisation of the strains within the human gut to maintain the natural equanimity of intestinal microflora during antibiotic therapy (Bacha *et al*., 2010).

An in-depth analysis of LAB resistance towards aminoglycosides has not been reported. However, over the last few years, LAB isolated from farm origin have portrayed resistance to streptomycin, gentamicin and kanamycin. Their resistance mechanism is linked to debilitated transport and enzymatic inactivation by three principal aminoglycoside-modifying enzymes (AMEs) as N-acetyltransferases (AACs), O-phosphotransferases (APHs), and O-nucleotidyltransferases (ANTs) encoded by MGE like transposons and insertion sequences (Jaimee and Halami, 2016, Álvarez-Cisneros and Ponce-Alquicira, 2018). *Leuconostoc* sp. has been reported to have varied resistance to aminoglycosides (inhibitors of protein synthesis) (Flórez *et al*., 2016). In the present study, it was observed that most of the isolates had exhibited intermediate resistance towards kanamycin and streptomycin and varied resistance towards gentamicin. These results were in partial agreement to results by Adimpong *et al*. (2012), Alegría *et al*. (2013) and Flórez *et al*. (2016).

LAB, especially *Lactobacillus* sp. are reported to be resistant to erythromycin and chloramphenicol (Temmerman *et al*., 2003, D'Aimmo *et al*., 2007, Ammor *et al*., 2008), but in this study, none of the isolates were resistant to erythromycin and chloramphenicol. The results achieved are in accordance with studies done by Rojo-Bezares *et al*. (2006), Klare *et al*. (2007) and Beyan *et al*. (2011). All isolates investigated in this study showed complete susceptibility towards ampicillin, which is a cell wall inhibitor, however, 2 isolates were found to have intermediate resistance to this antibiotic. These results were in accordance with Ammor *et al*. (2007), Morandi *et al*. (2013) and Flórez *et al*. (2016).

LAB have been reported to have intrinsic resistance towards last-resort antibiotics such as colistin, fosfomycin and pipemidic acid. In the present study, all isolates had exhibited resistance to these three antibiotics. The antibiotic colistin is a combination of polymyxin E1 and E2 which are two bactericidal pentacationic lipopeptides. Whilst their mode of action isn't

fully clarified, it involves binding to lipopolysaccharides and phospholipids in the outer membrane of Gram-negative bacteria, resulting in membrane interference and cell death (WHO, 2018). Additionally, colistin is ineffective against Gram-positive bacteria due to the lack of an outer membrane that is prevalent in Gram-negative bacteria only (WHO, 2018).

Fosfomycin, an inhibitor of cell wall synthesis in both Gram-positive and Gram-negative bacteria belongs to the phosphonic acid derivative group. This antibiotic inhibits cell wall synthesis by impeding the initial step involving phosphoenolpyruvate synthetase (Michalopoulos *et al*., 2011, Raz, 2012). Resistance to fosfomycin is deployed by three mechanisms i.e. two of which are present on the chromosome whilst the third is plasmid-mediated (Michalopoulos *et al*., 2011). Resistance to fosfomycin may be caused by genetic mutations in chromosomally encoded transport systems, GlpT and UhpT. Mutations can occur in either or both of these transport systems. It is atypically attributed by a fosfomycin-modifying enzyme leading to products linked with no antibacterial activity (Horii *et al*., 1999, Beharry and Palzkill, 2005). Plasmid-encoded fosfomycin resistance (PEFR) is exerted by a gene located in a transposon. PEFR is due to modification of the antibiotic molecule by an enzyme called metalloglutathione transferase, a glutathione S-transferase that catalyses the formation of a covalent bond between the sulfhydryl residue of the cysteine in glutathione and C-1 of fosfomycin (Michalopoulos *et al*., 2011). Abriouel *et al*. (2015b), had reported that fosfomycin, sulphonamides and vancomycin resistance appeared to be a common trait amongst the genera *Leuconostoc* and *Weisella*. This trend for *Leuconostoc* species in the present study was in accordance with the author's findings. Pipemidic acid is an antibiotic that inhibits DNA synthesis and is a member of the pyridopyrimidine class of antibiotics. LAB has been reported to be intrinsically resistant to fosfomycin (Teuber *et al*., 1999), a finding that is in accordance with this present study.

The results achieved in this present study regarding the AR profiles of *Leu*. *mesenteroides*, *Leu*. *pseudomesenteroides* and *Lc*. *lactis* isolates from commercially available *aMasi* to different antibiotics (mainly inhibitors to cell wall synthesis and protein synthesis) partially agree with those obtained by other researchers, as previously evidenced. In some cases, the response to the different classes of antibiotics seems to depend on the species but, within the species, it was possible to observe a strain-specific response to the AR. However, some species showed resistance, higher than the standards outlined by CLSI, so it further necessitates to investigate this resistance on a molecular level as the present study could serve as informative criteria for selecting the resistance pattern among *Leuconostoc* sp. and *Lc*. *lactis* isolated from different commercial products, as there are possibilities that commercial LAB could have their resistant determinants on MGE such as plasmids which may transfer these genes to other pathogenic species in the gut. Presence of AR genes on plasmids in LAB has been reported (Radulović *et al*., 2012, Álvarez-Cisneros and Ponce-

Alquicira, 2018) along with their transfer to other species which further necessitates the screening of LAB for AR determinants intended for use in commercial products.

5.8 MOLECULAR SCREENING OF ANTIBIOTIC-RESISTANT GENES IN LACTIC ACID BACTERIA USING PCR

Phenotypic resistance was confirmed by doing PCR to screen for AR genes using gene-specific primer sets for the three *Leu. mesenteroides* isolates previously mentioned. The findings of this study had illustrated contradictory results to the phenotypic resistance results displayed in Table 4.3 and Figures 4.7 to 4.9 under Chapter 4.6. None of these isolates had yielded any amplicons for the five glycopeptide resistant genes encoding resistance for vancomycin and for the three aminoglycoside resistant genes encoding for gentamicin resistance.

With respect to resistance towards the glycopeptides encoding resistance towards vancomycin, all *van* genes (*van*A, *van*B, *van*D, *van*E and *van*X) screened in this present study were absent. This finding is in accordance with results achieved by Morandi *et al*. (2013). There could be three possible reasons for this occurrence viz. (a) inaptness of the primer causing the primer to not bind to the target of the specific gene, (b) the gene itself within the bacterium could be absent or (c) the bacterial strain could exhibit phenotypic resistance but be genotypically susceptible (Radulović *et al*., 2012). *Van*A and *van*B genes are situated in the chromosome but can also be transported and harboured by plasmids (Patel, 2000, Morandi *et al*., 2013). These genes have high-level vancomycin resistance and possess acquired genetic determinants which prompt the production of an atypical ligase accountable for the synthesis of the depsipeptide D-alanyl-D-lactate subsequently resulting in poor binding of vancomycin. Whilst *van*A- or *van*B-type vancomycin resistance maintains the normal pentapeptide peptidoglycan cell wall precursor pathway in enterococci, some of its components are hydrolysed (Patel, 2000, Eliopoulos and Gold, 2001).

A type of vancomycin resistance that is different from the previous two types (*van*A- and *van*B) discussed is the *van*D-type of resistance. This type of vancomycin resistance has an unlikelihood of transferability despite it being linked with the synthesis of depsipeptide D-Ala-D-Lac (Depardieu and Courvalin, 2017). The *van*E-type of vancomycin resistance, on the other hand, encodes for low-level resistance and has been described in *E. faecalis* which is contemplated to be linked with a probable D-Ala:D-Ser ligase that is similarly present in *van*C-type enterococci. Both of these types (*van*D- and *van*E- types) of vancomycin resistance are not frequently implicated in clinical isolates of vancomycin-resistant enterococci (VRE). Moreover, these resistant gene types and are not the cause of glycopeptide resistance in the more prevalent *van*A- and *van*B-type VRE isolates (Patel, 2000).

Genera belonging to the LAB group have a long history of resistance towards vancomycin. The most common genera include *Enterococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. Vancomycin resistance in *Lb*. *casei*, *Lb*. *plantarum*, *Leu*. *mesenteroides* and *P*. *pentosaceus* are linked with the existence of a pentapeptide peptidoglycan precursor that terminates in D-Ala-D-Lac. The peptidoglycan precursor structure in *Leu*. *mesenteroides* (MurNAc-L-Ala-D- Glu-L-Lys-[L-Ala]-D-Ala-D-Lac), and in *Lb*. *plantarum* (MurNAc- L-Ala-D- Glu-L-m-Dpm- D-Ala-D-Lac), differ from that in enterococci, but still terminate in D-Ala-D-Lac. It is not fully understood if one or two ligases exist in these bacteria (Patel, 2000).

A study by Ferain *et al*. (1996), had found a *Lb*. *plantarum* strain to exhibit deficiency for D- and L-lactate dehydrogenase activities which produced only trace amounts of D- and L-lactate. These changes were found to greatly affect the peptidoglycan synthesis pathway with a detectable new precursor ending in D-alanine in addition to that terminating in D-lactate. Moreover, the bacterium had greatly heightened the susceptibility towards vancomycin. It was not completely understood whether the presence of the D-alanine-terminating precursor in the mutant was a consequence of a second ligase or whether a single ligase of heterogeneous substrate selectivity which specifically used D-lactate. However, since the differentiation of the ligase genes from intrinsically vancomycin-resistant lactobacilli and *Leu*. *mesenteroides* have shown that their ascertained amino acid sequences are closely related to each other as opposed to the vancomycin-susceptible strain of *Lb*. *leichmannii*, the last-mentioned reason could probably have been the situation.

Vancomycin resistance in *Leuconostoc* spp. is not transmittable to enterococci using filter mating experiments (Patel, 2000). Despite the fact that *Leuconostoc* spp. typically harbour several plasmids, some researchers have shown that plasmid-free strains of *Leuconostoc* spp. obtained by plasmid curing using curing agents such as ethidium bromide and novobiocin can retain their resistance to glycopeptides (Handwerger *et al*., 1994). Given the fact that glycopeptide resistance in LAB such as *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. are linked with the presence of peptidoglycan precursors terminating in D-Ala-D-Lac in enterococci with *van*A- and *van*B-mediated vancomycin resistance (Billot-Klein *et al*., 1994, Handwerger *et al*., 1994), the D-Ala:D-Lac ligase and D-lactate hydrogenase enzymes implicated in the manufacturing of altered precursors in *Leuconostoc* spp. and *Lactobacillus* spp. are dissimilar to *van*A or *van*B and *van*H or *van*H_B, respectively (Dartois *et al*., 1995, Elisha and Courvalin, 1995, Park and Walsh, 1997, Marshall *et al*., 1998). In a study by Tynkkynen *et al*. (1998), three sets of *van*A primers were used to amplify a PCR product in *Lb*. *rhamnosus* GG, however, no PCR product was amplified and enterococcal *van*A, *van*B, *van*H, *van*Z, *van*Y, *van*S and *van*R genetic material did not hybridise with DNA of *Lb*. *rhamnosus* GG.

More in-depth research has revealed that the ranks of identity between the D-Ala:D-Lac ligase in *Leuconostoc* spp. and *Lactobacillus* spp. and *van*A/B are 26-35% (Marshall *et al*., 1998). Differences occur in the primary sequences of vancomycin-resistant LAB for *van*A, *van*B and *van*D D-Ala:D-Lac ligases and D-Ala:D-Ser ligases and D-Ala:D-Ala ligases for *van*C, particularly in the omega loop region, which is essential to catalysis (Evers *et al*., 1996). This indicates that the evolution towards D-Ala-D-Lac formation had transpired independently in ligases from LAB and *van*A, *van*B and *van*D enterococci. Lactate is abundant in LAB and the use of D-lactate instead of D-alanine could demonstrate metabolic stress. The presence of glycopeptide-producing organisms in the environment could have also placed selective pressure and played a significant role in the development of D-Ala:D-Lac ligases in LAB (Evers *et al*., 1996).

For the aforementioned reasons, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* and the intrinsically resistant vancomycin-resistant *Enterococcus* spp. are not the root cause of the prevalence of glycopeptide resistance genes in VRE. Overall, the absence of the *van* genes in this present study augments the hypothesis that glycopeptide-resistant *Leuconostoc* strains are mediated by a mechanism that differs from that affiliated with *Enterococcus* spp. Furthermore, from a food safety perspective, the absence of the transmissible *van*A gene permits the safe usage of these *Leu*. *mesenteroides* isolates to be used in commercial dairy production.

With regards to resistance towards the aminoglycosides, three gentamicin resistance genes (*aac*(6')-*aph*(2''), *aph*(2'')-Ib and *aph*(3'')-IIIa) were screened during the PCR optimisation process and no amplicons were yielded (figures are not shown). Out of the 3 bacteria, *Leu*. *mesenteroides* MF0988088.1, *Leu*. *mesenteroides* MG953247.1 and *Leu*. *mesenteroides* KY660935.1, the PCR screening of these genes were in accordance with the phenotypically susceptible results displayed in Table 4.3 and Figure 4.7 under Chapter 4.6 (Phenotypic antibiotic susceptibility testing of LAB) in the latter two bacteria whilst, the former bacterium had exhibited a contradictory result whereby, the bacterium had exhibited phenotypic resistance but was genotypically susceptible. This could be due to the fact that the appropriate genes were excluded in the test patterns, or unknown resistance genes may be present. Limited studies have been done on aminoglycoside resistance in *Leuconostoc* spp. therefore, the results in this present study were in agreement with those achieved by Guo *et al*. (2017) whereby, the resistance genes for gentamicin were not detectable in *Lactobacillus* spp. Moreover, the results in this study were contradictory to those found by Vakulenko *et al*. (2003) and Rojo-Bezares *et al*. (2006) whereby, the presence of the *aac*(6')-*aph*(2'')-Ib, *aph*(2'')-Ib and *aph*(3'')-IIIa genes were present in *Lactobacillus* spp. and *Enterococcus* spp., respectively.

5.9 LIMITATIONS OF THIS STUDY

The outcome of this study was limited by the use of culture-dependent techniques. In the past, prior to modern-day techniques, the detection and identification of bacterial isolates from food were predominantly determined by using phenotypic and biochemical methods (Fguiri *et al*., 2015, Franco-Duarte *et al*., 2019). Despite this, taxonomists are mindful of the fact that phenotypic techniques have the disadvantage of imprecisely reflecting true bacterial relationships. Moreover, these methods are often time-consuming, labour intensive and may inconsistently provide researchers with unequivocal results. These outdated conventional methods are also insufficient to accurately identify and monitor proliferation and dynamics of several species and/or strains in complex bacterial communities (Giraffa and Carminati, 2008). Therefore, culture-dependent/conventional methods are often considered as substandard for examining dynamic ecological systems in fermented milk microflora (Giraffa, 2004).

The use of modern-day molecular techniques within polyphasic means are accessible to gain information about microorganisms prevalent in dairy products; providing an outlook to differentiate between species whose cells are viable at the moment of analysis from those previously present, but now inactive due to factors such as: (i) technological stress, (ii) microbial antagonism, (iii) depletion of specific nutrients and (iv) adverse environmental conditions that occur during the manufacturing process of fermented milk (Serrazanetti *et al*., 2013). Furthermore, molecular methods for identifying LAB have increased significantly leading to an increase in the number of LAB identified. Additionally, these molecular methods have also been applied in food microbial ecology. Such methods include PCR-based methods, *in situ* methods, metagenomics and next-generation sequencing which can be utilised due to them being more convenient than the conventional methods as they are faster, more specific and preceding cultivation of microorganisms is not necessary (Giraffa and Carminati, 2008). In contrast to conventional methods, the identification and characterisation of unculturable or slow-growing pathogens are easier to implement. These methods also provide more stable characteristics than conventional methods using phenotypic characteristics. However, the limitations of these molecular techniques have been questioned due to the extent of their database, the need for complex procedures, trained personnel, specialised and expensive equipment (Fguiri *et al*., 2015).

With regards to AR of LAB, this study was limited to the use of phenotypic methods (disc-diffusion method) which allowed for screening of AR in LAB. This method was chosen because it is one of the most well-established phenotypic methods in determining AR in bacteria and is still widely used today. Furthermore, it was chosen because of its cost-effectiveness, convenience and efficacy in determining AR in LAB. Despite this, the assessment of LAB phenotypic resistance to antibiotics should be assessed using acclaimed

techniques that permit the identification of MIC for the most commonly used antibiotics. Some methods to determine the MIC in LAB are the broth microdilution method, E-test and Kirby-Bauer test (agar diffusion method) (Álvarez-Cisneros and Ponce-Alquicira, 2018).

Currently, cut-off values are known for the genera *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Streptococcus*. A widely used method to evaluate MIC for a substantial number of strains and antibiotics is the broth microdilution method. This method has some disadvantages specifically for those antibiotics for which a strain could rapidly acquire resistance to (Kushiro *et al*., 2009). MIC evaluation in LAB, however, is to a certain degree inconsistent among researchers. This is mainly attributed to the lack of culture media that can ensure acceptable growth of LAB without producing biased results. It is, therefore, essential that complementary analyses involve the identification of AR genes by employing microarrays and PCR methods. Identifying the location of genes also permits researchers to further investigate their potential transfer, whilst gene sequencing can present evidence of their bacterial taxa and identity of the genes, which could aid in the traceability of their genomes (Álvarez-Cisneros and Ponce-Alquicira, 2018).

Another limitation to this study was the fact that the LAB isolates were not identified to strain level. This made it very difficult to decipher if the actual antibiotic-resistant gene was present in the bacterium in the first place. The use of Multilocus Sequencing Typing (MLST) is still widely used for identifying bacteria to strain level and could be employed in future studies (Larsen *et al*., 2012). More recently, functional metagenomics has become a significant strategy in the examination of AR genes since they can be employed to identify and characterise novel AR genes, inclusive of those not previously linked with AR. This method has also been recently applied to investigate resistance in pure bacterial groups and in more complex samples i.e. food where an array of resistance systems exist, also accounting for viable and non-viable bacteria (Álvarez-Cisneros and Ponce-Alquicira, 2018).

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

LAB were the predominating population in all 10 commercial *aMasi* samples analysed. Given the fact that milk is a highly nutritious medium, it supports the growth of a broad range of microorganisms, however, considering the fact that LAB is acid-tolerant and can metabolise lactose as a carbon source, it strengthened the hypothesis of LAB being the predominating population in a fermented milk medium. However, the use of culture-dependent techniques had limited the study in providing researchers with a 'true' bacterial community within these samples. Molecular methods such as 'profile fingerprinting' e.g. DGGE and other metagenomic or next-generation sequencing could be applied in future studies to give researchers a better representation of the bacterial communities that exist within fermented food samples.

In terms of the bacterial quality of the commercial *aMasi* products, despite LAB being the predominating population, *Bacillus* sp. was present in all 10 samples. This indicates that this could probably be a challenge in eradicating these spore-forming bacteria in the dairy industry and that CIP needs to be implemented more often and most importantly, more effectively. With respect to the relationship of LAB to the selected physicochemical properties, it seemed most likely that the LAB present in these samples were under stressed environments. It was characteristic of the fermented milk medium to have a low pH and titratable acidity and for the medium to have a high a_w and moisture content. Classification and regression tree analysis was successful in illustrating the likelihood of LAB to be present in *aMasi* samples with respect to the LAB count and pH levels and can be used in future studies to determine similar outcomes. Culture-dependent methods are regarded as substandard for studying the dynamics of LAB in a food microbial ecology and that the use of PCR-based methods or metagenomics should be employed to better understand the microbial dynamics of LAB in a fermented milk matrix.

The LAB isolates in this study were only identified to species level. This made it difficult to decipher if the chosen primers that were readily available in literature, were successful in amplifying the target region in LAB or not. At strain level, it would have been much easier to identify if a bacterium possessed a certain gene. MLST to identify bacteria to strain level could help in future studies to be able to choose suitable primers that will be successful in amplifying the target region in LAB. The present study showed that multiple AR is prevalent in different species of starter culture strains, which may pose a food safety concern. LAB that exhibit phenotypic resistance to antibiotics should also be evaluated on a molecular level to monitor their resistance. The presence of such a variety of expressed AR genes in probiotic isolates is a worrying trend. The impact of the interactions of these bacteria with pathogenic strains and their transfer of these AR genes is yet to be assessed. This can be determined using filter-mating experiments to determine the frequency at which AR genes are transferred from

one bacterium to another. Furthermore, antibiotic sensitivity is an important criterion in the safety assessment for the evaluation of food-grade and potential food-grade LAB.

The use of simple antibiotic PCR for screening AR genes will be useful in future studies to determine if certain antibiotic-resistant genes are present or absent. Furthermore, the use of imaging, proteomic and genomic techniques could be employed in future studies to provide an integrative approach to evaluate the risk of AR attributed to the long-term consumption of foods rich with LAB and its impact on human health.

REFERENCES

ABDI, H. & WILLIAMS, L. J. 2010. Principal component analysis. *WIREs Computational Statistics,* 2**,** 433-459.

ABRIOUEL, H., CASADO MUÑOZ, M. D. C., LAVILLA LERMA, L., PÉREZ MONTORO, B., BOCKELMANN, W., PICHNER, R., KABISCH, J., CHO, G.-S., FRANZ, C. M. A. P., GÁLVEZ, A. & BENOMAR, N. 2015a. New insights in antibiotic resistance of *Lactobacillus* species from fermented foods. *Food Research International,* 78**,** 465-481.

ABRIOUEL, H., LERMA, L. L., CASADO MUÑOZ, M. D. C., MONTORO, B. P., KABISCH, J., PICHNER, R., CHO, G.-S., NEVE, H., FUSCO, V., FRANZ, C. M. A. P., GÁLVEZ, A. & BENOMAR, N. 2015b. The controversial nature of the *Weissella* genus: technological and functional aspects versus whole genome analysis-based pathogenic potential for their application in food and health. *Frontiers in Microbiology,* 6**,** 1197. DOI: 10.3389/fmicb.2015.01197.

ADIMPONG, D. B., NIELSEN, D. S., SØRENSEN, K. I., DERKX, P. M. F. & JESPERSEN, L. 2012. Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. *BMC Microbiology,* 12**,** 75. DOI: 10.1186/1471-2180-12-75.

AGUIRRE, M. & COLLINS, M. D. 1993. Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology,* 75**,** 95-107.

AKABANDA, F., OWUSU-KWARTENG, J., TANO-DEBRAH, K., PARKOUDA, C. & JESPERSEN, L. 2014. The use of lactic acid bacteria starter culture in the production of *Nunu*, a spontaneously fermented milk product in Ghana. *International Journal of Food Science,* 2014**,** 721067. DOI: 10.1155/2014/721067.

AKABANDA, F., OWUSU-KWARTENGI, J., GLOVER, R. L. K. & TANO-DEBRAH, K. 2010. Microbiological characteristics of Ghanaian traditional fermented milk product, *Nunu*. *Nature and Science,* 8**,** 178-187.

AL BAYSSARI, C., DABBOUSSI, F., HAMZE, M. & ROLAIN, J.-M. 2015. Emergence of carbapenemase-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in livestock animals in Lebanon. *Journal of Antimicrobial Chemotherapy,* 70**,** 950-951.

ALEGRÍA, Á., DELGADO, S., FLÓREZ, A. B. & MAYO, B. 2013. Identification, typing, and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses. *Dairy Science & Technology,* 93**,** 657-673.

ÁLVAREZ-CISNEROS, Y. M. & PONCE-ALQUICIRA, E. 2018. Antibiotic resistance in lactic acid bacteria. *In:* KUMAR, Y. (ed.) *Antimicrobial Resistance - A Global Threat.* Intech Open.

AMMOR, M. S., BELÉN FLÓREZ, A. & MAYO, B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology,* 24**,** 559-570.

AMMOR, M. S., FLÓREZ, A. B., VAN HOEK, A. H., DE LOS REYES-GAVILÁN, C. G., AARTS, H. J., MARGOLLES, A. & MAYO, B. 2008. Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of Molecular Microbiology and Biotechnology,* 14**,** 6-15.

ANBUKKARASI, K., UMAMAHESWARI, T., HEMALATHA, T., NANDA, D. K., SINGH, P. & SINGH, R. 2014. Preparation of low galactose yogurt using cultures of Gal(+) *Streptococcus thermophilus* in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Food Science and Technology,* 51**,** 2183-2189.

ANUKAM, K. C. & REID, G. 2009. African traditional fermented foods and probiotics. *Journal of Medicinal Food,* 12**,** 1177-1184.

AOAC 2000a. AOAC official method 926.08 *Moisture in Cheese - First Action 1926 (Method I), 33.7.03.* Washington, DC: Association of Analytical Chemists.

AOAC 2000b. AOAC official method 930.28 - Lactose in milk. *Gravimetric method - Munson-Walker First Action 1930, 33.2.22.* Washington, DC: Association of Analytical Chemists.

AOAC 2000c. AOAC official method 974.05 - Acidity of milk. *Titrimetric Method - First Action 1947, 33.2.06.* Washington, DC: Association of Analytical Chemists.

AOAC 2002. AOAC official method 2000.18 - Fat content of raw and pasteurized whole milk. *Gerber Method by Weight (Method I) First Action 2000, 33.2.27A.* 16th ed. Washington DC: Association of Analytical Chemists.

ASHRAF, R. & SHAH, N. P. 2011. Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. *International Food Research Journal,* 3**,** 837-853.

AURAND, L. W., WOODS, A. E. & WELLS, M. R. 1987. *Food Composition and Analysis,* New York, Springer Science & Business Media.

AWAISHEH, S. S. 2012. Probiotic food products classes, types, and processing. *In:* RIGOBELO, E. C. (ed.) *Probiotics.* InTech Open.

AWOLUSI, O. O., NASR, M., KUMARI, S. & BUX, F. 2017. Principal component analysis for interaction of nitrifiers and wastewater environments at a full-scale activated sludge plant. *International Journal of Environmental Science and Technology,* 15**,** 1477**–**1490.

AXELSSON, L. 2004. Lactic acid bacteria: classification and physiology. *In:* SALMINEN, S. & VON WRIGHT, A. (eds.) *Lactic Acid Bacteria: Microbiological and Functional Aspects.* 3rd ed. New York: Marcel Dekker, Inc.

BACHA, K., MEHARI, T. & ASHENAFI, M. 2010. Antimicrobial susceptibility patterns of LAB isolated from *Wakalim*, a traditional Ethiopian fermented sausage. *Journal of Food Safety,* 30**,** 213-223.

BANDLER, D. K. & SINGH, R. P. 2018. Dairy products. Encyclopaedia Britannica, Inc.

BEHARE, P., PRAJAPATHI, J. B. & SINGH, R. 2016. Types and applications of lactic starters. *In:* PUNIYA, A. K. (ed.) *Fermented Milk and Dairy Products.* Boca Raton: CRC Press.

BEHARRY, Z. & PALZKILL, T. 2005. Functional analysis of active site residues of the fosfomycin resistance enzyme FosA from *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry,* 280**,** 17786-17791.

BELL, V., FERRÃO, J., PIMENTEL, L., PINTADO, M. & FERNANDES, T. 2018. One Health, fermented foods, and gut microbiota. *Foods,* 7**,** 195. DOI: 10.3390/foods7120195.

BESTER, L. A. & ESSACK, S. Y. 2010. Antibiotic resistance via the food chain: fact or fiction? *South African Journal of Science,* 106**,** 1-5. DOI: 10.4102/sajs. v106i9/10.281.

BEUKES, E. M. 1999. *Lactic acid bacteria in South African indigenous fermented milks and the evaluation of selected strains for application in the manufacturing of cultured milk.* MSc, University of Pretoria.

BEUKES, E. M., BESTER, B. H. & MOSTERT, J. F. 2001. The microbiology of South African traditional fermented milks. *International Journal of Food Microbiology,* 63**,** 189-197.

BEYAN, A., KETEMA, T. & BACHA, K. 2011. Antimicrobial susceptibility pattern of lactic acid bacteria isolated from *Ergo*, a traditional Ethiopian fermented milk, Jimma, South West Ethiopia *Ethiopian Journal of Education and Science,* 7**,** 9-17.

BHADORIA, P. B. S. & MAHAPATRA, S. C. 2011. Prospects, technological aspects and limitations of probiotics - a worldwide review. *European Journal of Food Research & Review,* 2**,** 23-42.

BHUNIA, A. K. 2018. *Bacillus cereus* and *Bacillus anthracis*. *Foodborne Microbial Pathogens: Mechanisms and Pathogenesis.* New York, NY: Springer New York.

BILLOT-KLEIN, D., GUTMANN, L., SABLÉ, S., GUITTET, E. & VAN HEIJENOORT, J. 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. *Journal of Bacteriology,* 176**,** 2398-2405.

BLEUVEN, C. & LANDRY, C. R. 2016. Molecular and cellular bases of adaptation to a changing environment in microorganisms. *Proceedings. Biological sciences,* 283**,** 1-9.

BMI. 2018. *Buttermilk and Maas in South Africa* [Online]. Available: https://www.bmi.co.za/wp-content/uploads/2018/09/Buttermilk-and-Maas-Media-Feedback-Report-2018.pdf [Accessed 28 December 2018].

BRADLEY, R. L. J. 2010. Moisture and total solids analysis. *In:* NIELSEN, S. S. (ed.) *Food Analysis. Part 2: Compositional Analysis of Foods.* 4th ed. New York: Springer Science & Business Media.

BROOKS, A. N., TURKARSLAN, S., BEER, K. D., LO, F. Y. & BALIGA, N. S. 2011. Adaptation of cells to new environments. *WIREs Systems Biology and Medicine,* 3**,** 544-561.

BULL, M., PLUMMER, S., MARCHESI, J. & MAHENTHIRALINGAM, E. 2013. The life history of *Lactobacillus acidophilus* as a probiotic: a tale of revisionary taxonomy, misidentification and commercial success. *FEMS Microbiology Letters,* 349**,** 77-87.

BURNS, P., PATRIGNANI, F., SERRAZANETTI, D., VINDEROLA, G. C., REINHEIMER, J. A., LANCIOTTI, R. & GUERZONI, M. E. 2008. Probiotic Crescenza cheese containing *Lactobacillus casei* and *Lactobacillus acidophilus* manufactured with high-pressure homogenized milk. *Journal of Dairy Science,* 91**,** 500-512.

BYAPPANAHALLI, M. N., NEVERS, M. B., KORAJKIC, A., STALEY, Z. R. & HARWOOD, V. J. 2012. Enterococci in the environment. *Microbiology and Molecular Biology Reviews: MMBR,* 76**,** 685-706.

BYLUND, G. 2003. *Dairy Processing Handbook*, Tetra Pak Processing Systems AB.

CAHILL, S. M., DESMARCHELIER, P., FATTORI, V., BRUNO, A. & CANNAVAN, A. 2017. Global perspectives on antimicrobial resistance in the food chain. *Food Protection Trends,* 37**,** 353-360.

CAMPEDELLI, I., FLÓREZ, A. B., SALVETTI, E., DELGADO, S., ORRÙ, L., CATTIVELLI, L., ALEGRÍA, Á., FELIS, G. E., TORRIANI, S. & MAYO, B. 2015. Draft genome sequence of three antibiotic-resistant *Leuconostoc mesenteroides* strains of dairy origin. *Genome Announcements,* 3**,** e01018-15. DOI: 10.1128/genomeA.01018-15.

CANCINO-PADILLA, N., FELLENBERG, M. A., FRANCO, W., IBÁÑEZ, R. A. & VARGAS-BELLO-PÉREZ, E. 2017. Foodborne bacteria in dairy products: detection by molecular techniques. *Ciencia e Investigación Agraria***,** 44, 215-229.

CANNON, J. P., LEE, T. A., BOLANOS, J. T. & DANZIGER, L. H. 2005. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. *European Journal of Clinical Microbiology & Infectious Diseases,* 24**,** 31-40.

CARRUTH, L., ROESS, A. A., TEREFE, Y., HOSH, F. M. & SALMAN, M. D. 2017. Antimicrobial resistance and food safety in Africa. *The Lancet,* 17**,** 575-576.

ÇATALOLUK, O. & GOGEBAKAN, B. 2004. Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. *FEMS Microbiology Letters,* 236**,** 7-12.

CHELULE, P. K., MOKGATLE, M. M. & ZUNGU, L. I. 2015. South African rural community understanding of fermented foods preparation and usage. *Journal of Education and Health Promotion,* 4**,** 82. DOI: 10.4103/2277-9531.171795.

CHEN, J. & NOVICK, R. P. 2009. Phage-mediated intergeneric transfer of toxin genes. *Science,* 323**,** 139-141.

CHERIF-ANTAR, A., MOUSSA**–**BOUDJEMÂA, B., DIDOUH, N., MEDJAHDI, K., MAYO, B. & FLÓREZ, A. B. 2016. Diversity and biofilm-forming capability of bacteria recovered from stainless steel pipes of a milk-processing dairy plant. *Dairy Science & Technology,* 96**,** 27-38.

CIANI, M., COMITINI, F. & MANNAZZU, I. 2008. Fermentation. *In:* JØRGENSEN, S. E. & FATH, B. D. (eds.) *Encyclopedia of Ecology.* Oxford: Academic Press.

CLEREMONT, D., CHESNEAU, O., DE CESPÉDÈS, G. & HORAUD, T. 1997. New tetracycline resistance determinants coding for ribosomal protection in streptococci and nucleotide sequence of *tet*(T) isolated from *Streptococcus pyogenes* A498. *Antimicrobial Agents and Chemotherapy,* 41**,** 112-116.

CLSI 2012. *Performance standards for antimicrobial susceptibility testing: twenty-second informational supplement,* Clinical Laboratory Standards Institute, Wayne, PA.

COLOMER-LLUCH, M., JOFRE, J. & MUNIESA, M. 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS One,* 6**,** e17549. DOI: 10.1371/journal.pone.0017549.

COURVALIN, P. 2006. Vancomycin resistance in Gram-positive cocci. *Clinical Infectious Diseases,* 42**,** S25-S34.

D'AIMMO, M. R., MODESTO, M. & BIAVATI, B. 2007. Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology,* 115**,** 35-42.

DALU, J. M. & FERESU, S. B. 1996. Survival of *Listeria monocytogenes* in three Zimbabwean fermented milk products. *Journal of Food Protection,* 59**,** 379-383.

DARSANAKI, R. K., ALIABADI, M. A. & CHAKOOSARI, M. M. D. 2013. Antibiotic resistance of lactic acid bacteria *Scientific Journal of Microbiology,* 11**,** 201-206.

DARTOIS, V., PHALIP, V., SCHMITT, P. & DIVIÈS, C. 1995. Purification, properties and DNA sequence of the D-lactate dehydrogenase from *Leuconostoc mesenteroides* subsp. *cremoris*. *Research in Microbiology,* 146**,** 291-302.

DASHKO, S., ZHOU, N., COMPAGNO, C. & PIŠKUR, J. 2014. Why, when, and how did yeast evolve alcoholic fermentation? *FEMS Yeast Research,* 14**,** 826-832.

DEEPNARAIN, N., NASR, M., KUMARI, S., STENSTRÖM, T. A., REDDY, P., PILLAY, K. & BUX, F. 2019. Decision tree for identification and prediction of filamentous bulking at full-scale activated sludge wastewater treatment plant. *Process Safety and Environmental Protection,* 126**,** 25-34.

DELCOUR, J., FERAIN, T., DEGHORAIN, M., PALUMBO, E. & HOLS, P. 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie van Leeuwenhoek,* 76**,** 159-184.

DEPARDIEU, F. & COURVALIN, P. M. 2017. Glycopeptide-resistance in enterococci. *In:* MAYERS, D., SOBEL, J., OUELLETTE, M., KAYE, K. & MARCHAIM, D. (eds.) *Antimicrobial Drug Resistance.* Springer, Cham.

DEPARDIEU, F., PERICHON, B. & COURVALIN, P. 2004. Detection of the *van* alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *Journal of Clinical Microbiology,* 42**,** 5857-5860.

DEVIRGILIIS, C., BARILE, S. & PEROZZI, G. 2011. Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes & Nutrition,* 6**,** 275-284.

DLAMINI, B. C. & BUYS, E. M. 2009. Adaptation of *Escherichia coli* O157:H7 to acid in traditional and commercial goat milk *amasi*. *Food Microbiology,* 26**,** 58-64.

DU PLOOY, Z., SCHÖNFELDT, H. C. & HALL, N. 2017. The role of traditional foods in food-based dietary guidelines – A South African case study on maas (cultured milk). *Food Chemistry,* 238**,** 22-28.

EGERVÄRN, M., LINDMARK, H., ROOS, S., HUYS, G. & LINDGREN, S. 2007. Effects of inoculum size and incubation time on broth microdilution susceptibility testing of lactic acid bacteria. *Antimicrobial Agents and Chemotherapy,* 51**,** 394-396.

EKWANZALA, M. D., DEWAR, J. B., KAMIKA, I. & MOMBA, M. N. B. 2018. Systematic review in South Africa reveals antibiotic resistance genes shared between clinical and environmental settings. *Infection and Drug Resistance,* 11**,** 1907-1920.

EL SAMRA, G. H. 2017. Climate change, food security, food safety and nutrition. *Egyptian Journal of Occupational Medicine,* 41**,** 217-236.

ELIOPOULOS, G. M. & GOLD, H. S. 2001. Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clinical Infectious Diseases,* 33**,** 210-219.

ELISHA, B. G. & COURVALIN, P. 1995. Analysis of genes encoding D-alanine: D-alanine ligase-related enzymes in *Leuconostoc mesenteroides* and *Lactobacillus* spp. *Gene,* 152**,** 79-83.

ENGLEN, M. D. & KELLEY, L. C. 2000. A rapid DNA isolation procedure for the identification of *Campylobacter jejuni* by the polymerase chain reaction. *Letters in Applied Microbiology,* 31**,** 421-426.

ERGINKAYA, Z., TURHAN, E. U. & TATLI, D. 2018. Determination of antibiotic resistance of lactic acid bacteria isolated from traditional Turkish fermented dairy products. *Iranian Journal of Veterinary Research,* 19**,** 53-56.

ERKMEN, O. & BOZOGLU, T. F. (eds.) 2016. *Food preservation by reducing water activity*.

EVERS, S., CASADEWALL, B., CHARLES, M., DUTKA-MALEN, S., GALIMAND, M. & COURVALIN, P. 1996. Evolution of structure and substrate specificity in D-Alanine:D-Alanine ligases and related enzymes. *Journal of Molecular Evolution,* 42**,** 706-712.

EWNETU, D. & MIHRET, A. 2010. Prevalence and antimicrobial resistance of *Campylobacter* isolates from humans and chickens in Bahir Dar, Ethiopia. *Foodborne Pathogens and Disease,* 7**,** 667-670.

FAO. 2018. *State of food security and nutrition* [Online]. Available: http://www.fao.org/state-of-food-security-nutrition/2018/en/ [Accessed 03 November 2019].

FAO. 2019. *Gateway to dairy production and products - health hazards* [Online]. Available: http://www.fao.org/dairy-production-products/products/health-hazards/en/ [Accessed 01 March 2019].

FERAIN, T., HOBBS, J. N., JR., RICHARDSON, J., BERNARD, N., GARMYN, D., HOLS, P., ALLEN, N. E. & DELCOUR, J. 1996. Knockout of the two *ldh* genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. *Journal of Bacteriology,* 178**,** 5431-5437.

FGUIRI, I., ATIGUI, M., ZIADI, M., ARROUM, S. & KHORCHANI, T. 2015. Biochemical and molecular identification of lactic acid bacteria isolated from camel milk in Tunisia. *Emirates Journal of Food and Agriculture,* 27**,** 716-720.

FISCHER, J., RODRÍGUEZ, I., SCHMOGER, S., FRIESE, A., ROESLER, U., HELMUTH, R. & GUERRA, B. 2012. *Escherichia coli* producing VIM-1 carbapenemase isolated on a pig farm. *Journal of Antimicrobial Chemotherapy,* 67**,** 1793-1795.

FLÓREZ, A. B., AMMOR, M. S., ÁLVAREZ-MARTÍN, P., MARGOLLES, A. & MAYO, B. 2006. Molecular analysis of *tet*(W) gene-mediated tetracycline resistance in dominant intestinal *Bifidobacterium* species from healthy humans. *Applied and Environmental Microbiology,* 72**,** 7377-7379.

FLÓREZ, A. B., CAMPEDELLI, I., DELGADO, S., ALEGRÍA, Á., SALVETTI, E., FELIS, G. E., MAYO, B. & TORRIANI, S. 2016. Antibiotic susceptibility profiles of dairy *Leuconostoc*, analysis of the genetic basis of atypical resistances and transfer of genes *in vitro* and in a food matrix. *PLoS One,* 11. DOI: 10.1371/journal.pone.0145203.

FLÓREZ, A. B. & MAYO, B. 2017. Antibiotic resistance-susceptibility profiles of *Streptococcus thermophilus* isolated from raw milk and genome analysis of the genetic basis of acquired resistances. *Frontiers in Microbiology,* 8. DOI: 10.3389/fmicb.2017.02608.

FLOROU-PANERI, P., CHRISTAKI, E. & BONOS, E. 2013. Lactic acid bacteria as source of functional ingredients. *In:* KONGO, M. (ed.) *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes.* Intech Open.

FOUNOU, L. L., RASPAIL, C. F. & SABIHA, Y. E. 2016. Antibiotic resistance in the food chain: a developing country-perspective. *Frontiers in Microbiology,* 7. DOI: 10.3389/fmicb.2016.01881.

FOX, P. F., UNIACKE-LOWE, T., MCSWEENEY, P. L. H. & O'MAHONY, J. A. 2015a. Lactose. *Dairy Chemistry and Biochemistry.* Switzerland: Springer.

FOX, P. F., UNIACKE-LOWE, T., MCSWEENEY, P. L. H. & O'MAHONY, J. A. 2015b. Water in milk and dairy products. *Dairy Chemistry and Biochemistry.* 2nd ed. Switzerland: Springer.

FRAIMOW, H. S., JUNGKIND, D. L., LANDER, D. W., DELSO, D. R. & DEAN, J. L. 1994. Urinary Tract Infection with an *Enterococcus faecalis* isolate that requires vancomycin for growth. *Annals of Internal Medicine,* 121**,** 22-26.

FRANCO-DUARTE, R., ČERNÁKOVÁ, L., KADAM, S., KAUSHIK, K. S., SALEHI, B., BEVILACQUA, A., CORBO, M. R., ANTOLAK, H., DYBKA-STĘPIEŃ, K., LESZCZEWICZ, M., RELISON TINTINO, S., ALEXANDRINO DE SOUZA, V. C., SHARIFI-RAD, J., COUTINHO, H. D. M., MARTINS, N. & RODRIGUES, C. F. 2019. Advances in chemical and biological methods to identify microorganisms - from past to present. *Microorganisms,* 7**,** 1-32.

FRANZ, C. M. A. P., HUCH, M., MATHARA, J. M. & ABRIOUEL, H. 2014. African fermented foods and probiotics. *International Journal of Food Microbiology,* 190**,** 84-96.

FRIEDLANDER, A., NIR, S., RECHES, M. & SHEMESH, M. 2019. Preventing biofilm formation by dairy-associated bacteria using peptide-coated surfaces. *Frontiers in Microbiology,* 10. DOI: 10.3389/fmicb.2019.01405.

FUKAO, M. & YAJIMA, N. 2012. Assessment of antibiotic resistance in probiotic lactobacilli. *In:* PANA, M. (ed.) *Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium.* Intech Open.

FYMAT, A. L. 2017. Antibiotics and antibiotic resistance. *Biomedical Journal of Scientific and Technical Research,* 1**,** e1-e16.

GAD, G. F. M., ABDEL-HAMID, A. M. & FARAG, Z. S. H. 2014. Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. *Brazilian Journal of Microbiology,* 45**,** 25-33.

GÄNZLE, M. G. 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science,* 2**,** 106-117.

GEBREYES, W. A., DUPOUY-CAMET, J., NEWPORT, M. J., OLIVEIRA, C. J. B., SCHLESINGER, L. S., SAIF, Y. M., KARIUKI, S., SAIF, L. J., SAVILLE, W., WITTUM, T., HOET, A., QUESSY, S., KAZWALA, R., TEKOLA, B., SHRYOCK, T., BISESI, M., PATCHANEE, P., BOONMAR, S. & KING, L. J. 2014. The Global One Health paradigm: challenges and opportunities for tackling infectious diseases at the human, animal, and

environment interface in low-resource settings. *PLoS Neglected Tropical Diseases,* 8**,** e3257. DOI: 10.1371/journal.pntd.0003257.

GEROVA, V. 2019. *Popular Aussie cheeses recalled over potential E. coli contamination* [Online]. 10 daily. Available: https://www.google.com/amp/s/10daily.com.au/amp/news/austraila/a191115ruwit/popular-ausie-cheeses-recalled-over-potential-e-coli-contamination-20191115 [Accessed 18 November 2019].

GHOSH, T., BENIWAL, A., SEMWAL, A. & NAVANI, N. K. 2019. Mechanistic insights into probiotic properties of lactic acid bacteria associated with ethnic fermented dairy products. *Frontiers in Microbiology,* 10. DOI: 10.3389/fmicb.2019.00502.

GIRAFFA, G. 2004. Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiology Reviews,* 28**,** 251-260.

GIRAFFA, G. & CARMINATI, D. 2008. Molecular techniques in food fermentation: principles and applications. *In:* COCOLIN, L. & ERCOLINI, D. (eds.) *Molecular Techniques in the Microbial Ecology of Fermented Foods.* New York, NY: Springer New York.

GOFF, H. D. 2018a. *Milk as a growth medium* [Online]. Available: https://www.uoguelph.ca/foodscience/book-page/milk-growth-medium [Accessed 12 December 2018].

GOFF, H. D. 2018b. *pH* [Online]. Available: https://www.uoguelph.ca/foodscience/book-page/ph-0 [Accessed 26 March 2019].

GOFF, H. D. 2018c. *Starter cultures* [Online]. Canada: University of Guelph. Available: https://www.uoguelph.ca/foodscience/book-page/starter-cultures [Accessed 10 December 2018].

GOFF, H. D. 2018d. *Titratable acidity* [Online]. Available: https://www.uoguelph.ca/foodscience/book-page/titratable-acidity [Accessed 26 March 2019].

GOPAL, N., HILL, C., ROSS, P. R., BERESFORD, T. P., FENELON, M. A. & COTTER, P. D. 2015. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. *Frontiers in Microbiology,* 6**,** 1418. DOI: 10.3389/fmicb.2015.01418.

GRANATO, D., DE ARAÚJO CALADO, V. M. & JARVIS, B. 2014. Observations on the use of statistical methods in Food Science and Technology. *Food Research International,* 55**,** 137-149.

GROHMANN, E., MUTH, G. & ESPINOSA, M. 2003. Conjugative plasmid transfer in Gram-positive bacteria. *Microbiology and Molecular Biology Reviews: MMBR,* 67**,** 277-301.

GROVER, S., RASHMI, H. M., SRIVASTAVA, A. K. & BATISH, V. K. 2012. Probiotics for human health - new innovations and emerging trends. *Gut Pathogens,* 4**,** 15. DOI: 10.1186/1757-4749-4-15.

GUEIMONDE, M., SÁNCHEZ, B., DE LOS REYES-GAVILÁN, C. & MARGOLLES, A. 2013. Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology,* 4**,** 1-6. DOI: 10.3389/fmicb.2013.00202.

GUERZONI, M. E., VERNOCCHI, P., NDAGIJIMANA, M., GIANOTTI, A. & LANCIOTTI, R. 2007. Generation of aroma compounds in sourdough: effects of stress exposure and lactobacilli–yeasts interactions. *Food Microbiology,* 24**,** 139-148.

GUO, H., PAN, L., LI, L. & LU, J. 2017. Characterization of antibiotic resistance genes from *Lactobacillus* isolated from traditional dairy products. *Journal of Food Science,* 82**,** 724-730.

HANDWERGER, S., PUCCI, M. J., VOLK, K. J., LIU, J. & LEE, M. S. 1994. Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic peptidoglycan precursors that terminate in lactate. *Journal of Bacteriology,* 176**,** 260-264.

HASSAN, A. & AMJAD, I. 2010. Nutritional evaluation of yoghurt prepared by different starter cultures and their physiochemical analysis during storage *African Journal of Biotechnology,* 9**,** 2913-2917.

HATI, S., MANDAL, S. & PRAJAPATHI, J. B. 2013. Novel starters for value added fermented dairy products. *Current Research in Nutrition and Food Science,* 1**,** 83-91.

HELLER, K. J. 2001. Probiotic bacteria in fermented foods: product characteristics and starter organisms. *The American Journal of Clinical Nutrition,* 73**,** 374s-379s.

HERMAN, L., CHEMALY, M., COCCONCELLI, P. S., FERNANDEZ, P., KLEIN, G., PEIXE, L., PRIETO, M., QUEROL, A., SUAREZ, J. E., SUNDH, I., VLAK, J. & CORREIA, S. 2019. The qualified presumption of safety assessment and its role in EFSA risk evaluations: 15 years past. *FEMS Microbiology Letters,* 366. fny260. DOI: 10.1093/femsle/fny260.

HICKEY, C. D., SHEEHAN, J. J., WILKINSON, M. G. & AUTY, M. A. E. 2015. Growth and location of bacterial colonies within dairy foods using microscopy techniques: a review. *Frontiers in Microbiology,* 6. DOI: 10.3389/fmicb.2015.00099.

HOLZAPFEL, W. H. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology,* 75**,** 197-212.

HOQUE, M. Z., AKTER, F., HOSSAIN, K. M., RAHMAN, M. S. M., BILLAH, M. M. & ISLAM, K. M. D. 2010. Isolation, identification, and analysis, of probiotic properties of *Lactobacillus* sp. from selective regional yoghurts. *World Journal of Dairy and Food Sciences,* 5**,** 39-46.

HORII, T., KIMURA, T., SATO, K., SHIBAYAMA, K. & OHTA, M. 1999. Emergence of fosfomycin-resistant isolates of shiga-like toxin-producing *Escherichia coli* O26. *Antimicrobial Agents and Chemotherapy,* 43**,** 789-793.

HÖRMANN, S., SCHEYHING, C., BEHR, J., PAVLOVIC, M., EHRMANN, M. & VOGEL, R. F. 2006. Comparative proteome approach to characterize the high-pressure stress response of *Lactobacillus sanfranciscensis* DSM 20451T. *Proteomics,* 6**,** 1878-1885.

HUTKINS, R. W. & NANNEN, N. L. 1993. pH homeostasis in lactic acid bacteria. *Journal of Dairy Science,* 76**,** 2354-2365.

HUYS, G., D'HAENE, K., COLLARD, J.-M. & SWINGS, J. 2004. Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. *Applied and Environmental Microbiology,* 70**,** 1555-1562.

IFIC. 2019. *Background on carbohydrates & sugars* [Online]. Available: https://foodinsight.org/background-on-carbohydrates-sugars/ [Accessed 10 March 2019].

ISO 1998. Microbiology of food and animal feeding stuffs. *Horizontal method for the enumeration of mesophilic lactic acid bacteria — Colony-count technique at 30 °C, ISO 15214:1998.* Switzerland: International Organization for Standardization.

ISO 2013. Microbiology of the food chain - Horizontal method for the enumeration of microorganisms. *Part 2: Colony count at 30 °C by the surface plating technique, ISO 4833-2:2013.* Switzerland: International Organization for Standardization.

JACOBSEN, L., WILCKS, A., HAMMER, K., HUYS, G., GEVERS, D. & ANDERSEN, S. R. 2007. Horizontal transfer of *tet*(M) and *erm*(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnotobiotic rats. *FEMS Microbiology Ecology,* 59**,** 158-166.

JAIMEE, G. & HALAMI, P. M. 2016. Emerging resistance to aminoglycosides in lactic acid bacteria of food origin - an impending menace. *Applied Microbiology and Biotechnology,* 100**,** 1137**–**1151.

JAMES, C. S. 1995. Theory of analytical methods for specific food constituents. *In:* JAMES, C. S. (ed.) *Analytical Chemistry of Foods.* Springer - Science+Business Media, BV. : United Kingdom.

JORGENSEN, J. H. & FERRARO, M. J. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases,* 49**,** 1749-1755.

JOSE, N. M., BUNT, C. R. & HUSSAIN, M. A. 2015. Implications of antibiotic resistance in probiotics. *Food Reviews International,* 31**,** 52-62.

JOUBERT, L. 2013. Food security: the optimal diet for people and the planet. *South African Medical Journal,* 103**,** 809-810.

KAJFASZ, J. K. & QUIVEY, R. G. J. 2011. Responses of lactic acid bacteria to acid stress. *In:* TSAKALIDOU, E. & PAPADIMITRIOU, K. (eds.) *Stress responses to lactic acid bacteria.* New York: Springer Science and Business Media.

KARENZI, E., MASHAKU, A., NSHIMIYIMANA, A. M. & MUNYANGANIZI, B. 2013. Kivuguto traditional fermented milk and the dairy industry in Rwanda. A review. *Biotechnologie, Agronomie, Société et Environnement,* 17**,** 383-391.

KASTNER, S., PERRETEN, V., BLEULER, H., HUGENSCHMIDT, G., LACROIX, C. & MEILE, L. 2006. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. *Systematic and Applied Microbiology,* 29**,** 145-155.

KAYITESI, E., BEHERA, S. K., PANDA, S. K., BHEKI, D. & MULABA-BAFUBIANDI, A. F. 2017. *Amasi* and *Mageu* - Expedition from ethnic Southern African Foods to cosmopolitan markets. *In:* RAMESH, C. R. & MONTET, D. (eds.) *Fermented Foods Part II: Technological Intervention.* CRC Press.

KEARNS, D. B. 2010. A field guide to bacterial swarming motility. *Nature Reviews. Microbiology,* 8**,** 634-644.

KLARE, I., KONSTABEL, C., MÜLLER-BERTLING, S., REISSBRODT, R., HUYS, G., VANCANNEYT, M., SWINGS, J., GOOSSENS, H. & WITTE, W. 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. *Applied and Environmental Microbiology,* 71**,** 8982-8986.

KLARE, I., KONSTABEL, C., WERNER, G., HUYS, G., VANKERCKHOVEN, V., KAHLMETER, G., HILDEBRANDT, B., MÜLLER-BERTLING, S., WITTE, W. & GOOSSENS, H. 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *Journal of Antimicrobial Chemotherapy,* 59**,** 900-912.

KONGO, J. M. 2013. Lactic acid bacteria as starter-cultures for cheese processing: past, present and future developments. *In:* KONGO, J. M. (ed.) *Lactic Acid Bacteria – R & D for Food, Health and Livestock Purposes.* Intech Open.

KUMAR, R. & KAUSHIK, J. K. 2016. Molecular biology of adaption of starter lactic acid bacteria to dairy system. *In:* PUNIYA, A. K. (ed.) *Fermented Milk and Dairy Products.* Boca Raton: CRC Press.

KUMAR, S. G., ADITHAN, C., HARISH, B. N., SUJATHA, S., ROY, G. & MALINI, A. 2013. Antimicrobial resistance in India: a review. *Journal of Natural Science, Biology, and Medicine,* 4**,** 286-291.

KUMARI, S. & SARKAR, P. K. 2016. *Bacillus cereus* hazard and control in industrial dairy processing environment. *Food Control,* 69**,** 20-29.

KUSHIRO, A., CHERVAUX, C., COOLS-PORTIER, S., PERONY, A., LEGRAIN-RASPAUD, S., OBIS, D., ONOUE, M. & VAN DE MOER, A. 2009. Antimicrobial susceptibility testing of lactic acid bacteria and bifidobacteria by broth microdilution method and Etest. *International Journal of Food Microbiology,* 132**,** 54-58.

LABADARIOS, D., STEYN, N. P. & NEL, J. 2011. How diverse is the diet of adult South Africans? *Nutrition Journal,* 10**,** 33. DOI: 10.1186/1475-2891-10-33.

LANCIOTTI, R., PATRIGNANI, F., IUCCI, L., SARACINO, P. & GUERZONI, M. E. 2007. Potential of high pressure homogenization in the control and enhancement of proteolytic and fermentative activities of some *Lactobacillus* species. *Food Chemistry,* 102**,** 542-550.

LARSEN, M. V., COSENTINO, S., RASMUSSEN, S., FRIIS, C., HASMAN, H., MARVIG, R. L., JELSBAK, L., SICHERITZ-PONTÉN, T., USSERY, D. W., AARESTRUP, F. M. & LUND, O. 2012. Multilocus Sequence Typing of Total-Genome-Sequenced Bacteria. *Journal of Clinical Microbiology,* 50**,** 1355-1361.

LAULUND, S., WIND, A., DERKX, P. M. F. & ZULIANI, V. 2017. Regulatory and safety requirements for food cultures. *Microorganisms,* 5**,** 28. DOI: 10.3390/microorganisms5020028.

LAVEFVE, L., MARASINI, D. & CARBONERO, F. 2019. Microbial ecology of fermented vegetables and non-alcoholic drinks and current knowledge on their impact on human health. *In:* TOLDRÁ, F. (ed.) *Advances in Food and Nutrition Research.* Academic Press.

LEBRETON, F., WILLEMS, R. J. L. & GILMORE, M. S. 2014. *Enterococcus* diversity, origins in nature, and gut colonization. *In:* GILMORE, M. S., CLEWELL, D. B., IKE, Y. & SHANKAR, N. (eds.) *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection* Boston: Massachusetts Eye and Ear Infirmary.

LEE, J.-H. & O'SULLIVAN, D. J. 2010. Genomic insights into bifidobacteria. *Microbiology and Molecular Biology Reviews: MMBR,* 74**,** 378-416.

LEKOFA, M. 2009. *The survival of microbial pathogens in dairy products.* MS, University of the Free State.

LEMCKE, R. & BÜLTE, M. 2000. Occurrence of the vancomycin-resistant genes *van*A, *van*B, *van*C1, *van*C2 and *van*C3 in *Enterococcus* strains isolated from poultry and pork. *International Journal of Food Microbiology,* 60**,** 185-194.

LERNER, A., MATTHIAS, T. & AMINOV, R. 2017. Potential effects of horizontal gene exchange in the human gut. *Frontiers in Immunology,* 8**,** 1630. DOI: 10.3389/fimmu.2017.01630.

LIPTÁKOVÁ, D., MATEJČEKOVÁ, Z. & VALÍK, Ľ. 2017. *Lactic acid bacteria and fermentation of cereals and pseudocereals*, InTech Open.

LIU, C., ZHANG, Z.-Y., DONG, K., YUAN, J.-P. & GUO, X.-K. 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. *Biomedical and Environmental Sciences,* 22**,** 401-412.

MARSHALL, C. G., LESSARD, I. A., PARK, I. & WRIGHT, G. D. 1998. Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrobial Agents and Chemotherapy,* 42**,** 2215-2220.

MASCO, L., VAN HOORDE, K., DE BRANDT, E., SWINGS, J. & HUYS, G. 2006. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. *Journal of Antimicrobial Chemotherapy,* 58**,** 85-94.

MATHUR, S. & SINGH, R. 2005. Antibiotic resistance in food lactic acid bacteria - a review. *International Journal of Food Microbiology,* 105**,** 281-295.

MATSHEKA, M. I., MAGWAMBA, C. C., MPUCHANE, S. & GASHE, B. A. 2013. Biogenic amine producing bacteria associated with three different commercially fermented beverages in Botswana. *African Journal of Microbiology Research,* 7**,** 342-350.

MCKEVITH, B. & SHORTT, C. 2003. Fermented milks | Other Relevant Products. *In:* CABALLERO, B. (ed.) *Encyclopedia of Food Sciences and Nutrition.* 2nd ed. Oxford: Academic Press.

MENDELSON, M. 2015. Role of antibiotic stewardship in extending the age of modern medicine. *SAMJ: South African Medical Journal,* 105**,** 414-419.

MERCK. 2019. *Plate count agar* [Online]. Available: http://www.merckmillipore.com/ZA/en/product/Plate-Count-agar,MDA_CHEM-105463#anchor_BRO [Accessed 23 March 2019].

MICHALOPOULOS, A. S., LIVADITIS, I. G. & GOUGOUTAS, V. 2011. The revival of fosfomycin. *International Journal of Infectious Diseases,* 15**,** e732-e739.

MOKOENA, M. P., MUTANDA, T. & OLANIRAN, A. O. 2016. Perspectives on the probiotic potential of lactic acid bacteria from African traditional fermented foods and beverages. *Food & Nutrition Research,* 60**,** 29630. DOI: 10.3402/fnr.v60.29630.

MONTANARI, C., SADO KAMDEM, S. L., SERRAZANETTI, D. I., ETOA, F.-X. & GUERZONI, M. E. 2010. Synthesis of cyclopropane fatty acids in *Lactobacillus helveticus* and *Lactobacillus sanfranciscensis* and their cellular fatty acids changes following short term acid and cold stresses. *Food Microbiology,* 27**,** 493-502.

MORAČANIN, S. V., DJUKIĆ, D., ZDOLEC, N., MILIJAŠEVIĆ, M. & MAŠKOVIĆ, P. 2017. Antimicrobial resistance in fermented foods. *Journal of Hygienic Engineering and Design,* 18**,** 25-35.

MORAES, P. M., PERIN, L. M., JÚNIOR, A. S. & NERO, L. A. 2013. Comparison of phenotypic and molecular tests to identify lactic acid bacteria. *Brazilian Journal of Microbiology,* 44**,** 109-112.

MORANDI, S., CREMONESI, P., SILVETTI, T. & BRASCA, M. 2013. Technological characterisation, antibiotic susceptibility and antimicrobial activity of wild-type *Leuconostoc* strains isolated from north Italian traditional cheeses. *Journal of Dairy Research,* 80**,** 457-466.

MOYANE, J. N. & JIDEANI, A. I. O. 2013. The physicochemical and sensory evaluation of commercial sour milk (*amasi*) products. *African Journal of Food Science,* 7**,** 56-62.

MOYANE, J. N., JIDEANI, A. I. O. & AIYEGORO, O. A. 2013. Antibiotics usage in food-producing animals in South Africa and impact on human: antibiotic resistance. *African Journal of Microbiology Research,* 7**,** 2990-2997.

MULLAN, W. M. A. 2014. Starter cultures | Importance of Selected Genera. *In:* BATT, C. A. & TORTORELLO, M. L. (eds.) *Encyclopedia of Food Microbiology.* 2nd ed. Oxford: Academic Press.

MUNITA, J. M. & ARIAS, C. A. 2016. Mechanisms of antibiotic resistance. *Microbiology Spectrum,* 4**,** 1-37. DOI: 10.1128/microbiolspec.VMBF-0016-2015.

NAWAZ, M., WANG, J., ZHOU, A., MA, C., WU, X., MOORE, J. E., CHERIE MILLAR, B. & XU, J. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Current Microbiology,* 62**,** 1081-1089.

NDAGIJIMANA, M., VALLICELLI, M., COCCONCELLI, P. S., CAPPA, F., PATRIGNANI, F., LANCIOTTI, R. & GUERZONI, M. E. 2006. Two 2[5H]-furanones as possible signaling molecules in *Lactobacillus helveticus*. *Applied and Environmental Microbiology,* 72**,** 6053-6061.

NELSON, R. R. S. 1999. Intrinsically vancomycin-resistant Gram-positive organisms: clinical relevance and implications for infection control. *Journal of Hospital Infection,* 42**,** 275-282.

NOVASINA 2010. LabSwift-a_w operating instructions. Switzerland: Novasina AG.

NYCHAS, G. J. E. & PANAGOU, E. 2011. Microbiological spoilage of foods and beverages. *In:* KILCAST, D. & SUBRAMANIAM, P. (eds.) *Food and Beverage Stability and Shelf Life.* Woodhead Publishing.

O'MAHONY, M., MITCHELL, E., GILLBERT, R. J., HUTCHINSON, D. N., BEGG, N. T., RODHOUSE, J. C. & MORRIS, J. E. 1990. An outbreak of foodborne botulism associated with contaminated hazelnut yoghurt. *Epidemiological Infections,* 104**,** 389-395.

O'NEILL, J. 2016. *Tackling drug-resistant infections globally: final report and recommendations* [Online]. Available: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf [Accessed 15 December 2018].

OGIER, J.-C., CASALTA, E., FARROKH, C. & SAÏHI, A. 2008. Safety assessment of dairy microorganisms: the *Leuconostoc* genus. *International Journal of Food Microbiology,* 126**,** 286-290.

OSTROV, I., HAREL, A., BERNSTEIN, S., STEINBERG, D. & SHEMESH, M. 2016. Development of a method to determine the effectiveness of cleaning agents in removal of biofilm derived spores in milking system. *Frontiers in Microbiology,* 7**,** 1498. DOI: 10.3389/fmicb.2016.01498.

OSVIK, R. D., SPERSTAD, S., BREINES, E., HAREIDE, E., GODFROID, J., ZHOU, Z., REN, P., GEOGHEGAN, C., HOLZAPFEL, W. & RINGØ, E. 2013. Bacterial diversity of *aMasi*, a South African fermented milk product, determined by clone library and denaturing gradient gel electrophoresis analysis. *African Journal of Microbiology Research,* 7**,** 4146-4158.

OUOBA, L. I. I., LEI, V. & JENSEN, L. B. 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. *International Journal of Food Microbiology,* 121**,** 217-224.

OWUSU-KWARTENG, J., WUNI, A., AKABANDA, F., TANO-DEBRAH, K. & JESPERSEN, L. 2017. Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus sensu lato* isolated from dairy farms and traditional dairy products. *BMC Microbiology,* 17**,** 65. DOI: 10.1186/s12866-017-0975-9.

PAPADIMITRIOU, K., ALEGRÍA, Á., BRON, P. A., DE ANGELIS, M., GOBBETTI, M., KLEEREBEZEM, M., LEMOS, J. A., LINARES, D. M., ROSS, P., STANTON, C., TURRONI, F., VAN SINDEREN, D., VARMANEN, P., VENTURA, M., ZÚÑIGA, M., TSAKALIDOU, E. & KOK, J. 2016. Stress physiology of lactic acid bacteria. *Microbiology and Molecular Biology Reviews: MMBR,* 80**,** 837-890.

PARK, I. & WALSH, C. T. 1997. D-Alanyl-D-lactate and D-alanyl-D-alanine synthesis by D-alanyl-D-alanine ligase from vancomycin resistant *Leuconostoc mesenteroides*. Effects of a phenylalanine 261 to tyrosine mutation. *The Journal of Biological Chemistry,* 272**,** 9210-9214.

PATEL, A. R., SHAH, N. P. & PRAJAPATI, J. B. 2012. Antibiotic resistance profile of lactic acid bacteria and their implications in food chain. *World Journal of Dairy and Food Sciences,* 7**,** 202-211.

PATEL, R. 2000. Enterococcal-type glycopeptide resistance genes in non-enterococcal organisms. *FEMS Microbiology Letters,* 185**,** 1-7. DOI: 10.1111/j.1574-6968.2000.tb09032.x.

PAVLOVIC, M., HÖRMANN, S., VOGEL RUDI, F. & EHRMANN MATTHIAS, A. 2008. Characterisation of a piezotolerant mutant of *Lactobacillus sanfranciscensis*. *Zeitschrift für Naturforschung B,* 63**,** 791-797.

PETERSON, E. & KAUR, P. 2018. Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology,* 9. DOI: 10.3389/fmicb.2018.02928.

PIURI, M., SANCHEZ-RIVAS, C. & RUZAL, S. M. 2003. Adaptation to high salt in *Lactobacillus*: role of peptides and proteolytic enzymes. *Journal of Applied Microbiology,* 95**,** 372-379.

PIURI, M., SANCHEZ-RIVAS, C. & RUZAL, S. M. 2005. Cell wall modifications during osmotic stress in *Lactobacillus casei*. *Journal of Applied Microbiology,* 98**,** 84-95.

PRADO, M. R., BLANDÓN, L. M., VANDENBERGHE, L. P. S., RODRIGUES, C., CASTRO, G. R., THOMAZ-SOCCOL, V. & SOCCOL, C. R. 2015. Milk kefir: composition, microbial cultures, biological activities, and related products. *Frontiers in Microbiology,* 6**,** 1177. DOI: 10.3389/fmicb.2015.01177

PRESTERL, E., KNEIFEL, W., K MAYER, H., ZEHETGRUBER, M., MAKRISTATHIS, A. & GRANINGER, W. 2001. Endocarditis by *Lactobacillus rhamnosus* due to yogurt ingestion? *Scandinavian Journal of Infectious Diseases,* 33**,** 710-714.

PRICE, L. B., STEGGER, M., HASMAN, H., AZIZ, M., LARSEN, J., ANDERSEN, P. S., PEARSON, T., WATERS, A. E., FOSTER, J. T., SCHUPP, J., GILLECE, J., DRIEBE, E., LIU, C. M., SPRINGER, B., ZDOVC, I., BATTISTI, A., FRANCO, A., ZMUDZKI, J., SCHWARZ, S., BUTAYE, P., JOUY, E., POMBA, C., PORRERO, M. C., RUIMY, R., SMITH, T. C., ROBINSON, D. A., WEESE, J. S., ARRIOLA, C. S., YU, F., LAURENT, F., KEIM, P., SKOV, R. & AARESTRUP, F. M. 2012. *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. *mBio,* 3**,** e00305-11. DOI: 10.1128/mBio.00305-11.

QUIGLEY, L., O'SULLIVAN, O., STANTON, C., BERESFORD, T. P., ROSS, R. P., FITZGERALD, G. F. & COTTER, P. D. 2013. The complex microbiota of raw milk. *FEMS Microbiology Reviews,* 37**,** 664-698.

QUINTO, E. J., JIMÉNEZ, P., CARO, I., TEJERO, J., MATEO, J. & GIRBÉS, T. 2014. Probiotic lactic acid bacteria: a review. *Food and Nutrition Sciences,* 5**,** 1765-1775.

RADULOVIĆ, Z., PETROVIĆ, T. & BULAJIĆ, S. 2012. Antibiotic susceptibility of probiotic bacteria *In:* PANA, M. (ed.) *Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium.* InTech Open.

RAKIB, M. R. H., KABIR, M. A. & AMANULLAH, S. M. 2017. Starter cultures used in the production of probiotic dairy products and their potential applications: a review. *Chemical and Biomolecular Engineering,* 2**,** 83-89.

RAZ, R. 2012. Fosfomycin: an old-new antibiotic. *Clinical Microbiology and Infection,* 18**,** 4-7.

REID, G., FOOD, AGRICULTURAL ORGANIZATION OF THE UNITED, N. & THE, W. H. O. 2005. The importance of guidelines in the development and application of probiotics. *Current Pharmaceutical Design,* 11**,** 11-16.

REIS, J. A., PAULA, A. T., CASAROTTI, S. N. & PENNA, A. L. B. 2012. Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Engineering Reviews,* 4**,** 124-140.

ROGERS, P., CHEN, J.-S. & ZIDWICK, M. J. 2013. Organic acid and solvent production: acetic, lactic, gluconic, succinic, and polyhydroxyalkanoic acids. *In:* ROSENBERG, E., DELONG, E. F., LORY, S., STACKEBRANDT, E. & THOMPSON, F. (eds.) *The Prokaryotes: Applied Bacteriology and Biotechnology.* Berlin, Heidelberg: Springer Berlin Heidelberg.

ROJO-BEZARES, B., SÁENZ, Y., POETA, P., ZARAZAGA, M., RUIZ-LARREA, F. & TORRES, C. 2006. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. *International Journal of Food Microbiology,* 111**,** 234-240.

ROSTAL, M. K., ROSS, N., MACHALABA, C., CORDEL, C., PAWESKA, J. T. & KARESH, W. B. 2018. Benefits of a One Health approach: an example using Rift Valley fever. *One Health,* 5**,** 34-36.

SABTU, N., ENOCH, D. A. & BROWN, N. M. 2015. Antibiotic resistance: what, why, where, when and how? *British Medical Bulletin,* 116**,** 105-113.

SANDROU, D. K. & ARVANITOYANNIS, I. S. 2000. Implementation of Hazard Analysis Critical Control Point (HACCP) to the dairy industry: current status and perspectives. *Food Reviews International,* 16**,** 77-111.

SCHELLACK, N., BENJAMIN, D., BRINK, A., DUSE, A., FAURE, K., GOFF, D., MENDELSON, M., MEYER, J., MIOT, J., PEROVIC, O., POPLE, T., SULEMAN, F., VAN VUUREN, M. & ESSACK, S. 2017. A situational analysis of current antimicrobial governance, regulation, and utilization in South Africa. *International Journal of Infectious Diseases,* 64**,** 100-106.

SCHUTTE, L. M. 2013. *Isolation and identification of the microbial consortium present in fermented milks from Sub-Saharan Africa.* MS, University of Stellenbosch.

SERRAZANETTI, D. I., GOTTARDI, D., MONTANARI, C. & GIANOTTI, A. 2013. Dynamic stresses of lactic acid bacteria associated to fermentation processes. *In:* KONGO, M. (ed.) *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes.* Intech Open.

SERRAZANETTI, D. I., GUERZONI, M. E., CORSETTI, A. & VOGEL, R. 2009. Metabolic impact and potential exploitation of the stress reactions in lactobacilli. *Food Microbiology,* 26**,** 700-711.

SERRAZANETTI, D. I., NDAGIJIMANA, M., SADO-KAMDEM, S. L., CORSETTI, A., VOGEL, R. F., EHRMANN, M. & GUERZONI, M. E. 2011. Acid stress-mediated metabolic shift in *Lactobacillus sanfranciscensis* LSCE1. *Applied and Environmental Microbiology,* 77**,** 2656-2666.

SHAO, Y., ZHANG, W., GUO, H., PAN, L., ZHANG, H. & SUN, T. 2015. Comparative studies on antibiotic resistance in *Lactobacillus casei* and *Lactobacillus plantarum*. *Food Control,* 50**,** 250-258.

SHARMA, C., GULATI, S., THAKUR, N., SINGH, B. P., GUPTA, S., KAUR, S., MISHRA, S. K., PUNIYA, A. K., GIL, J. P. S. & PANWAR, H. 2017. Antibiotic sensitivity pattern of

indigenous lactobacilli isolated from curd and human milk samples. *3 Biotech,* 7**,** 14. DOI: 10.1007/s13205-017-0682-0.

SHARMA, P., TOMAR, S. K., SANGWAN, V., GOSWAMI, P. & SINGH, R. 2015. Antibiotic resistance of *Lactobacillus* sp. isolated from commercial probiotic preparations. *Journal of Food Safety,* 36**,** 38-51.

SHENDURSE, A. M. & KHEDKAR, C. D. 2016. Lactose. *In:* CABALLERO, B., FINGLAS, P. M. & TOLDRÁ, F. (eds.) *The Encyclopedia of Food and Health.* Oxford: Academic Press.

SHIBY, V. K. & MISHRA, H. N. 2013. Fermented milks and milk products as functional foods - a review. *Critical Reviews in Food Science and Nutrition,* 53**,** 482-496.

SHISANA, O., LABADARIOS, D., REHLE, T., SIMBAYI, L., ZUMA, K., DHANSAY, A., REDDY, P., PARKER, W., HOOSAIN, E., NAIDOO, P., HONGORO, C., MCHIZA, A., STEYN, N. P., DWANE, N., MAKOAE, M., MALULEKE, T., RAMLAGAN, S., ZUNGU, N., EVANS, M. G., JACOBS, L., FABER, M. & TEAM SOUTH AFRICA-. 2014. *South African National Health and Nutritional Examination Survey (SANHENES-1),* Cape Town, HSRC Press.

SIMATENDE, P. 2016. *Microbial ecology and diversity of Swazi traditional fermented foods.* PhD, University of KwaZulu-Natal.

SOLOMON, E., BERG, L. & MARTIN, D. W. 2010. Biology. 9th ed. Canada: Cengage Learning.

SONG, A. A.-L., IN, L. L. A., LIM, S. H. E. & RAHIM, R. A. 2017. A review on *Lactococcus lactis*: from food to factory. *Microbial Cell Factories,* 16**,** 55. DOI: 10.1186/s12934-017-0669-x.

SONG, Y.-Y. & LU, Y. 2015. Decision tree methods: applications for classification and prediction. *Shanghai Archives of Psychiatry,* 27**,** 130-135.

SOUTH AFRICAN DEPARTMENT OF AGRICULTURE, FORESTRY & FISHERIES. 2015. Agricultural Product Standards Act, 1990 (Act No. 119 of 1990) - Regulations relating to the classification, packing and marking of dairy products and imitation dairy products intended for sale in the Republic of South Africa *In:* DEPARTMENT OF AGRICULTURE, FORESTRY & FISHERIES. (ed.). South Africa.

SOUTH AFRICAN DEPARTMENT OF HEALTH. 2015a. Antimicrobial resistance document. *In:* SOUTH AFRICAN DEPARTMENT OF HEALTH (ed.). South Africa.

SOUTH AFRICAN DEPARTMENT OF HEALTH. 2015b. Antimicrobial resistance national strategy framework 2014-2024. *In:* SOUTH AFRICAN DEPARTMENT OF HEALTH (ed.). South Africa.

STEYN, N. P. & OCHSE, R. 2013. "Enjoy a variety of foods": as a food-based dietary guideline for South Africa. *South African Journal of Clinical Nutrition,* 26**,** S13-S17.

STILES, M. E. & HOLZAPFEL, W. H. 1997. Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology,* 36**,** 1-29.

SUMPRADIT, N., CHONGTRAKUL, P., ANUWONG, K., PUMTONG, S., KONGSOMBOON, K., BUTDEEMEE, P., KHONGLORMYATI, J., CHOMYONG, S., TONGYOUNG, P., LOSIRIWAT, S., SEESUK, P., SUWANWAREE, P. & TANGCHAROENSATHIEN, V. 2012. Antibiotics smart use: a workable model for promoting the rational use of medicines in Thailand. *Bulletin of the World Health Organization,* 90**,** 905-913.

SURONO, S. & HOSONO, A. 2011. Fermented Milks | Starter Cultures. *In:* FUQUAY, J. W. (ed.) *Encyclopedia of Dairy Sciences.* 2nd ed. San Diego: Academic Press.

TADESSE, B. T., ASHLEY, E. A., ONGARELLO, S., HAVUMAKI, J., WIJEGOONEWARDENA, M., GONZÁLEZ, I. J. & DITTRICH, S. 2017. Antimicrobial resistance in Africa: a systematic review. *BMC Infectious Diseases,* 17**,** 616. DOI: 10.1186/s12879-017-2713-1.

TANG, K. L., CAFFREY, N. P., NÓBREGA, D. B., CORK, S. C., RONKSLEY, P. E., BARKEMA, H. W., POLACHEK, A. J., GANSHORN, H., SHARMA, N., KELLNER, J. D. & GHALI, W. A. 2017. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *The Lancet Planetary Health,* 1**,** e316-e327.

TEMMERMAN, R., POT, B., HUYS, G. & SWINGS, J. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *International Journal of Food Microbiology,* 81**,** 1-10.

TEREFE, N. S. 2016. Food fermentation. *Reference Module in Food Science.* Elsevier.

TEUBER, M., MEILE, L. & SCHWARZ, F. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek,* 76**,** 115-137.

TIRLONI, E., GHELARDI, E., CELANDRONI, F., BERNARDI, C. & STELLA, S. 2017. Effect of dairy product environment on the growth of *Bacillus cereus*. *Journal of Dairy Science,* 100**,** 7026-7034.

TOOMEY, N., BOLTON, D. & FANNING, S. 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. *Research in Microbiology,* 161**,** 127-135.

TOOMEY, N., MONAGHAN, A., FANNING, S. & BOLTON, D. J. 2009. Assessment of antimicrobial resistance transfer between lactic acid bacteria and potential foodborne pathogens using *in vitro* methods and mating in a food matrix. *Foodborne Pathogens and Disease,* 6**,** 925-933.

TROLLER, J. A. & STINSON, J. V. 1981. Moisture requirements for growth and metabolite production by lactic acid bacteria. *Applied and Environmental Microbiology,* 42**,** 682-687.

TYNKKYNEN, S., SINGH, K. V. & VARMANEN, P. 1998. Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (*van*) genes. *International Journal of Food Microbiology,* 41**,** 195-204.

ULLAH, H. & ALI, S. 2017. Classification of anti-bacterial agents and their functions. *In:* KUMAVATH, R. N. (ed.) *Antibacterial Agents.* Intech Open.

VAKULENKO, S. B., DONABEDIAN, S. M., VOSKRESENSKIY, A. M., ZERVOS, M. J., LERNER, S. A. & CHOW, J. W. 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrobial Agents and Chemotherapy,* 47**,** 1423-1426.

VAN DE GUCHTE, M., SERROR, P., CHERVAUX, C., SMOKVINA, T., EHRLICH, S. D. & MAGUIN, E. 2002. Stress responses in lactic acid bacteria *Antonie van Leeuwenhoek* 82**,** 187-216.

VAN HIJUM, S. A. F. T., VAUGHAN, E. E. & VOGEL, R. F. 2013. Application of state-of-art sequencing technologies to indigenous food fermentations. *Current Opinion in Biotechnology,* 24**,** 178-186.

VAN HOEK, A. H. A. M., MAYRHOFER, S., DOMIG, K. J., FLÓREZ, A. B., AMMOR, M. S., MAYO, B. & AARTS, H. J. M. 2008. Mosaic tetracycline resistance genes and their flanking regions in *Bifidobacterium thermophilum* and *Lactobacillus johnsonii*. *Antimicrobial Agents and Chemotherapy,* 52**,** 248-252.

VANNINI, L., NDAGIJIMANA, M., SARACINO, P., VERNOCCHI, P., CORSETTI, A., VALLICELLI, M., CAPPA, F., COCCONCELLI, P. S. & GUERZONI, M. E. 2007. New signaling molecules in some Gram-positive and Gram-negative bacteria. *International Journal of Food Microbiology,* 120**,** 25-33.

VERNOCCHI, P., NDAGIJIMANA, M., SERRAZANETTI, D., GIANOTTI, A., VALLICELLI, M. & GUERZONI, M. E. 2008. Influence of starch addition and dough microstructure on fermentation aroma production by yeasts and lactobacilli. *Food Chemistry,* 108**,** 1217-1225.

VERRAES, C., VAN BOXSTAEL, S., VAN MEERVENNE, E., VAN COILLIE, E., BUTAYE, P., CATRY, B., DE SCHAETZEN, M.-A., VAN HUFFEL, X., IMBERECHTS, H., DIERICK, K., DAUBE, G., SAEGERMAN, C., DE BLOCK, J., DEWULF, J. & HERMAN, L. 2013. Antimicrobial resistance in the food chain: a review. *International Journal of Environmental Research and Public Health,* 10**,** 2643-2669.

VON WRIGHT, A. & AXELSSON, L. 2011. Lactic acid bacteria: an introduction. *In:* LAHTINEN, S., OUWEHAND, A. C., SALMINEN, S. & VON WRIGHT, A. (eds.) *Lactic Acid Bacteria: Microbiological and Functional Aspects.* 4th ed. Boca Raton: CRC Press.

VORSTER, H. H., BADHAM, J. B. & VENTER, C. S. 2013a. An introduction to the revised food-based dietary guidelines for South Africa. *South African Journal of Clinical Nutrition,* 26**,** S5-S12.

VORSTER, H. H., LOVE, P. & BROWNE, C. 2001. Development of food-based dietary guidelines for South Africa - the process. *South African Journal of Clinical Nutrition,* 14**,** S3-S6.

VORSTER, H. H., WENHOLD, F. A. M., WRIGHT, H. H., WENTZEL-VILJOEN, E., VENTER, C. S. & VERMAAK, M. 2013b. Have milk, maas or yoghurt every day. *South African Journal of Clinical Nutrition,* 26**,** S57-S65.

WALLET, F., DESSEIN, R., ARMAND, S. & COURCOL, R. J. 2002. Molecular diagnosis of endocarditis due to *Lactobacillus casei* subsp. *rhamnosus*. *Clinical Infectious Diseases,* 35**,** e117-e119.

WANG, H. H., WANG, H. H., MANUZON, M., LEHMAN, M. & WAN, K. 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. *FEMS Microbiology Letters,* 254**,** 226-231.

WEBER, M., LIEDTKE, J., PLATTES, S. & LIPSKI, A. 2019. Bacterial community composition of biofilms in milking machines of two dairy farms assessed by a combination of culture-dependent and –independent methods. *PLoS One,* 14**,** e0222238. DOI: 10.1371/journal.pone.0222238.

WHO (ed.) 2014. *Antimicrobial resistance,* Geneva, Switzerland: WHO.

WHO 2018. GLASS - The detection and reporting of colistin resistance. Geneva, Switzerland: WHO.

WHO. 2019. *Food Safety* [Online]. WHO. Available: https://www.who.int/news-room/fact-sheets/detail/food-safety [Accessed 31 October 2019].

ZAGO, M., FORNASARI, M. E., CARMINATI, D., BURNS, P., SUÀREZ, V., VINDEROLA, G., REINHEIMER, J. & GIRAFFA, G. 2011. Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology,* 28**,** 1033-1040.

ZAMAN, S. B., HUSSAIN, M. A., NYE, R., MEHTA, V., MAMUN, K. T. & HOSSAIN, N. 2017. A review on antibiotic resistance: alarm bells are ringing. *Cureus,* 9**,** e1403. DOI: 10.7759/cureus.1403.