Probiotic and technological properties of lactic acid bacteria from bovine origin

By

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Submitted in partial fulfilment of the requirements of the degree

Master of Science

Food Science

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria, South Africa

November 2020

DECLARATION

I, Michael Kudzanai Mungwari declare that the thesis/ dissertation, which I at this moment submit for the degree MSc Food Science at the University of Pretoria, is my work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Michael Kudzanai Mungwari

November 2020

ACKNOWLEDGEMENTS

I would like to express my gratitude and sincerely appreciate:

My supervisor Prof Elna. M. Buys for her support and enormous contribution throughout the study. Working with her enlightened me professionally.

Dr Richard Nyanzi and Dr Srinivasan Krishnamoorthy for help to operate laboratory equipment and carrying out experimental work.

My study colleagues and lab mates. Special thanks to Mr Serlom Agbemavor for his help in statistical and data analysis. It is deeply appreciated.

My mother, Mrs T. Mukasa and Uncle Mr Mutsongodza, for their financial and moral support and advice throughout the study.

DEDICATION

The work is dedicated to my parents and the Almighty God for opening opportunities to further my studies.

ABSTRACT

Probiotic and technological properties of lactic acid bacteria from bovine origin

By

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Lactic acid bacteria are generally regarded as safe microorganisms, and they have been used as food additives and therapeutic supplements in functional foods. In 2013 the world market for probiotics was USD 36 million and increased to 35.9 billion in 2018. The probiotic preference is because of extreme interest in probiotic yoghurt and development in the utilisation of functional foods. This has led to new bacterial strains being distinguished and added to pharmaceutical and nutrition products. Most of the probiotics in use are obtained from the gastrointestinal tract of healthy human individuals. However, there are other probiotics in use which are of non- human origin. The probiotics which are intended to be used in humans require proof that they work in human hosts. Identification of probiotics at the strain level, evaluation of biosafety by antibiotic susceptibility testing and absence of virulence factors as well as an assessment of probiotic characteristics must be carried out before use in humans.

Therefore, the current study focused on characterising probiotic lactic acid bacteria from bovine and dairy sources for application in yoghurt. *Lactobacillus acidophilus* (strain D) and *Lactobacillus rhamnosus* (strain V) were from the dairy origin, *Lactobacillus plantarum* (strain VLL1) and *Lactobacillus pentosus* (strain LIP), were from the bovine origin and *Lactobacillus acidophilus* ATCC 4536 from American Type Culture Collection. The first phase of the study

aimed at identifying and characterising the lactic acid bacteria. The identification was done using phenotypic methods which included Gram staining, catalase test, oxidase test, and carbon dioxide gas production from glucose. The isolates were gram-positive, catalase-negative, and oxidase negative, which proved them to be lactic acid bacteria. They were also able to produce carbon dioxide gas from glucose. The production of carbon dioxide gas classified the bacteria as heterofermentative, and ethanol, and lactic acid were produced as by-products of glucose metabolism.

The second phase included safety assessment and determination of probiotic properties. The safety assessment included haemolytic activity, lipase production, gelatinase production, and antibiotic resistance. The probiotic properties which were determined included acid and bile resistance, bile salt hydrolase activity and antimicrobial activity. The antimicrobial activity was against enteroinvasive *Escherichia coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), *Candida albicans* ATCC 10231 (C1), *C. albicans* 1051255 (C4), and *C. albicans* M0826 (C7). On exposure to the acidic environment, the lactic acid bacteria significantly reduced their viability at pH 2. However, they were able to survive pH 5 and pH 3. The isolates were able to survive a 0.3% bile salt concentration and released the bile salt hydrolase enzymes. The lactic acid bacteria were able to inhibit diarrheagenic *E.coli* and pathogenic *Candida* spp. On antibiotic susceptibility testing, the strains were resistant to vancomycin and kanamycin and susceptible to gentamicin, clindamycin, erythromycin, tetracycline, ampicillin, and chloramphenicol. The isolates from bovine origin were moderately sensitive to tetracycline. The isolates were gelatinase and lipase negative, but they showed α- hemolysis which is the partial hydrolysis of red blood cells.

The third phase was to determine the fate of the bovine and dairy probiotic strains when used with regular fermenting bacteria during fermentation as well as storage. *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* were selected for yoghurt processing. The yoghurt was stored for 28 days under refrigeration at 4 °C. The probiotic cells were selectively enumerated using MRS- bile agar. The cell population levels of the probiotics were positively affected in relation to the control up to day 28 of storage at 4 °C. Overall, this study exemplifies the probiotic potential of bovine and dairy isolates, where the isolates from bovine origin have slightly increased tolerance to human gastrointestinal conditions such as acid and bile tolerance, pathogenic inhibition, α-haemolytic and negative to lipase and gelatinase enzyme activities.

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CHAPTER 1: PROBLEM STATEMENT

In 2001 the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) experts defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". The classifying of microorganisms as probiotics should follow an appropriate criterion which takes into account their safety and technological features (Zielińska & Kolożyn-Krajewska, 2018). The significant factors for microorganisms to be considered as probiotics are that they should be microorganisms identified at strain level and alive during administration, possess natural antibiotic resistance genes, lack virulence factors, resistant to digestive enzymes, bile acids, gastric acidity, antimicrobial activity against pathogens, and the administered microbes should exhibit beneficial effects on the target host. In around 1900 Elie Metchnikoff came up with the idea of probiotics. Consumption of live bacteria in fermented milk and yoghurt (*Lactobacillus bulgaricus*) improved some biological characteristics of the gastrointestinal tract (de Simone, 2019). Probiotics are presently viewed as an excellent possibility to cooperate in functional foods. New bacterial strains are distinguished and added to nutrition and pharmaceutical products. The world market for probiotics was estimated at USD 36 million in 2013 (de Simone, 2019). In 2016 the value increased to USD 35.9 billion with the European continent contributing large with an annual growth rate of 20% (Zielińska & Kolożyn-Krajewska, 2018).

The probiotic preference may be due to extreme interest in probiotic yoghurt and developments in the utilisation of functional foods (Jose *et al*., 2015). Probiotics come in various formulations, including medicinal items such as tablets, and dairy products which include milk, cream, yoghurt, and cheese (Lugli *et al.*, 2019). The beneficial effects exhibited by probiotics include modulating the immune system, antitumor action, cholesterol and lactose intolerance reduction, and stool transplantation (Rigobelo & de Ávila, 2012). The recommended characteristics of probiotics include producing bacteriocins which are antimicrobial substances, adhering to human intestinal walls, colonising the intestine, and resistance to acid and bile salts to survive and grow in the intestines (Anadón *et al*., 2016). Some probiotic strains of non-human origin such as *Saccharomyces cerevisiae* var. *boulardii* and *Bifidobacterium animalis* subsp. *lactis* were tested (Zielińska & Kolożyn-Krajewska, 2018). A certain number of strains were isolated from Habin sausages and evaluated for *in vitro* probiotic properties. The strains included *L. fermentum, L. curvatus, L. brevis,* and *Pediococcus pentosaceus.* The strains were able to thrive in the human intestine and possessed antioxidant action (Han *et al*., 2017). Bengoa *et al*. (2018) stated that isolates from kefir grains which included *L. paracasei* were exposed to acid and bile tension and evaluated for *in vitro* adhesion. They observed that in the gastrointestinal passage, they increased adherence to epithelial and mucin cells.

The famous species of probiotics are considered safe for the human population by the European Food Safety Authority (Kothari *et al*., 2019). A scientific and technical guide was developed, which focuses on the clarification of using health claims. The reason for forming the technical guideline is for health claims to be authentic and authorised on the European level, and to guarantee that health claims depend on logical proof (Zielińska & Kołożyn-Krajewska, 2018). The probiotic bacteria found in the human gut can produce vitamins and supply them to the human body (Srinivasan & Buys, 2019). Vitamins are micronutrients required by living cells to perform biochemical reactions and are enzyme precursors (Gu & Li, 2016). The gut microbiota produces vitamin K, thiamine, pyridoxine, cobalamin, and folate. These vitamins are present in fermented foods in large quantities. Increased levels of pyridoxine, niacin, vitamin B12, folate, and riboflavin have resulted from LAB fermentation in cheese, yoghurt, and various fermented foods (Gu & Li, 2016).

The bovine isolates can grow in the gastrointestinal tract, while dairy isolates can grow in food processing conditions (Jose *et al*., 2015). Probiotics offer a wide range of health benefits, but consumers are now health-conscious. The health benefits of probiotics are both species-specific and strain-specific. Although the *Lactobacillus* and *Bifidobacterium* of food and dairy origin are mostly in use as probiotics, there is a need to keep on finding new probiotic strains with health benefits. This study is focusing on characterising potential probiotics isolated from bovine and dairy species and evaluating their technological suitability in yoghurt processing.

CHAPTER 2: LITERATURE REVIEW

2.1 THE DEFINITION AND HISTORY OF PROBIOTICS

The word "probiotic" when derived in Latin means "for life". The first individuals to put forward the scientific suggestion about the use of bacteria as probiotics were Metchnikoff (1903) and Tissier (1906). However, the concept of "probiotics" remained not widespread till 1960, to suggest "substances created by microorganisms which promote the growth of other microorganisms". Several authors brought up different suggestions for the definition of probiotics. Fuller (1989) stated that probiotics are "live microbial feed supplements that beneficially affect the host animal by improving its intestinal balance". Havenaar and Ten Brink (1992) defined them as "viable mono or mixed culture of bacteria which when applied to animals or man beneficially affects the host by improving the properties of the indigenous flora". The "Food and Agriculture Organization/ World Health Organization (FAO/WHO), Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria" (2001) defined probiotics "as live microorganisms which when administered in adequate amounts confer a health benefit on the host". In 2002 the International Life Science described probiotics as "a live microbial food ingredient that, when taken up in adequate amounts, confers health benefits on the consumers" and "live microorganisms which, when ingested in sufficient numbers, provide the consumer with one or more proven health benefits".

2.1.1 REGULATIONS FOR THE USE OF PROBIOTICS

Huys *et al*. (2013) suggested that the FAO/ WHO consultation only focused their idea of probiotic discussion as an inclusion of the food but left out the term biotherapeutics agents, and microorganisms which provide health benefits even when the source is not food. They are very critical considerations that were documented for probiotics to be used in foods. Besides being capable of thriving through the passage of the digestive tract, bile and acid-tolerant, withstanding digestive enzymes, ability to colonise the gut, probiotics must provide health benefits to the host by either growing or showing activity in the human body. Therefore, each strain should be verified for its potential not to lose viability and to be active on the target site (Huys *et al.*, 2013). A guideline which focuses on evaluating probiotics in food was generated by FAO/ WHO (2002). The above definition of probiotics put forward by FAO/ WHO and the guidelines were recommended to be officially adopted for referring a microbial strain as probiotic. For probiotics the minimum requirements are the evaluation of the identity of the strain (strain, species, genus level), *in vitro* test to ascertain probiotic characteristics for example bile and acid tolerance and antimicrobial activity, safety assessment to prove the probiotic strain in its delivery form is safe without contamination, and assessment of health effects in the target host through *in vivo* studies.

According to Huys *et al.* (2013), it should be considered that besides the identification of the strain, surviving in the gastrointestinal tract, adhering and colonising the lining of the epithelium, the ability of the strain to withstand industrial processing and storage is essential. This brings in the concept of the food matrix as well as the manufacturing conditions used to prepare the food and ability of the strain to remain viable until the consumption of the food. This gives an idea of the quantity of food to be taken for a suggested health outcome and provides an essential insight into a fair number of active cells, respectively. According to Laulund *et al*. (2017), a few concepts were analysed by the food and drug administration and grouped microorganisms in the criteria generally regarded as safe (GRAS). The ideas include safety aspects, taxonomy, antibiotic resistance, the ability to secrete pathogenicity toxins, and historical evidence of food safety.

There is a long history for the safety of *Lactobacillus* used in fermented foods. However, antibiotic resistance presents a threat to GRAS status, especially to *Lactobacillus* that have transferable mobile genes since guidelines to determine the type of resistance in microorganisms applied in food processing are not presented. However, on the other hand, the safety of *Lactobacillus* that have probiotic or starter culture potential is regulated by the European union commission, through European food safety authority that came up with procedures since 2003 to assign a qualified presumption of safety to the organisms (Laulund *et al*., 2017). The QPS status is founded on evidence that is realistic and competent to permit tight restrictions. It may be analogous to the generally regarded as safe status but containing more regulatory guidelines in verifying the safety of the bacteria. The EFSA BIOHAZARD Panel (Biological Hazards) gives the bacterium the QPS status. It must take into consideration the following aspects such as taxonomic identity regarding the genus level, scientific evidence supporting the safety of *Lactobacillus* based on historical use, and the strains must be evaluated if they have pathogenicity factors and the knowledge regarding how the bacterium is used as being part of the food chain (Fraqueza, 2015; Laulund *et al.*, 2017). According to Fraqueza (2015), the lactic acid bacteria species which have been assigned the QPS status are *Streptococcus thermophilus*, *Pediococcus pentosaceus*, *P. dextrinicus*, *P. acidilactici*, *Lactococcus lactis*, *Leuconostoc pseudomesenteroides*, *L. mesenteroides*, *L. lactis*, *L. citreum*, *L. curvatus*, *L. alimentarius*, *L. brevis*, *L. fermentum*, and *L. plantarum*.

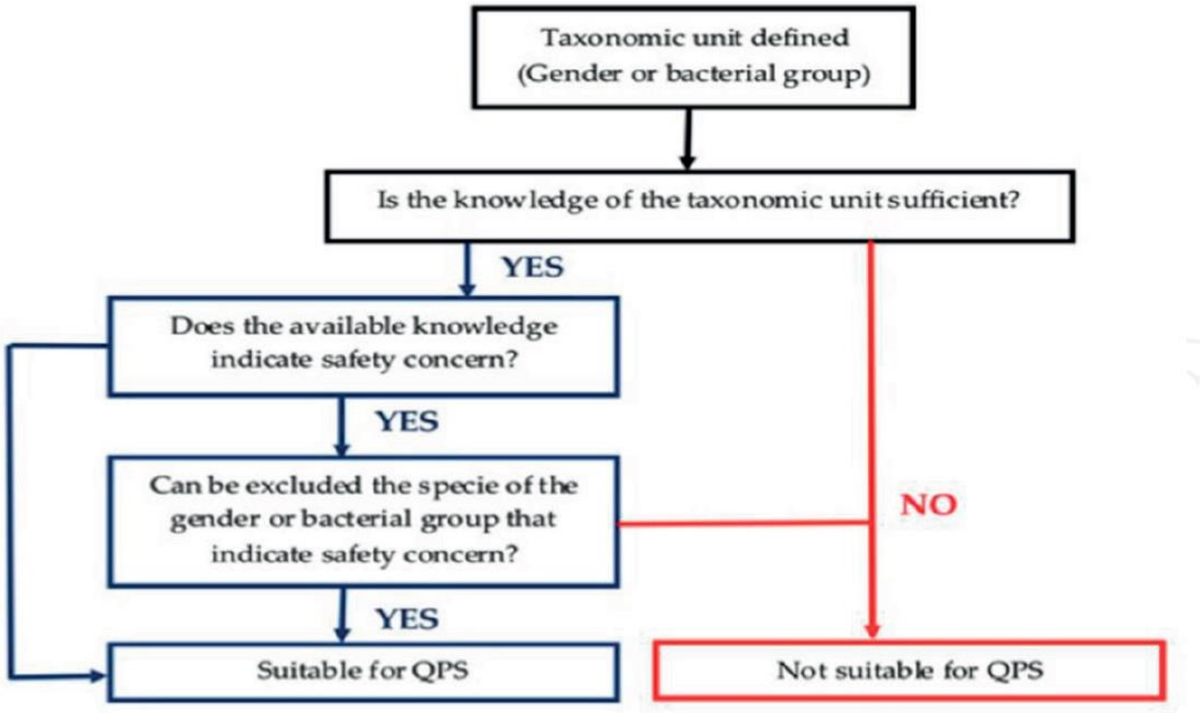

Figure 2.1: Guidelines used to assign qualified presumption safety status to lactic acid bacteria (Laulund *et al.*, 2017)

2.1.2 PROBIOTICS USED IN FOODS

Nowadays, most of the probiotics on the market are in the form of foodstuffs and drugs. The food industry has made use of species such as *Lactobacillus*, *Pediococcus*, and *Leuconostoc*, and consumption of foods containing these strains and their metabolites has taken place over a long period (Mayra-Makinen, 1998). According to Anadón *et al.* (2016), most of the widely used probiotics in human health are bifidobacteria, lactobacilli and some strains which are non-pathogenic, but mainly of human origin. However, other sources have been recorded, as shown in figure 2.1.

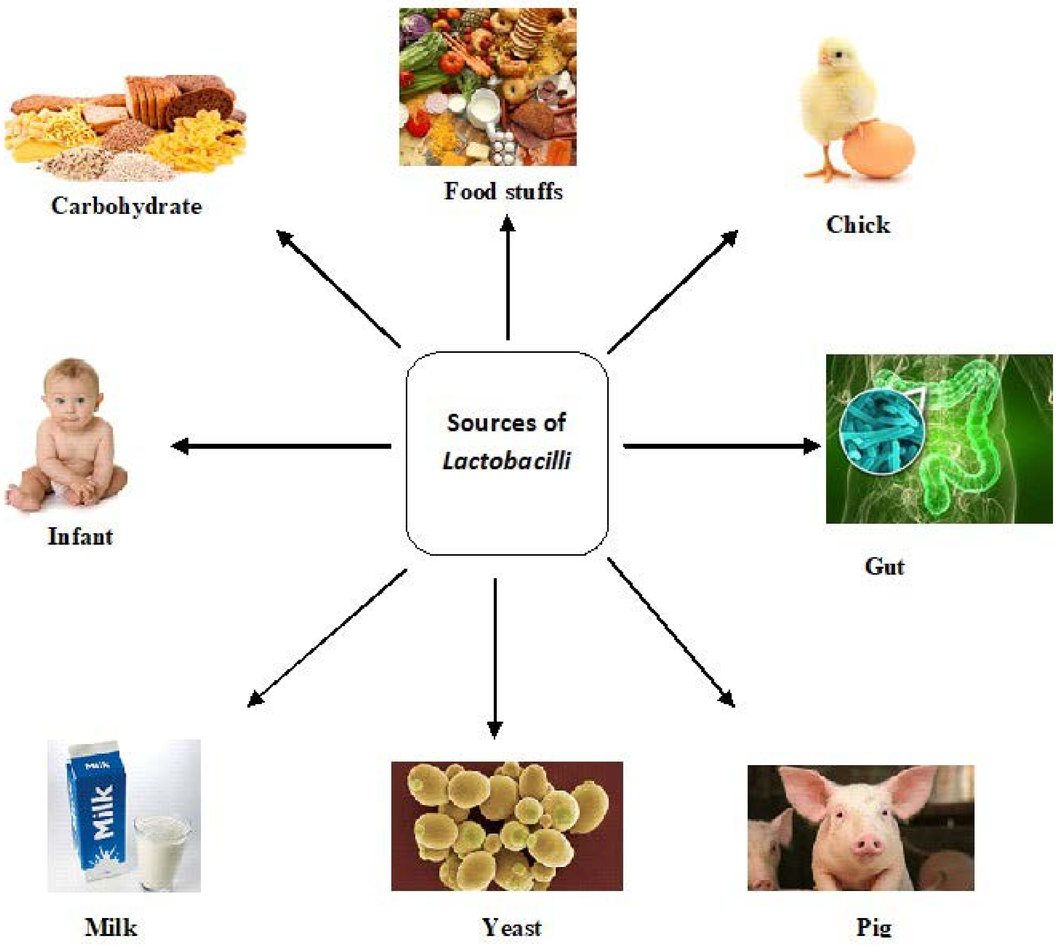

Figure 2.2: The different sources of lactic acid bacteria used as probiotics (Arshad *et al*., 2018)

The microorganisms, which include *L. rhamnosus*, *L. salivarius*, *L. reuteri*, *L. casei*, *L. acidophilus*, and *L. plantarum*, are mainly used as probiotics (Anadón *et al.*, 2016). Huys *et al.* (2013) suggested that *Lactobacillus* is an associate which belong to the class of lactic acid bacteria that are found on feed materials and raw food naturally. *Bifidobacterium* also produces lactic acid but is not considered as an associate of the lactic acid bacteria because of its remote phylogenetic position. Other members such as *Pediococcus*, *Lactococcus*, and *Enterococcus*, are to a lesser extent used as probiotics. Lactobacilli play a role in maintaining a healthy digestive system as well as keeping the gut microflora in balance. The main reasons for the use of lactobacilli are because the bacteria have competitive exclusion; they attach easily on the intestine epithelial cells and boost the immune system (Arshad *et al.*, 2018). Microorganisms used in humans as probiotics illustrated in Table 2.1 (Huys *et al.*, 2013).

Table 2.1: Microorganisms applied in humans as probiotics (Huys *et al.*, 2013)

Lactobacillus	*Bifidobacterium*
L. salivarius	*B. adolescents*
L. rhamnosus	*B. breve*
L. reuteri	*B. bifidum*
L. plantarum	*B. animalis* subsp. *lactis*
L. johnsonii	*B. longum* subsp. *longum*
L. gasseri	*B. longum* subsp. *Infantis*
L. gallinarium	*B. animalis* subsp. *lactis*
L. fermentum	
L. crispatus	
L. acidophilus	
L. casei/ paracasei	
Sporolactobacillus inulins	
Pediococcus	
P. pentosaseus	
P. acidilactici	
Oenococcus oeni	
L. mesenteroides subsp. *cremoris*	
Leuconostoc	
L. citreum	
Enterococcus faecium	
Lactococcus lactis subsp. *lactis*	

According to Martín Rosique *et al*. (2008), the antagonistic effect of lactobacilli has led them to be applied for the protection of the vaginal and intestinal mucosa. As early as 1892, Doderlein had already discovered the value possessed by lactobacilli in the human vaginal microbiota. The vaginal pH is around 4. It has an inhibitory effect on all microorganisms, which cause vaginal pathology. The vagina is protected against urogenital infections by this microflora. Hamid and Abulfazl (2012), explained the role of lactobacilli in inhibiting pathogenic microorganisms which include *Helicobacter, Shigella*, and *Salmonella.* Apart from that, there have been many health benefits linked to lactic acid bacteria such as lactose intolerance reduction, efficient immune response, and obscuring colon cancer. *Lactobacillus* has a beneficial role in acting as biotherapeutic agents in humans. (Abriouel *et al*., 2015). Figure 2.3 illustrates the biotherapeutic effect of lactobacilli (Arshad *et al*., 2018).

According to Sears *et al*. (2011), in this case, they prevent and treat conditions such as immune system stimulation, lactosemal absorption, prevention of diarrhoea, and cholesterol reduction. The use of *L. acidophilus* in milk has been encouraged for the treatment of constipation whereas *L. bulgaricus* has been found to produce anticarcinogenic substances (Alm, 1983; Cheplin & Rettger, 1920; Graf, 1983). There are three categories of lactobacilli anticarcinogenic features which are suppression of tumour cells, the destruction of carcinogens, and the inhibition of bacteria that have the responsibility of releasing carcinogens (Bogdonovi *et al*., 1962; Goldin & Gorbach, 1977; Reddy *et al*., 1973; Rowland & Grasso, 1975). The concentration of blood cholesterol was lowered in humans who were fed skimmed and fermented milk yoghurt (Grunewald, 1982; Mann, 1977; Nair & Mann, 1977) while the urogenital tract of females is protected by vaginal bifidobacteria or lactobacilli (Mccarthy *et al*., 1988; Morishita *et al*., 1981)

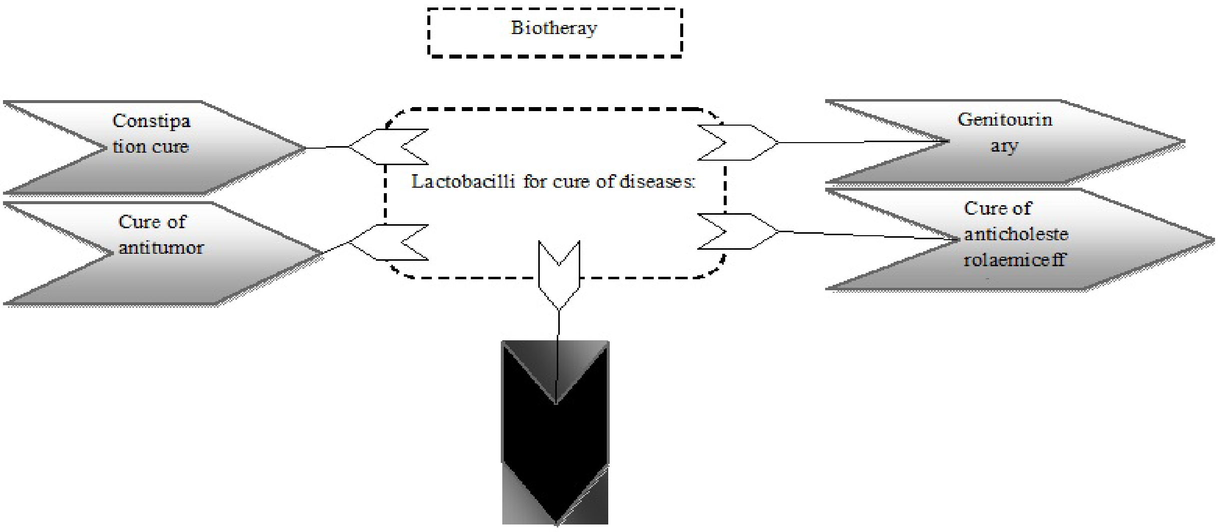

Figure 2.3: Bio-therapeutic effect of lactobacilli (Arshad *et al.*, 2018)

2.2 CLASSIFICATION AND TAXONOMY OF *LACTOBACILLUS*

According to De Angelis and Gobbetti (2016), the genus *Lactobacillus* is associated with the phylum Firmicutes. The species is followed by class Bacilli, followed by order II

Lactobacillales, and family Lactobacillaceae. According to Inglin *et al*. (2015), the genus has got nearly 180 species namely *L. amyylovorus*, *L. amylotrophicus*, *L. amylophilus*, *L. amylolyticus*, *L. alimentarius*, *L. algidus*, *L agilis*, *L. acidophilus*, *L. acidipiscis*, *L. acidifarinae*, and *L. acetotlerans*. Figure 2.4 illustrates the species found in the genus *Lactobacillus* and their sources (Arshad *et al*., 2018).

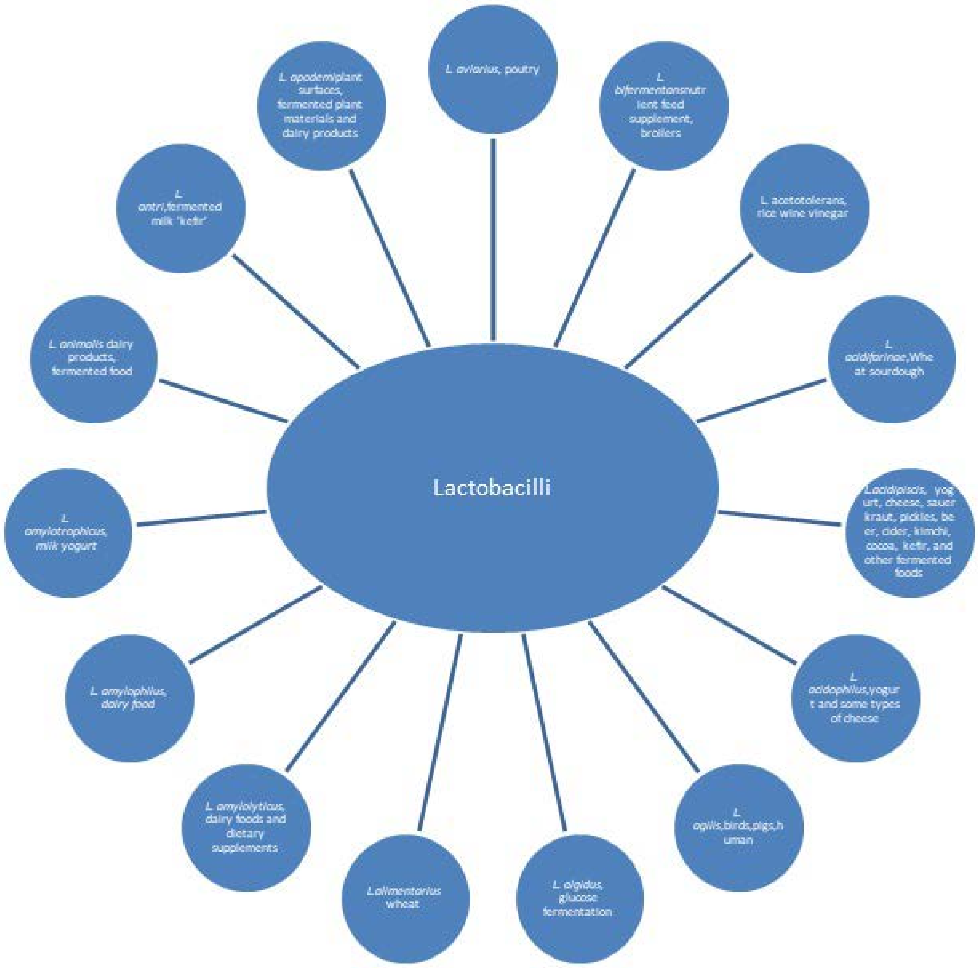

Figure 2.4: The genus *Lactobacillus* and their sources (Arshad *et al*., 2018)

The organisms are gram-positive, and their shape is in the form of rods. They do not produce spores and lack the enzyme catalase. The bacteria need a wide range of nutrients for their metabolism, which includes fatty acids, vitamins, nucleic acids, and carbohydrates (De Angelis & Gobbetti, 2016). According to Gopal (2011) in 1970, *L. acidophilus* was fully described by Hanset and Mocquot. They came up with a neotype strain (ATCC 4536) according to the guidelines of the International Subcommittee on Taxonomy of Lactobacilli and Closely Related Organisms. Six homologous groups were differentiated in 1980. The differentiation was based on DNA hybridization techniques, and the *L. acidophilus* was classified as B- 2, B- 1, A- 4, A- 3, A- 2, and A- 1. The strains in group A have 20- 30% homology, B- 2 and B- 1 have very low homology. Individual strains in different groups have got DNA- DNA homology values ranging from 20- 50%. This brought up *L. acidophilus* definition as (neotype strain ATCC 4536), *L. johnsonii, L. gasseri, and L. gallinarium.* The 16S rRNA sequences of lactobacilli first reported in 1991 facilitated coming up with the phylogenetic closeness of the different species. *L. acidophilus* is closer to *L. helveticus* phylogenetically. There is a slight relationship between physiologically or morphologically founded subdivision of lactobacilli and their phylogenetic closeness. It has been put forward that the genus *Lactobacillus* and related genera must be divided into three groups and *L. acidophilus* together with *L. delbrueckii* and the other obligately homofermentative lactobacilli are all in group 1. Microarray analyses have been done for *L. acidophilus* complex for inter- and intraspecies diversity was conducted and perfected with multilocus sequence analysis, comparative genomics and DNA typing. There has been a high similarity pattern among the *L. acidophilus* complex even though there are high different genomic G+C contents. This has been demonstrated by using modern whole-genome founded techniques.

De Angelis and Gobbetti (2016) highlighted that the genus *Lactobacillus* has got many bacteria which are characterised with a broad range of G + C ratios and few DNA- DNA homology among all their species. There is a close relationship between the genus and the genera *Leuconostoc* and *Pediococcus*. However, there is a broad distance relationship to *Carnobacterium, Streptococci*, and the aerobic *Bacillus* species. The genus is divided into three groups based on the carbohydrate fermentation pathways. The first group are obligate homofermentative lactobacilli that metabolise hexoses yielding lactic acid, though they do not act on pentoses or gluconate. The second group is facultative heterofermentative lactobacilli which metabolise hexoses almost to lactic acid or under conditions in which glucose is limited to lactic acid, formic acid, ethanol, and pentoses are metabolised to lactic and acetic acid. The third group are obligate heterofermentative lactobacilli which act upon hexoses yielding lactic acid, carbon dioxide, acetic acid, ethanol, and pentoses to lactic acid and acetic acid. Several lactobacilli have been reclassified. This is because of the examination of lactobacilli using methods based on genetics, which includes 16S rRNA sequencing and DNA- DNA homology. The lactobacilli species which are validly described up to now were investigated using 16S rRNA sequencing for their phylogenetic relatedness. This allowed allocation of *Lactobacillus* into the groups *L. salivarius, L. sakei*, *L. reuteri*, *L. plantarum, L. delbrueckii, L. casei, and L. buchnerii,* as shown in figure 2.5 (Hammes & Hertel, 2006).

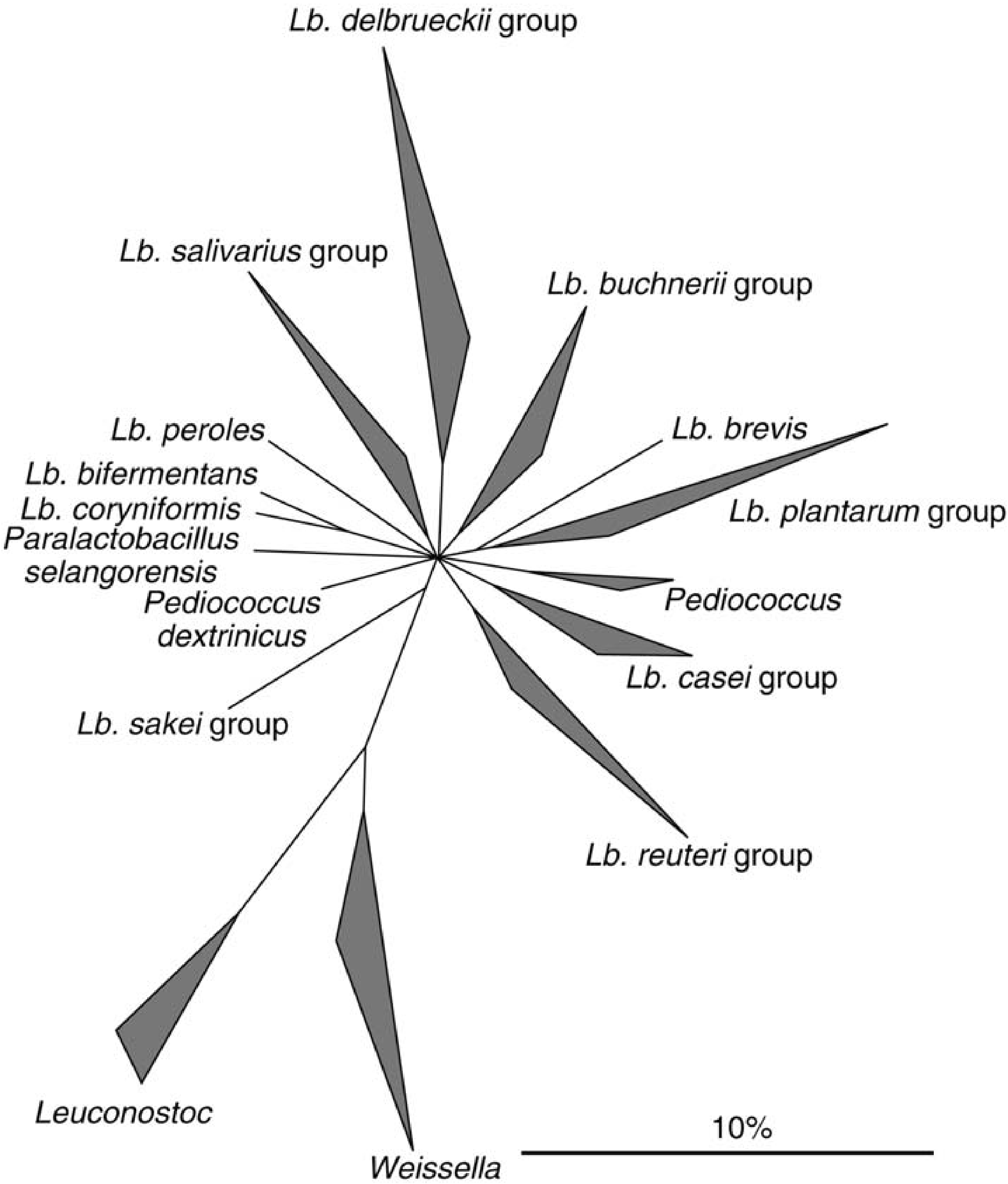

Figure 2.5: Phylogenetic tree showing the Lactobacillaceae family. The tree is a result of the analyses of at least 90% 16S rRNA sequences of the species (Hammes & Hertel, 2006)

Since the relationship among the groups was not successfully resolved ambiguously, the branching is shown through multifurcations which originate from one ancestor. However, *L. brevis* and *L. perolens* and the associated species *L. bifermentants* and *L. coryneformis* are distinctly positioned among lactobacilli. The G+C content contained in the species is widespread entirely among the subgroups. This event is explained by the changes encountered in codon convention stemming from the genetic code, which is degenerative. All the lactobacilli contained in the *L. buchneri* group are only obligately heterofermentative, except for the lactobacilli in the *L. homohiochii* are facultatively heterofermentative. As for lactobacilli in the *L. casei* group are obligately homofermentative and facultatively heterofermentative bacteria at the same time. The rest is allocated to the extremely related species *L. rhamnosus, L. paracasei, and L. casei*. There is still more controversy regarding their taxonomic status. The lactobacilli in the *L. delbrueckii* group are obligate homofermentative bacteria, and their G+C content is less than 40mol %. The *L. delbrueckii* has got three subspecies. These species are difficult to differentiate using rRNA sequencing analysis.

The *L. plantarum* group has got 12 *Lactobacillus* species, and the three forms of carbohydrate fermentation are present. Within the group, they are several species which showed very high 16S rRNA sequence resemblance which is *L. pentosus, L. plantarum, and L. paraplantarum* (99.7%- 99.9%), *L. kimchii* and *L. paralimentarius* (99.9%); and *L. mindensis* and *L. farciminis* (99.9%). However, there is a similarity in the G+C content of the species. The lactobacilli in the *L. reuteri* group are entirely obligate heterofermentative bacteria. The members within the group showed differences in their DNA composition (36- 45 mol%). The *L. durianis* and *L. vaccinostercus* showed 16S rRNA sequence similarity which was very high (99.7%) but showed differences in G+C content (36 and 43 mol%) when compared with other related *Lactobacillus* genus species. The difference in G+C content is unique. All the lactobacilli in the *L. sakei* group are facultatively heterofermentative, and the group is tiny. The *L. salivarius* group contains bacteria which are obligate homofermentative and facultative heterofermentative. Another 16S rRNA sequence similarity was shown by *L. animalis* and *Lb. murinus* (99.7%) including also *L. cypricasei and L. acidipiscis* (99.7%). There are two subspecies contained in *L. salivarius* group. The two subspecies could not be differentiated with rRNA sequence analysis.

2.2.1 ENUMERATION AND ISOLATION OF *LACTOBACILLUS*

The media which is used for the growth of lactobacilli must be able to meet their vast nutritional supplies and their need to favour microaerophilic conditions. De Man, Rogosa (MRS) agar is best suitable for enumerating lactobacilli non- selectively, though all-purpose tween (APT) agar is also appropriate to be used for the growth of lactobacilli. Anaerobic lactobacilli need a reduction in oxygen tension by the introduction of 0.05% cysteine. The agar plates are then anaerobically exposed to incubation for 48 hours at 30 °C to 37 °C. The aciduric nature of lactobacilli is essential for their selective enumeration and distinguishing them from other bacteria. Rogosa agar (SL or LBS agar) is the most suitable agar for selective enumeration of lactobacilli from dairy products. The pH of the medium should be regulated to about 5.3- 5.0. This is done for preventing the growth of *Streptococci*, *Enterococci*, and *Lactococci*. When the pH of MRS agar is brought to 5, lactobacilli can also be selectively enumerated. The plates containing both the media are incubated anaerobically at 37 °C for three days or at 30 °C for five days. However, all the media are not entirely selective because yeasts, *Pediococcus*, and *Leuconostoc* can grow at pH 5. A microscopic examination must be done to confirm if the colonies will be lactobacilli (De Angelis & Gobbetti, 2016).

According to Gopal (2011), selective media must be used for isolating *L. acidophilus* when it is a constituent of mixed genera of microorganisms. Rogosa acetate medium is predominately used for this purpose. The medium will allow other lactobacilli to grow, such as *Enterococci, Pediococci,* and *Bifidobacteria* as it is not entirely selective. The addition of a concentration of 100ug mL^{-1} of cycloheximide can contribute to the elimination of yeasts and moulds. The growth of *L. acidophilus* is assisted by supplementation with tomato juice, sodium acetate, and bile ox gall. When isolating *L. acidophilus* from the vagina, faces, and the mouth 0.05% (w/v) cysteine should be added to the medium. When subjected to anaerobic conditions, lactobacilli can grow better. The growth is more optimum under anaerobic conditions of 90% nitrogen and 10% carbon dioxide, whether in agar or broth. When the pH of MRS agar is adjusted to 5.4, *L. acidophilus* can be enumerated from yoghurts and various dairy products. The International Dairy Federation recommended the method as the best.

The growth of *S. thermophilus* is permitted under the conditions, but some lactobacilli available also grow in the medium if present in the product. When there is the introduction of probiotic lactobacilli to a referred dairy product, it is of vital importance to check for strain identification on the isolation medium. The identity of the strains can be confirmed by DNA based methods or carbohydrate fermentation patterns. To preserve the *L. acidophilus* cultures for short periods, they can be grown on MRS agar stab and stored at around 4 °C once growth is observed. For longer-term storage, freeze-drying can be used. Cultures grow, and they are put in sterile skim milk, then they are dried for storage under nitrogen. They can be stored for 10- 20 years in this form.

2.2.2 METHODS FOR IDENTIFICATION OF LACTOBACILLI

According to (Gopal, 2011), traditionally the classification of lactobacilli is carried out on phenotypic characteristics such as morphology, the growth at various temperatures, glucose fermentation, the configuration of lactic acid, and the form of proteins inside the cell wall or the entire cell (SDS- PAGE). The recent phenotypic identification incorporates studying huge fermentative profile (95 different carbon sources) using the Biolog system and spectroscopic methods such as matrix-assisted laser desorption ionization-time- of – flight mass spectrometry (MALDI- TOF- MS). However, there are some limitations experienced with phenotypic methods which include poor reproducibility caused by the elasticity of bacterial growth and the expression of genes that are affected by environmental factors. To identify lactobacilli efficiently genotypic methods are used, and the polyphasic approach, the simultaneous use of phenotypic and genotypic is considered the most efficient identification method (Gopal, 2011). Genotyping methods are applied at the clonal level to identify and differentiate the lactobacilli strains. The use of rRNA technology has been used for bacterial identification because of the availability of 16S and 23S rRNA sequence data set.

All *Lactobacillus* species have completed or partial 16S rRNA sequences. For the most reliable strain identification virtual analysis of the 16S rRNA sequences may be used. On the other hand, for the differentiation of closely related species, the 16S rRNA is too well conserved. Among the genus *Lactobacillus*, many species have a high similarity of 16S rRNA sequence which include *L. animalis* and *L. murinus* (99.7%), *L. mindensis* and *L. farciminis* (99.9%), *L. kimchii* and *L. paralimentarius* (99.9%) and *L. plantarum*, *L. pentosus*, and *L. paraplantarum* (99.7–99.9%). Considering the information above a reliable way to find more insight into the taxonomic location of an unknown *Lactobacillus* isolate, virtual analysis of partial 16S rRNA sequences (roughly the first 900 bases) must be done. Virtual analysis of 16S rRNA and 23S rRNA sequences has revealed evolutionary few preserved sections that have a diagnostic value for species, genus, or group organisms which are phylogenetically related. In fluorescent *in situ* hybridization (FISH) where exact rRNA sequences are sensed within morphologically intact cells, specific rRNA probes were already in use. Cultivation- dependent techniques are used for intraspecific and genotyping lactobacilli which are isolated from many foods which are fermented and from the human gastrointestinal tract. The methods include DNA fingerprinting techniques like pulsed-field gel electrolysis (PFGE) of rare- cutting restriction enzyme fragments, ribotyping, randomly amplified polymorphic DNA (RAPD), and fragment length polymorphism (AFLP). For the culture-independent analysis of the variety of multifaceted microbial communities in dairy products, molecular biological methods have been developed. Figure 2.6 shows the molecular approaches applied singularly or simultaneously to analyze the microbial communities in cheese (Randazzo *et al*., 2009).

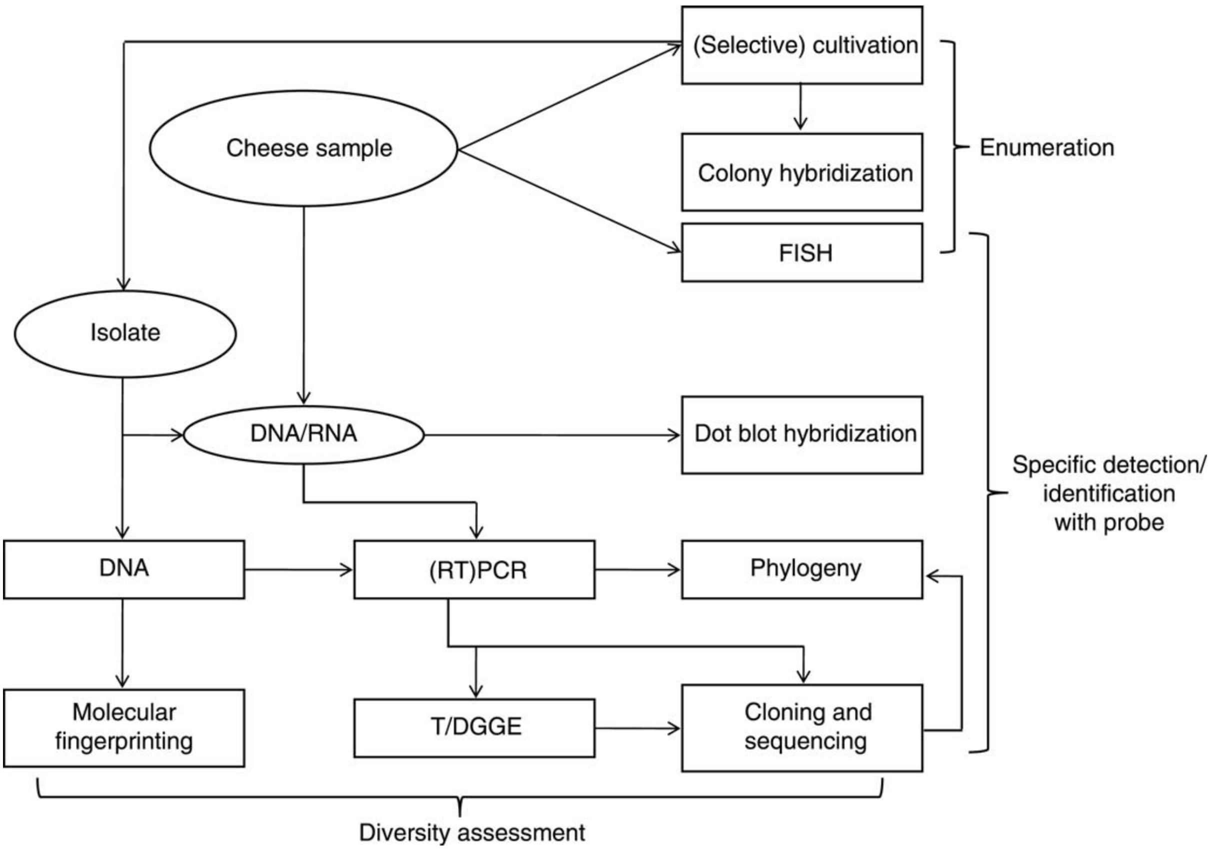

Figure 2.6: Flow chart showing molecular approaches for analysing of microbial communities present in cheese (Randazzo *et al*., 2009).

There is the extraction of total DNA or rRNA from the cheese samples, and the amplification of the DNA or rRNA fragments with the use of universal primers. Denaturing gradient gel electrophoresis (DDGE) or temporal temperature gradient electrophoresis (TTGE) is used to separate PCR amplicons which have the same size but different sequences. Real-time (RT) PCR which is then followed by denaturing gradient gel electrophoresis (DDGE) of the 16S rRNA gene is used to evaluate species which are metabolically active during the manufacturing of cheese. Terminal- restriction fragment length polymorphism (T- RFLP) and single-strand conformation polymorphism (SSCP) analyses are founded on exact target positions for restriction enzymes and single-stranded DNA secondary structure. These techniques are not affected by the physiological form of the cells, and during cell growth, they remain constant. Hence, they have revolutionized the detection of DNA/ RNA in microbiological ecological studies. The choice of the most comprehensive method of typing depends on the cost, capacity of throughput, and fingerprints producibility.

2.3 GROWTH CONDITIONS AND MORPHOLOGY

2.3.1 MICROSCOPIC MORPHOLOGY

Morphologically *L. acidophilus* appears as gram-positive rods which do not form spores and have round ends which measure approximately 0.6–0.9 ×1.5–6 µm. They appear in pairs, single or short chains (Gopal, 2011).

2.3.2 CULTURE MORPHOLOGY

The colonies which appear on agar are usually (2- 5mm). They have well distinct margins, glistening, smooth, convex, and opaque lacking pigments (Gopal, 2011).

2.3.3 GROWTH CONDITIONS

Optimum growth of *Lactobacillus* is witnessed at a temperature of 45 °C, in a media which is slightly acidic at a pH range of about 6.4- 4.5. Even though some strains are impartially aerotolerant, growth is mostly optimum when conditions are anaerobic. The members belonging to *L. acidophilus* are used to grow on organic substrates. In other words, they are fastidious organisms. They do not need carbohydrates as the only source of carbon, but vitamins, nucleotides, and amino acids are also required. Riboflavin, niacin, folic acid, and calcium pantothenate are essential. Vitamin B_{12}, thymidine, thiamine, and pyridoxal are not necessary (Gopal, 2011).

2.3.4 GENERAL CHARACTERISTICS

The species in *L. acidophilus* are mostly obligately homofermentative lactobacilli, but few of them are facultative heterofermenters. Since they have homofermentative metabolism and can grow above 45°C, they were initially grouped as lactic acid thermobacteria. 32- 37mol% is DNA G+C content. The organisms show a gram-positive profile. The cell wall appeared as thin sections displayed by electron micrographs. Lys- D- Asp is the cross-linkage that is present in the peptidoglycan cell wall (Gopal, 2011).

2.5 PROBIOTIC CHARACTERIZATION AND PROPERTIES

2.5.1 BOVINE AND DAIRY PROBIOTIC BACTERIA

The screening for potential probiotic characteristics for lactobacilli obtained from various ecological niches has been carried out systematically. Ideal bovine pH Is around 6-7. In the bovine digestive system, the bovine isolates can thrive because they are exposed to a wide range of stressful conditions (Jose *et al.*, 2015). Bovine isolates are subjected to encounter antibiotics as they are used in preventing and curing infections in animals. A typical example is a condition called postpartum metritis, which is observed in cows. When treating this condition, antibiotics have not been effective, and traces were found in milk (Abe *et al*., 1995; Otero *et al*., 2006). This means that bovine isolates are exposed to antibiotics most of the times and possession of non- transferrable resistance will aid in *vivo* survival. Bovine isolates have been reported to inhibit *Listeria monocytogenes* and *S. aureus,* which are gram-positive bacteria more than dairy isolates (Jose *et al.*, 2015). This commonly is not expected as the bovine isolates will probably co-exist together with the pathogens or must strive *in vivo* in cases of infection by the pathogens of the host environment. Dairy isolates have been reported to have poor adherence properties when compared to bovine isolates (Jose *et al.*, 2015).

2.5.2 ACID TOLERANCE

Probiotics should be able to thrive when subjected to the gastric environment for them to access the small intestine, colonise the host and impart health benefits. *Lactobacillus* species have been considered to be inherently acid resistant (Tannock, 2004). Even though changes might be encountered among species and strains, the organisms show higher sensitivity when subjected to pH values less than 3 (Jin *et al*., 1998; Rönkä *et al*., 2003). Henceforth, tolerance to acidity is considered when selecting potential probiotic strains. A constant gradient that exists between cytoplasmic and extracellular pH attributes to the acid tolerance of lactobacilli. The maximum value is attained by the internal pH cells because of inhibition of cellular functions (Kashket, 1987). According to Cotter and Hill (2003), gram-positive organisms have a mechanism that protects them against acidic conditions called F_0F_1-ATPase. The F_0F_1-ATPase has a multiple- subunit of enzymes which consist of (F_1) which is a catalytic portion with incorporated subunits α, β, γ, δ, and ε for the metabolism of ATP and an essential membrane portion (F_0) with the a, b, and c subunits that act as a membranous channel for proton translocation (Sebald *et al*., 2012). The function of the F_0F_1-ATPase within organisms that do not have a respiratory chain is to create a proton motive force, through proton exclusion. The F_0F_1-ATPase is significantly thought to upsurge the intercellular pH against a low extracellular pH. The regulation occurs at a transcriptional level where F_0F_1-ATPase is brought at a low pH (Fortier *et al*., 2003). The improved survival chances of probiotic lactobacilli when subjected to acidic conditions when glucose is present have been previously reported, though the mechanisms were not fully established (Charalampopoulos *et al*., 2003). Also, there have been reports which illustrate the capability of lactic acid bacteria to metabolize glucose at low pH, even though at lower rates (Hong *et al*., 1999; Venkatesh *et al*., 1993).

2.5.3 BILE SALT TOLERANCE

Bile is a yellow-green aqueous solution made up of molecules such as cholesterol, phospholipids, biliverdin, and bile acids (Begley *et al*., 2006; Carey & Duane, 1994). Bile is synthesized inside the liver by hepatocytes and transported to the gall bladder for storage. In the gall bladder, it is concentrated and later released inside the duodenum (Begley *et al*., 2006). Bile functions in fat digestion emulsifying and making lipids soluble. According to Begley *et al*. (2005), this detergent property of bile contributes to its antagonism activity which destroys bacterial membranes. Bile which is secreted in the small intestines has the capacity of reducing the existence of bacteria by disrupting the cell membranes, which are made up of fatty acids and lipids, these alterations do not only affect the viability and cell permeability, but also interaction that is observed between the probiotic cells and the environment (Succi *et al*., 2005). Kurdi *et al*. (2006) stated that the lipophilic nature possessed by the steroid ring makes the cell membranes to be targeted by these molecules.

The molecules disintegrate the way the lipid layers are arranged and the proton motive force resulting in cell inhibition and death. According to Sánchez *et al*. (2013), unconjugated forms can diffuse inside cells since they are weak acids in nature. Upon reaching the cellular environment, they dissociate and cause the cytoplasm to be more acidic. Begley *et al.* (2005) highlighted that the other harmful effects of bile acids include misfolding of proteins and alterations in the metabolism of sugar. Resistance to bile salts is a well-thought-out standard of selecting strains with probiotic potential (Boke *et al*., 2010). 0.15- 0.3% is the typical concentration of bile salts which has been approved for selecting probiotic strains which can be used in humans (Begley *et al.*, 2006). A concentration gradient of bile salts which ranges from 40 mM and 1mM, which is about a percentage range of 2% and 0.05% is kept in the human intestine. Characterisation of the microbial community present in the gut is achieved by these bile salts (Islam *et al*., 2011). Bile salt tolerance is a vital characteristic of probiotic bacteria. This is because it affects the probability of survival when subjected to the small intestine and the extent to which the probiotic roles can be performed. Lactobacilli can adapt when exposed to bile salts, even though inherent bile tolerance is strain-dependent (Begley *et al.*, 2006).

2.5.3.1 MECHANISMS OF BILE SALT TOLERANCE

The most common mechanisms for bile resistance in bacteria are bile salt hydrolysis, compositional variations in the cell wall, and active efflux of bile salts as shown in figure 2.7 (Ruiz *et al*., 2013). When bile salts and acids accumulate in the cytoplasm, their toxicity is counteracted by efflux pumps (Piddock, 2006). Several multidrug transporters (MDRs) are part of the ATP- binding cassette that facilitate bile tolerance in bifidobacteria and lactobacilli strains. There is a total of four carriers in *L. acidophilus* NCFM LBA1679, LBA1446, LBA 0552, LBA 1429 (Pfeiler & Klaenhammer, 2009), two are found in *B. lomgum, BL0920 and* *ctr* (Gueimonde *et al*., 2009; Price *et al*., 2006), one in *L. reuteri* ATCC 55730, *lr1584*, and one are found in *B. breve*, *Bbr_0838* (Ruiz *et al*., 2012). Indeed, any fault in LBA1446, LBA1679, LBA0552, and LBA1429 in the strain *L. acidophilus* NCFM will more likely increase sensitivity in mutant strains to certain antibiotics and bile (Pfeiler & Klaenhammer, 2009). In *L. johnsonii* a functional taurocholic and cholic acid antiporter belonging to the leading CbsT2 superfamily facilitator has been identified through here is no functional genetic means that has contributed for its role in bile tolerance (Elkins & Savage, 2003). The removal of both conjugated and free bile acids has been found in *L. reuteri,* and ATP is the driving force behind the removal activity (Bustos *et al*., 2011).

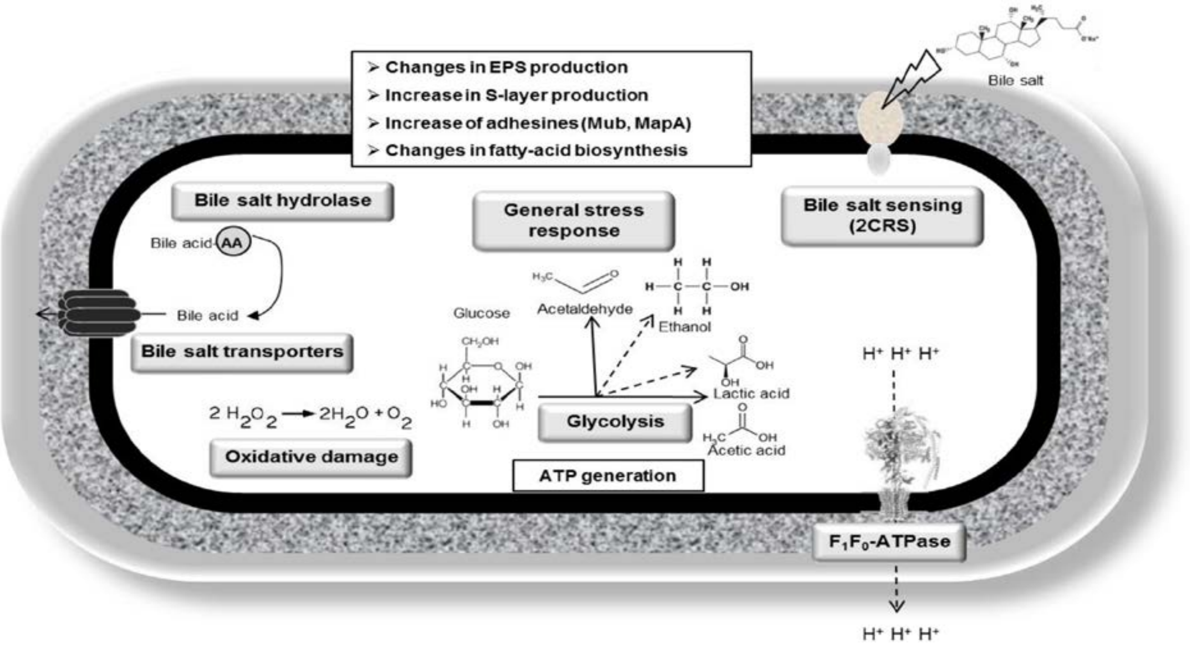

Figure 2.7: Mechanisms for bile resistance characterised and identified in lactobacilli (Ruiz *et al.*, 2013)

2.5.4 BILE SALT HYDROLASE ACTIVITY

In the gut, passive diffusion absorbs bile acids both conjugated and unconjugated, for them to be incorporated in the terminal ileum active transport is employed (Carey & Duane, 1994). The bile acids are reabsorbed, and they go in the portal bloodstream for uptake by hepatocytes where they are conjugated and secreted as bile. The intestinal bacteria modify 5% of the total bile acid concentration, that is 0.3g to 0.6g because it eludes epithelial absorption. Before further modifications are possible, deconjugation is the most critical transformation that must occur (Batta *et al*., 1990). Bile salt hydrolase enzymes facilitate deconjugation by hydrolysing the amide bond from the steroid core liberating glycine/ taurine moiety. Unconjugated or deconjugated bile acids are the end products. Bile salt hydrolase activity was identified as possibly hazardous to the human host and no sufficient evidence to ascertain if BSH activity is an essential property in probiotics (Begley *et al.*, 2006). Oral intake of beverages or pharmaceutical preparations containing probiotic strains may significantly decrease levels of cholesterol (Begley *et al*., 2006).

A wild *Lactobacillus* strain contained in fermented milk was found to reduce levels of cholesterol in humans (Mann & Spoerry, 1974). After that, more experiments were carried out for the investigation of the cholesterol-lowering effect of *Lactobacillus* (Öner *et al*., 2014; Pan *et al*., 2011a; Wang *et al*., 2012). In an assessment done by Pereira and Gibson (2002) on how probiotics aid on cholesterol reduction, it was concluded that consumption of dairy foods which have been fermented with suitable strains of bacteria would reduce levels of cholesterol that will be circulating in the bloodstream. Nevertheless, the strains contained in dairy products which are fermented are not found in the human intestinal tract (Pereira & Gibson, 2002). Thus, a dietary approach for making long term reduced cholesterol effects is the daily intake of dairy products. Lactobacilli that have bile salt hydrolase activity can colonise and survive when subjected to the lower small intestines in which the enterohepatic cycle is executed. Therefore, for selecting probiotic strains which have cholesterol-lowering properties an essential criterion to consider is bile salt hydrolase activity since specific probiotic bifidobacteria and lactobacilli can enzymatically deconjugate bile acids (Tahri *et al*., 1997). Sanders (1999) stated that the rate of bile acid excretion and reduction in levels of serum cholesterol could be increased by bile salt hydrolase activity when colonic microbes use the mechanism.

2.5.5 ANTAGONISTIC ACTIVITY OF LACTIC ACID BACTERIA

The antimicrobial activity which is exhibited by *Lactobacillus* against pathogens and spoilage bacteria is a probiotic trait for maintaining the gut microflora in balance and eliminating pathogens in the gut (Zommiti *et al.*, 2018). Secretion of non- specific antimicrobial substances consists of the antimicrobial properties of probiotics. The antimicrobial substances include hydrogen peroxide, low molecular weight bacteriocins, and short-chain fatty acids (Cruz‐Guerrero *et al*., 2014). A varied range of antimicrobial substances are known to be produced by *Lactobacillus* and bacteriocin is widely inspected (Schnürer & Magnusson, 2005). Secretion of organic acids, that bring down the pH, thereby inhibiting the growth of other bacteria by creating a hostile environment is a mechanism used by *Lactobacillus* to inhibit pathogen growth. The organic acids prove to be toxic to other bacteria, thereby reducing competitive inhibition between probiotic bacteria and the pathogens for mucosal binding sites (Jose *et al.*, 2015). Chen *et al*. (2019) stated that several studies had highlighted many antimicrobial mechanisms shown by *Lactobacillus* such as the release of inhibitory compounds, nutrient competition, competition for binding sites, and immune stimulation. Additionally, *Lactobacillus* can produce formic acid, acetic acid, lactic acid that bring down the intestinal pH, which is the vital mechanism. Georgieva *et al*. (2015) further explained that using such mechanisms has enabled *Lactobacillus* to inhibit numerous bacterial pathogens which include *Shigella spp, Pseudomonas aeruginosa, Streptococcus mutants, Clostridium difficile,* and *Staphylococcus aureus*.

2.5.6 AUTO AGGREGATION AND COAGGREGATION ASSAY

An important mechanism which aids to colonisation is aggregation. Co- aggregation, involves genetically discrete bacteria attaching with the help of specific molecules (Rickard *et al*., 2003). Auto- aggregation means clumping of cells belonging to the similar species (Schembri *et al*., 2001). Probiotics should effectively aggregate on intestinal mucosa for them to provide health benefits. The adhesion capacity, which is the attachment of microorganisms to the intestinal epithelium has to do with autoaggregation. This is an essential case for their integration into the intestinal microbiota. (da Silva *et al*., 2019). Therefore, the autoaggregation capacity can be considered and be used as a parameter when selecting strains for probiotic potential (Xu *et al*., 2010). Autoaggregation is a vital bacterial property in numerous ecological niches, particularly in the mucosa for humans and animals because this is where probiotics demonstrate functional activities (Pessoa *et al*., 2017). Bouchard *et al*. (2015) stated that lactobacilli auto-aggregation capacity ranges from low to moderate. Probiotics have been shown to have the ability to co- aggregate with pathogenic microorganisms. During the process, they secrete antimicrobial substances which directly attack the pathogenic bacteria inhibiting their growth and finally killing them (Bao *et al*., 2010). Ocaña *et al*. (1999) stated that some uropathogens such as *E. coli* could aggregate with lactobacilli. This process, when combined with the release of antimicrobial substances that include bacteriocin, hydrogen and lactic acid can result in growth inhibition of the pathogens. Ekmekci *et al*. (2009) proved that the capacity to coaggregate with *E. coli* provides a defence mechanism against urogenital infections. Arief *et al*. (2015) highlighted that the extent to which probiotics co- aggregate against gram-positive bacteria is greatly influenced by similar cell wall orientation. They both possess a peptidoglycan layer, and the interaction is further strengthened by their hydrophobic nature.

2.6 SAFETY ASSESSMENT OF LACTIC ACID BACTERIA

2.6.1 ANTIBIOTIC RESISTANCE

Even though *Lactobacillus* are applied in fermented foods and are generally regarded as safe, roughly a quite number have shown inherent or acquired antibiotic resistance. Therefore, it is vital to screen out antibiotic resistance of LAB in various fermented foods (Clementi & Aquilanti, 2011; Pan *et al*., 2011b). Lactic acid bacteria carry antibiotic resistance determinants. This resistance is passed via the food chain to humans (Rodriguez ‐ Alonso *et al*., 2009). Entirely several strains are not pathogenic; they can create a reservoir of genes conferring resistance to antibiotics which are transferrable to pathogens. Antibiotic resistance is shared among several lactic acid bacteria. The resistance determinants are inherent and non-transmissible (Zhou *et al*., 2005). Nevertheless, some strains, such as *L. reuteri, L. plantarum*, and *L. fermentum,* have antibiotic resistance genes that are plasmid-encoded and can be transferrable (Fons *et al*., 1997). The EFSA in conjunction with the "panel of additives and products or substance used in animal feed (FEEDAP)" came up with a procedure for identifying bacterial resistance that is acquired to chloramphenicol, tetracycline, clindamycin, erythromycin, gentamicin, streptomycin, kanamycin, and ampicillin (Álvarez-Cisneros & Ponce-Alquicira, 2018).

The lactobacilli that show acquired resistance when included in the food chain are facultative heterofermentative (*L.paracasei*, *L. rhamnosus*, and *L. plantarum*), obligate heterofermentative (*L. fermentum* and *L. reuteri*), obligate heterofermentative (*L. delbrueckii*, *L. acidophilus*, and *L. helveticus*), *Leuconostoc* spp., *L. lactis*, *Pediococcus* spp., and *S. thermophilus* (Álvarez-Cisneros & Ponce-Alquicira, 2018; Gueimonde *et al*., 2013). *Lactobacillus* can be added to food products as a starter or probiotic cultures, or they can be found in fermented foods as part of their natural microbiota, but most of them are resistant to antibiotics. Table 2.2 show *Lactobacillus* isolated from traditional fermented foods that have acquired resistance (Brown-

Jaque *et al*., 2015; Ma *et al*., 2014; Vaningelgem *et al*., 2004; Wacher‐Rodarte *et al*., 2016) The strains which are isolated from traditional fermented foods have acquired resistance (Álvarez-Cisneros & Ponce-Alquicira, 2018). The *Lactobacillus* and *Enterococci* genera possess a potential health risk since they are found in food, humans and animals (Imperial & Ibana, 2016; Mazurkiewicz *et al*., 2005)

Table 2.2: Antibiotic-resistant lactic acid bacteria isolated from food (Álvarez-Cisneros & Ponce-Alquicira, 2018)

Lactic acid bacteria	Antibiotic resistance*	Food
Lactobacillus sakei *L. curvatus* *L. mesenteroides*	Vancomyicn, gentamicin, ampicillin, erythromycin, and tetracycline	Chorizo, fuet, and sausages
L. sakei *Pediococcus pentosaceus* *L. plantarum* *L. paraplantatum*	Streptomycin, gentamicin, and tetracycline	Italian sausage
L. sakei *L. plantarum*	Chloramphenicol, quinuprisitin-dalfopristin, lincomycin, erythromycin (*ermA*, *ermB*, *ermC*), rifampicin, tetracyclin (*tetM*, *tetO*, *tetS*, *tetW*, *tetK*, and *tetL* genes), gentamicin, vancomycin and penicillin	Traditional dry fermented sausages from the south Portugal
Enterococcus faecium *E. faecalis* *L. reuteri* *L. delbrueckii subsp. bulgaricus* *L. johnsonii* *L. plantarum*	Gentamicin, tetracycline (*tetM*), clindamycin, vancomycin (*VanA*), chloramphenicol (*cat gen*), ciprofloxacin, penicillin, and nitrofurantoin	Sausges

Lactococcus lactis K214	Chloramphenicol, streptomycin, and tetracycline (*tetS* and *tetM*), erythromycin (*ermT*)	Raw milk cheese
S. thermophilus	tetracycline (*tetS*), erythromycin (ermB), clindamycin, streptomycin, and neomycin	Raw milk
L. pentosus *L. pseudomesenteroides*	Amoxicillin, ampicillin, chloramphenicol, and gentamicin	Green olives
Bifidobacterium spp. *Lactobacillus spp.* *S. thermophilus*	Vancomycin, streptomycin, aztreonamine, gentamicin, ciprofloxacin, and gentamicin	Commercial probiotics

Strains that harbour plasmids for antibiotic resistance are regarded as inappropriate to be used as probiotics in humans or animals (Marteau & Salminen, 1997; Morelli & Wright, 1997; Saarela *et al*., 2000). Zhou *et al.* (2005) highlighted that patients who have unbalanced or significantly reduced microbiota as a result of administration of different microbial agents could benefit from intrinsically antibiotic-resistant probiotic strains. Lactobacilli are highly naturally resistant to vancomycin, streptomycin, ofloxacin, nitrofurantoin, metronidazole, gentamicin, kanamycin, fusidic acid, ciprofloxacin, and bacitracin (Danielsen & Wind, 2003). Since homofermentative lactobacilli are vancomycin sensitive, several strains of *L. salivarius, L. acidophilus, L. casei*, and *L. plantarum* are naturally resistant to this drug which is contributed by D-alanine ligase-related group of enzymes (Elisha & Courvalin, 1995). In a comparable study, an incidence of lactobacilli resistance to ciprofloxacin (26%), gentamicin (48%), tetracycline (43%), erythromycin (10.8%), and vancomycin (58%) was investigated in Turkish fermented dairy products (Erginkaya *et al*., 2018). Although there is evidence that confirms resistant determinants transmission, famous resistance genes in lactobacilli are tetracycline [tet(M)], erythromycin [erm(B)] and *cat* genes that encode for resistance to chloramphenicol (Moračanin *et al*., 2017).

2.6.1.1 MECHANISMS OF RESISTANCE IN LACTIC ACID BACTERIA

The mechanisms used by *Lactobacillus* to resist antibiotics are modification by enzymes, degradation by enzymes and efflux pumps operated by enzymes (Mazurkiewicz *et al.*, 2005).

There must be two elements available for the antibiotic- target interaction, the antibiotic must first identify the target and the amount of the antibiotic in the target site should be enough for inhibiting the growth of bacteria. A resistance mechanism aids to the failure of the antibiotics to stop bacterial growth due to insufficient antibiotic- target interaction which is classified as passive or active. There is transfer of passive mechanism to other cells by clonal transfer which involves alterations on the target site or a decrease in absorption of antimicrobials. The structure of the antibiotic is not affected, and this resistance is called inherent resistance. In the active mechanism, there is the reduction in the amount of intracellular antibiotic by alteration

of its structure with enzymes or with the performance of efflux pumps (Martínez & Baquero, 2014; Munita & Arias, 2016). Multidrug-resistant (MDR) efflux pumps which remove physically different compounds is the mechanism used by *Lactobacillus* to resist antibiotics (Gueimonde *et al.*, 2013; Mazurkiewicz *et al.*, 2005). *Lactobacillus* from pozol were analysed, and it was identified that strains such as *L. plantarum* and *L. lactis* have active flux pumps including the ABC type that is chromosomally encoded with LmrA transporter (*lmr*A gene) (Wacher‐Rodarte *et al.*, 2016). Poelarends *et al*. (2002) highlighted that the innate resistance of *L. lactis* to tetracyclines, quinolones (ciprofloxacin), macrolides (erythromycin), lincosamides (clindamycin), and aminoglycosides (kanamycin and gentamicin) is responsible for the presence of LmrA transporter. *L. pseudomesenteroides* and *L. pentosus* were resistant to kanamycin, cephalosporins, and streptomycin. These strains were isolated from fermented olives. This is because of the variance of the permeability of the cell wall as the main popular resistance mechanism. Both the strains have AcrAB- TolC system which is complex and participates in MDR efflux pumps for tetracycline, chloramphenicol, fluoroquinolones and β-lactams and other genes involved with *nor*A and *Mde* superfamily pumps which are encoded chromosomally to deliver fluoroquinolones and chloramphenicol resistance (Muñoz *et al*., 2014).

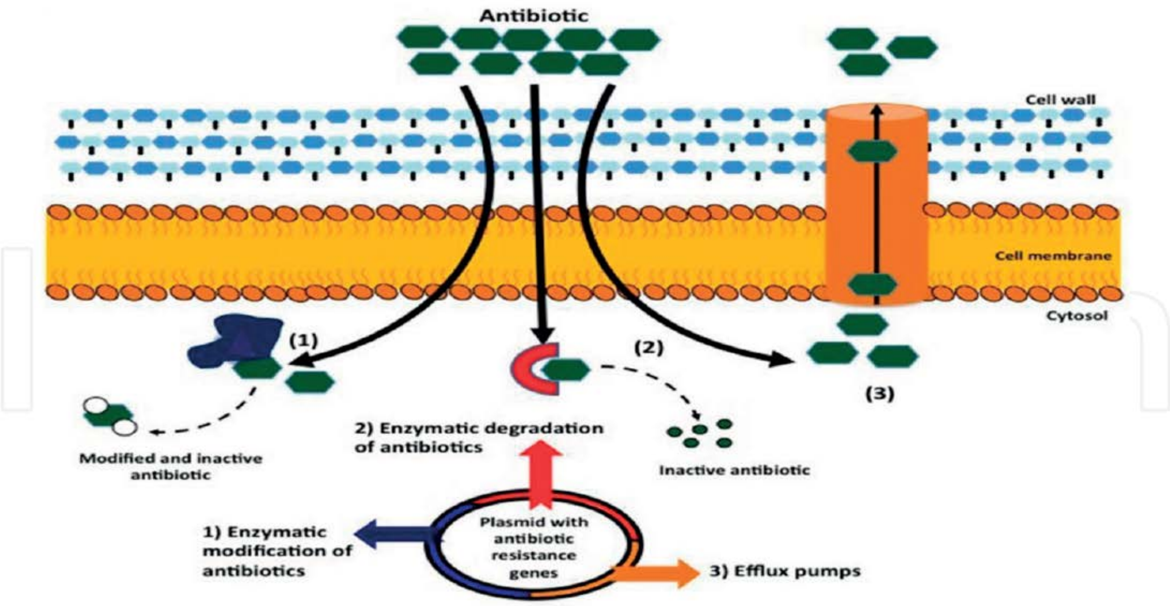

Figure 2.8: The mechanisms used by lactic acid bacteria to resist antibiotics, (1) modification by enzymes, (2) degradation by enzymes, and (3) efflux pumps (Mazurkiewicz *et al.*, 2005)

There are no reports of *Lactobacillus* resistance to aminoglycosides although resistance to streptomycin, kanamycin, and gentamicin have been shown by *Lactobacillus* isolated from farm origin. The mechanism of resistance is a result of reduced transport or inactivation of enzyme which modifies aminoglycosides (AMEs) as N- acetyltransferase (AACs), O-nucleotidyltransferase (ANTs) and O- phosphotransferase (APHs) encoded by transposons and insertion sequences which are mobile genetic elements (Jaimee & Halami, 2016). According to Álvarez-Cisneros and Ponce-Alquicira (2018), the bacterium species found in the genera *Bifidobacterium, Pediococcus, Lactobacillus*, and *Enterococcus* have both extrinsic or intrinsic resistance which is of concern as they can spread the resistance by horizontal transfer (between genera of bacteria) and or vertical transfer (between species).

2.6.2 LIPASE PRODUCTION

Lipolytic activity is detected when there is the presence of an opaque zone around the active colonies (Tanasupawat *et al*., 2015). A strain is non- virulent when there is an absence of lipase, gelatinase, and haemolytic activity, indicating its selection as a probiotic strain (Marroki & Bousmaha-Marroki, 2014).

2.6.3 HAEMOLYTIC ACTIVITY

When selecting probiotic strains, the absence of haemolytic activity is a well-thought safety criterion (Joint, 2002). The haemolytic reaction is usually recorded as α-haemolysis in which red blood cells are partially hydrolysed leading to the appearance of greening zones, β-haemolysis observed when red blood cells are broken down seen by clear zones around active bacterial colonies and γ-haemolysis where there is no reaction at all (Ruiz - Moyano *et al*., 2010). The presence of positive haemolytic activity destroys the underlying epithelial layer of the host organism (Tanasupawat *et al.*, 2015).

2.6.4 GELATINASE PRODUCTION

The ability of an organism to secrete gelatinase, which is a proteolytic enzyme is detected by gelatin hydrolysis test. Positive gelatinase activity which is regarded as the ability to breakdown gelatin contributes to the breakdown of the protective lining of the gastrointestinal tract (Marroki & Bousmaha-Marroki, 2014).

2.6.5 SCREENING OF VIRULENCE GENES AND ANTIBIOTIC RESISTANCE GENES

Virulence factors are molecules that facilitate a microorganism to colonize the host of specific animal species and upgrade its capability to cause sickness. Bacterial toxins, hydrolytic enzymes, and cell surface proteins that mediate the bacteria attachment are all virulence factors (Zommiti *et al.*, 2018). The safety considerations of lactic acid bacteria (LAB) include defining their possibility of being virulent (Joint, 2002). The virulence determinants which are associated with pathogenicity include antibiotics resistance, production and secretion of extracellular proteins such as haemolysin and gelatinase, and aggregation substances (Padmavathi *et al*., 2018; Yogurtcu & Tuncer, 2013). Heidari *et al*. (2016) stated that the pathogenicity of a strain is not indicated by the availability of resistance genes alone. However, the strain may become more dangerous when associated with the company of virulence factors. Chajęcka-Wierzchowska *et al*. (2017) highlighted that this is because genes responsible for the expression of antibiotic resistance and virulence factors are located on the same genetic elements. Colonisation alone is not regarded as pathogenicity, but when in combination with virulence factors and availability of possibly detrimental resistance genes. According to Hollenbeck and Rice (2012) and Strzelecki *et al*. (2011), the virulence factors that are responsible for colonisation include cell wall adhesion, aggregation substance, and collagen-binding protein.

2.7 TECHNOLOGICAL SUITABILITY OF *LACTOBACILLUS* STRAINS FOR PROBIOTIC YOGHURT PROCESSING

According to Mattila-Sandholm *et al*. (2002), probiotics must possess excellent technological properties. This will enable them to be incorporated into food products in which they do not have to lose functionality and viability or release unfriendly textures and flavours. Careful screening of technological suitability enables selection of probiotic strains which have the best food technology as well as manufacturing characteristics. Mattila-Sandholm *et al.* (2002) highlighted that there are numerous aspects to be put into play when selecting probiotics which include viability throughout processing, optimal sensory properties, stability in the product throughout storage and phage resistance. According to Saarela *et al.* (2000), before probiotics are added to functional foods and before being delivered to consumers, they must withstand industrial processing environments, endure and keep their functionality throughout storage in the food items in which they will be finally formulated lacking production of off- flavours and textures. The storage conditions and the materials used for packaging affect the quality of products incorporated with probiotic cultures (Saarela *et al.*, 2000).

2.7.1 ROLE OF *LACTOBACILLUS* IN FERMENTED DAIRY PRODUCTS

According to Gopal (2011), in the manufacturing of koumiss, miru- miru, and yoghurt, strains of *L. acidophilus* have been applied. In these products, a minimum level of viability should be maintained because they are purchased with a claim. The pH affects the survival and stability of *L. acidophilus* in these products. The higher the pH, the more *L. acidophilus* survives longer. The temperature at which *L. acidophilus* is exposed affects the rate of survival. *L. acidophilus* survives more at low temperatures which range from 5- 9 °C. When manufacturing yoghurt *L. acidophilus* and *S. thermophilus* are inoculated together at the same time. In terms of growth rate, *S. thermophilus* grows more than *L. acidophilus.* This is important because of the metabolites formed by *S. thermophilus,* which act as growth promoters to *L. acidophilus.* The growth promoters include substances such as carbon dioxide and formic acid. To have a product with a relative amount of *L. acidophilus*, there is a need to carefully monitor and control the ratio of these two species in the various milk formulations during fermentation. The stability of the *L. acidophilus* cells added to yoghurt is affected by hydrogen peroxide that is produced by lactobacilli found in the yoghurt cultures. There is much-improved stability of *L. acidophilus* by the introduction of reducing agents, mainly dithiothreitol, cysteine, and thioglycolate.

The reducing agents confer excellent stability by interacting with the sulphydryl (-SH) groups of the main enzymes involved in hydrogen peroxide pathway. If the milk is fermented with only *L. acidophilus* strains, the final product will lack the characteristic buttery flavour of yoghurt because of the insufficient production of acetaldehyde. The fermented milk will be dull and tart. For flavour to be improved, fruit juices can be added, and strawberry harms *L. acidophilus* viability. *L. acidophilus* can be delivered to humans using dairy products like milk powders. The products need a shelf life that is long and often experienced at higher temperatures. Water activity is essential because strains survive better at low water activity.

2.7.2 THE MANUFACTURING PROCESS FOR FERMENTED PRODUCTS

Optimal sensory properties should be brought by the probiotic culture that is used in fermented probiotic foods. In milk-based products, it is best to use a combination of probiotic bacteria appropriate for fermenting that product. *L. delbrueckii and S. thermophilus* are applied together with probiotic cultures to get the favourite flavour and consistency (Saarela *et al.*, 2000). The standard flavour characteristics of the species mainly used in probiotic preparations are shown in table 2.3 (Saarela *et al.*, 2000). Consumers have found products fermented with *L. delbrueckii* so much acidic and with a heavy acetaldehyde flavour (yoghurt flavour). So probiotic cultures that produce the desired flavour in the products in which they are applied have been made. The cultures include *S. thermophilus, Bifidobacterium* and *L. acidophilus* (Saarela *et al.*, 2000).

Table 2.3: Common flavour properties of strains mainly applied in probiotic mixtures (Saarela *et al.*, 2000).

Culture	Main flavour components
Lactobacillus. delbrueckii subsp. *bulgaricus*[b]	Lactate (D-), acetaldehyde, diacetyl
S. thermophilus[b]	Lactate (L+), acetaldehyde, diacetyl
Bifidobacterium spp[a]	Lactate (+), acetate
L. acidophilus[a]	Lactate (DL)

[a] Probiotic strain

[b] Non- probiotic strains

When selecting the proper starter cultures, the steadfast acid-forming ability is a significant attribute. However, the selection of probiotics should be based on their impact on human health and wellbeing. The probiotics are often not suitable to be used as a starter organism because of the environmental difference between the GI tract and the food (German *et al*., 1999; Oberman *et al*., 1985). The probiotics might give off- flavours as their growth rate might be too slow. This can be prevented by the usage of specific aseptic procedures as those used in the production of acidophilus milk that is fermented with probiotic levels reaching 10^9 CFU g^{-1}. Another method which can be employed for improving the suitability of the food to tolerate the probiotics is the addition of sources of energy such (glucose), growth-promoting factors (protein hydrolysates and yeasts extract), vitamins, minerals, and antioxidants (Saxelin *et al*., 1999). However, such adjustments are never enough for the probiotic to perform as a starter. This problem can be overcome by using a supportive starter together with a probiotic. In the manufacture of products containing probiotics, a temperature range of 37- 40 °C is employed since it is the temperature range at which probiotic strains multiply rapidly. The probiotics will be a component of the starter cultures and grow in a symbiotic relationship with some of the strains contained in the culture. In the Danish product "cultura" probiotic strains are applied into the fermented milk after fermentation. In some sweet milk products, probiotics are applied such that they will preserve their viability but inhibit them from multiplying. This is done by rapidly cooling the product (Saxelin *et al*., 1999).

2.7.2.1 INTERACTION BETWEEN PROBIOTICS AND STARTER BACTERIA

The quality of the fermented product is usually affected by the interaction between the probiotics and starter cultures. According to Fonden *et al*. (1999), good fermented products that have exceptional sensory characteristics and excellent bacterial survival rate have been produced by applying probiotic strains and starter cultures at the same time. The appropriate starter cultures include yoghurt cultures, *S thermophilus*, mesophilic starters combined with various strains of *Lactococcus*. A good mixture of a specific probiotic bacterium and starter cultures can be obtained by evaluating the influence of diverse starter cultures on the sensorial characteristics and survival rate of probiotic strains (Ishibashi & Shimamura, 1993; Samona *et al*., 1996). Some general properties should be followed in the screening process. During fermentation, probiotic bacteria must grow. Since optimum growth of probiotics is observed at 37 °C, there is a need for a thermophilic starter which is best suited for the job than a mesophilic one (Svensson, 1999). The starter culture should grow at a moderate rate to allow the probiotic bacteria to grow as well. The probiotic bacteria should be added earlier or simultaneously with the starter culture (Kailasapathy & Rybka, 1997; Reddy, 1989). If the probiotic strains are added after fermentation, there will be no growth (Hull *et al*., 1984). Starter cultures enrich conditions for the probiotic to grow through releasing substances which promote probiotic growth by minimising the pressure of oxygen. Selected specific strains of *S. thermophilus* enabled the growth of *Bifidobacterium*, which are sensitive to oxygen (Ishibashi & Shimamura, 1993).

2.8 HYPOTHESIS AND OBJECTIVES

2.8.1 HYPOTHESIS

1. Lactic acid bacteria from both bovine and dairy origin will have excellent probiotic properties. Guasch-Jané *et al*. (2006), explained that diverse fermented foods from various geographical locations provide different lactic acid bacteria which have potential probiotic properties. Ahn *et al*. (2002) stated that lactic acid bacteria are now extensively used in probiotic preparations because they improve the function of the immune system, modify the intestinal microflora balance, and inhibit harmful bacteria from growing.

2. The bovine and dairy-based lactic acid bacteria strains will produce bacteriocins and would exhibit natural antibiotic resistance. Some lactic acid bacteria can compete with other bacteria in the environment because they produce specific bacteriocins (Messaoudi *et al*., 2013). Probiotic strains with natural resistance to antibiotics could be valuable for re-establishing the gut microbiota after antibiotic treatment. This property is vital for survival after co-regulated medications (Gueimonde *et al.*, 2013).

3. The bovine and diary-based lactic acid bacteria can withstand the industrial manufacturing process and keep their viability from storage to consumption and even within the human gut. Mattila-Sandholm *et al.* (2002) stated that for probiotics to deliver health benefits to humans, they should have excellent technological properties for incorporation into manufactured food products without losing functionality, viability, and producing undesirable flavours or textures. The viability of probiotic bacteria in yoghurt is affected by storage temperature, pH, the content of oxygen, hydrogen peroxide, and concentration of lactic and acetic acids (Lankaputhra *et al*., 1996). Thus, there has been so much interest in investigating the viability of *Lactobacillus* up to consumption to guarantee that live microorganisms are delivered (Akalın *et al*., 2004).

2.8.2 OBJECTIVES

1. To evaluate and compare the probiotic characteristics of the dairy and bovine *Lactobacillus* isolates.

2. To determine the bacteriocinogenic potential, antibiotic susceptibility and resistance pattern of the isolates.

3. To determine the fate of the probiotic strains when used with regular fermenting bacteria during fermentation as well as storage.

CHAPTER 3: RESEARCH

3.1 BRIEF INTRODUCTION TO RESEARCH STUDY

The probiotic guidelines of FAO/WHO demonstrate that the bacteria should remain intact throughout the gastrointestinal tract and confer health benefits upon entering their site of action. Further, the probiotics are expected to survive under extreme physiological conditions. The dairy origin probiotics are proven to remain active over different food processing conditions, whereas the bovine origin probiotics can well be adapted to grow under gastrointestinal conditions. The study aimed at evaluating bovine and dairy origin lactic acid bacteria as potential probiotics for application in yoghurt as to the benefit of human application. The study was carried out in three phases. The first phase was to identify and characterise the selected bacteria using phenotypic methods. The second phase was to evaluate the probiotic characteristics as well as safety evaluation through microbiological methods such as antibiotic susceptibility test. The third phase was conducted to determine the techno-economic feasibility of the probiotic strains when used in a fermentation medium and viability during shelf life. Thus, the study objectives are as:

i) To evaluate and compare the probiotic characteristics of the bovine and dairy lactic acid bacteria isolates
ii) To determine the bacteriocinogenic potential, antibiotic susceptibility and resistance pattern of the isolates
iii) To determine the technological suitability of the probiotic strains when used in a regular fermentation product as well as storage

3.2 IDENTIFYING AND CHARACTERISING THE SELECTED LACTIC ACID BACTERIA FROM BOVINE AND DAIRY ORIGIN

ABSTRACT

Identifying and classifying lactic acid bacteria enables to have a clear distinction in their roles towards the fermentation process and to understand their functional roles in therapeutic and industrial applications. For this study, probiotics from bovine and dairy origin were isolated and characterised. The analysis was based on biochemical characteristics which include Gram staining, catalase test, oxidase test, and carbon dioxide gas production from glucose. The morphological characterization experiments such as Gram staining revealed that the bacterial isolates were of *Lactobacillus* and were able to produce carbon dioxide gas from glucose indicating its capacity to undergo heterofermentative metabolism. All the four strains, including the positive control were found to be catalase-negative, oxidase negative, and gram-positive. The bacterial isolates were identified as *L. plantarum*, *L. pentosus*, *L. acidophilus* and *L. rhamnosus*.

3.2.1 INTRODUCTION

There has been growing interest in microorganisms originating from food and animal sources because of the possibility of finding new probiotic candidates of nutritional significance (Anandharaj & Sivasankari, 2013). The goal to improve human nutrition has enabled several lactobacilli strains to be used as starter cultures in food formulations and supplementation approaches (Parvez *et al*., 2006). Probiotics are important because they prevent growth of pathogenic bacteria, modify the intestinal microflora balance, facilitate improved digestion, and improve the function of the immune system as well as resistance towards infection (Ahn *et al.* (2002). The world market for probiotics was estimated at USD 36 million in 2013 (de Simone, 2019). In 2016 the value increased to USD 35.9 billion with the European continent contributing large with an annual growth rate of 20% (Zielińska & Kolożyn-Krajewska, 2018).

The screening for potential probiotic characteristics for lactobacilli obtained from various ecological niches has been carried out systematically. The dairy origin probiotics are proven to remain active over different food processing conditions, whereas the bovine origin probiotics can well be adapted to grow under gastrointestinal conditions (Jose *et al*., 2015) Ideal bovine pH Is around 6-7. In the bovine digestive system, the bovine isolates can thrive because they are exposed to a wide range of stressful conditions (Jose *et al*., 2015). Bovine isolates are subjected to encounter antibiotics as they are used in preventing and curing infections in animals. Bovine isolates have been reported to inhibit *L. monocytogenes* and *S. aureus*, which are gram-positive bacteria more than dairy isolates (Jose *et al*., 2015). This commonly is not expected as the bovine isolates will probably co-exist together with the pathogens or must strive *in vivo* in cases of infection by the pathogens of the host environment. Dairy isolates have been reported to have poor adherence properties when compared to bovine isolates (Jose *et al*., 2015)

The species-level identification of probiotic cultures serves a functional purpose on the safety evaluation of the strains to prevent health hazards and undocumented claims on the description regarding the strains (Mohania *et al*., 2008). Since there are various strains of LAB which may affect the microenvironment of the human intestine, it is important to characterise and identify microorganisms available in a microbial ecosystem which might have protective effects (Mohania *et al*., 2008). Tannock *et al*. (1999) stated that it is challenging to identify LAB using phenotypic methods; because, in most situations, it requires the evaluation of bacterial characteristics outside of those reported in common fermentation assessments.

According to Holzapfel *et al*. (2001), identifying lactobacilli depends mainly upon phenotypic features such as morphology, carbohydrate fermentation, and growth determination at different temperatures. Physiological, morphological, and chemical principles are the main factors on which the conventional phenotypic identification of LAB is primarily dependent (Reginensi *et al*., 2013). Many methods which are DNA- based are present for typing and identifying LAB isolates, and modern taxonomic polyphasic methods consider both phenotypic and genotypic analysis (Temmerman *et al*., 2004). Failure to differentiate LAB species or subspecies, which are phylogenetically closely related is experienced when the LAB is identified using 16S rRNA gene sequencing (Temmerman *et al.*, 2004). According to Van Hoorde *et al*. (2008), variations are made difficult because of the graded and complex differences, even though phenotypic assessments offer indications of different metabolic abilities between species. Molecular techniques have resolved the ambiguities brought about by designation of specific new type strain-based solely on phenotypic characteristics. Callon *et al*. (2004) stated that a great transition from phenotypical to genotypical methods had been observed on which LAB identification now primarily focuses as the latter generate precise and sensitive results.

The isolating isolation and identifying identification of probiotic bacteria from the bovine and dairy origin and its comparison have not been carried out in South Africa. So, this study aimed at identifying and characterizing bacteria from dairy and the bovine origin using phenotypic methods.

3.2.2 MATERIALS AND METHODS

3.2.2.1 COLLECTION AND MAINTENANCE OF PROBIOTIC CULTURES

The lactic acid bacteria from bovine sources were obtained from the Department of Consumer and Food Sciences at the University of Pretoria. The cultures were labelled VLL1 (*L. plantarum*) and LIP (*L. pentosus*). Other *Lactobacillus* strains from dairy products were obtained from the Tshwane University of Technology. The cultures were labelled D (*L. acidophilus*) and V (*L. rhamnosus*). The reference culture labelled ATCC 4536 (*L. acidophilus*) was also obtained from the ATCC culture collection. The cultures were recovered carefully through inoculating into 9 mL of MRS broth (De Man *et al*., 1960) and incubated at 37 °C for 16 - 18 hours in aseptic conditions. Further, for preservation of bacteria, a sterilized freezing medium prepared with 25 mL of 14% aqueous bacteriological peptone solution, 25 mL of 14% aqueous dextrose solution, and 50 mL of 30% glycerol was used. The freezing medium (cryoprotectant) was placed in the refrigerator until it was needed to be used (Nyanzi, 2007). Gram staining was used to confirm the purity of the culture. 9 mL of MRS broth (Sigma, Modderfontein, South Africa) were introduced into capped test tubes, and single colonies of each *Lactobacillus* cultures on MRS agar (Sigma, Modderfontein, South Africa) was aseptically picked and transferred to the capped test tubes using a sterile inoculation loop. The MRS broth, which was inoculated, was incubated at 37 °C for 18 hours until turbid growth was observed. The matured cultures in MRS broth were vortexed, and 5 mL of each suspension was transferred aseptically into screw-capped plastic tubes which were sterilised. The cells were centrifuged at 4000 × g for 5 minutes at 5 °C using the NF400/ NF 400R Bench-top centrifuge and were concentrated into pellets. The supernatant was decanted, and the cell pellets were suspended in 2 mL of freezing medium and frozen at -80 °C.

3.2.2.2 GRAM STAINING

The bacterial cells were smeared and dispersed in the water drop. The slides were then air-dried. The specimen were heat-fixed over the flame to fix the bacteria. Few drops of crystal violet were added and left for 60 seconds followed by washing in running water. The slides were gently blotted with paper towels. A few drops of gram iodide were added for 60 seconds, followed by the decolouriser which was 95% alcohol for 30 seconds, and safranin for 60 seconds. In between these treatments, the slide was washed with running water and gently blotted with paper towels. A coverslip was placed on the slide, and immersion oil added. The slides were placed on the stage of the microscope, and the stained material was viewed using the 40X and 100X objectives.

3.2.2.3 CATALASE SLIDE TEST

A small amount of each bacterial colony was transferred to the surface of a clean, dry glass slide using a sterile loop. Two drops of 3% hydrogen peroxide were placed onto the slide for

60 seconds (Nelson & George, 1995). The liberation of gas bubbles was observed against a dark background to characterize the bacteria.

3.2.2.4 OXIDASE TEST

The oxidase test strips were moistened with sterile water in a petri dish. The overnight grown cultures of *Lactobacillus* strains were smeared onto the oxidase test stripes. The appearance of purple/blue colour was monitored within 60 seconds for confirming the presence or absence of oxidase enzymes (Tarrand & Gröschel, 1982).

3.2.2.5 CARBON DIOXIDE PRODUCTION FROM GLUCOSE

In order to determine the homofermentative and heterofermentative characteristics of the isolates, carbon dioxide production from glucose assay was performed using the method proposed by Müller (1990). 50 µL of overnight cultures were transferred into 9 mL of MRS-cysHCL broth in test tubes containing Durham tubes. 5 g of L- cysteine- hydrochloride-monohydrate were mixed with 1000 mL of MRS broth to make MRS- cysHCL broth. The tubes were then incubated anaerobically at 30 °C in anaerobic jars (Oxoid) containing AnaeroGen^{TM} 3.5L sachet (Thermo Scientific) for five days. After incubation, gas accumulation in Durham was taken as evidence for CO_2 production from glucose.

3.2.3 RESULTS

Table 3.1: Biochemical characterization of lactic acid bacteria strains from bovine and dairy sources

Strain	Gram staining	Oxidase test	Catalase test	Glucose fermentation
D	+	-	-	+
V	+	-	-	+
VLL1	+	-	-	+
LIP	+	-	-	+
ATCC 4536	+	-	-	+

+, positive reaction and -, negative reaction

All the five isolates were gram-positive as they appeared purple under microscopic examination. They were all rod-shaped. The bacterial isolates were catalase-negative as there was no rapid production of gas bubbles. This indicates they lack the enzyme catalase, which decomposes hydrogen peroxide (H_2O_2) liberating oxygen (O_2) and water (H_2O). All the five bacterial isolates were oxidase negative since phenylene-diamine on the diagnostic strips was not oxidised to produce a deep purple colour proving they do not secrete the enzyme cytochrome c oxidase which plays a role in the bacterial electron transport chain. Gas production is used to classify if the isolates are heterofermentative or homofermentative. All the isolates presented heterofermentative metabolism as they produced carbon dioxide gas from glucose.

3.2.4 DISCUSSION

The screening of lactobacilli from different ecological niche has been systematically carried out for picking a probiotic culture with superior functional benefits to human beings. This study is an effort to unravel and compare the probiotic culture characteristics of lactobacilli from dairy and bovine origin. The morphological characterization of bacterial isolates from the bovine and dairy sources as well as the reference culture through Gram staining indicate that all the strains were gram-positive. Further, they were identified as oxidase and catalase-negative. The genus *Lactobacillus* is a group of gram-positive, non-spore forming, catalase negative bacteria (MacFaddin, 2000). In this present study, many of the physiological and biochemical properties of the lactobacilli obtained from dairy sources were in conjunction with those reported by Cullimore (2010).

The production of gas is analysed as a functional tool for classifying the isolates as heterofermentative or homofermentative (da Silva *et al*., 2019). This classification plays a significant role in choosing the food matrix where the potential probiotic microorganisms may be incorporated (da Silva *et al*., 2019). The bacterial strains evaluated presented heterofermentative metabolism. According to De Almeida Júnior *et al*. (2015), about 12% of lactobacilli can produce carbon dioxide gas in glucose metabolism. The production of gas from the LAB is of technological importance in products that include cheese and kefir (Shah, 2000). In contrast, some obligatory homofermentative species were tested for use in humans as probiotics. The species include *L. gasseri*, *L. johnsonii*, *L. gallinarium*., *L. amylovarius*, *L. crispatus*, and *L. acidophilus* (Ferreira, 2012).

3.2.5 CONCLUSION

The lactobacilli strains of dairy and bovine origin were gram-positive, rod shaped, oxidase and catalase negative and heterofermentative. These conclusions meet those characterization qualities of lactobacilli species as identified by other researchers from environmental sources. Furthermore, the study isolates were in line with the ATCC *L. acidophilus* culture in morphological and biochemical classifications. The isolates were further subjected to probiotic characterization including the resistance to gut environmental conditions.

3.3 EVALUATION OF THE PROBIOTIC PROPERTIES OF THE DAIRY AND BOVINE *LACTOBACILLUS* ISOLATES

ABSTRACT

Lactobacillus strains from bovine and dairy origin were evaluated and compared for selected probiotic characteristics with the aim of assessing their safety for human use. The probiotic properties evaluated were acid and bile resistance, bile salt hydrolase activity, antimicrobial activity, antibiotic sensitivity, gelatinase production, lipase production, and hemolytic activity. All the four strains including the ATCC control showed moderate to better ability to survive in the acidic conditions at pH 5 and pH 3, even though they loss their viability significantly at pH 2. The isolates tolerated 0.3% bile salts, and they all secreted bile salt hydrolase enzymes. All the strains showed resistance to kanamycin and vancomycin. Besides, the lactobacilli strains showed susceptibility to gentamicin, clindamycin, erythromycin, tetracycline, ampicillin, and chloramphenicol. However, the isolates from bovine origin were moderately susceptible to tetracycline. In antimicrobial activity assay, the strains showed excellent antimicrobial activity against diarrheagenic *E. coli* and pathogenic *Candida* spp. Furthermore, the study isolates were lipase and gelatinase negative, but they have shown α- hemolysis which is the partial hydrolysis of red blood cells. The performance characteristics of all the four lactobacilli isolates were identified and compared. Though all the strains possess excellent probiotic potential, the rumen isolates have shown slightly increased tolerance to human gastrointestinal conditions.

3.3.1 INTRODUCTION

The probiotic bacteria of multiple foodstuffs and natural origins are commonly used in the formulation of commercial yoghurts in the recent periods. Most of the probiotic formulations have been based on strains of *L. acidophilus* (A) and *Bifidobacterium* (B) mainly known as A-B culture in addition to *L. bulgaricus* and *S. thermophilus* which are gaining momentum throughout the world market (Viljoen & Lourens-Hattingh, 2002). Numerous brands of bio-yoghurt, which contains AB cultures, have been sold in South Africa (Viljoen & Lourens-Hattingh, 2002). Since they are both unstable, the survival *L. acidophilus* and *B. bifidum* in bio- yoghurt before consumption have become vital (Viljoen & Lourens-Hattingh, 2002). The market for probiotic functional foods is increasing as society is becoming more health-conscious. The department of agriculture, forestry, and fisheries under the agricultural product standards act 1990, (ACT No, 119 OF 1990) on the regulations relating to the classification, packaging and making of dairy products and imitation dairy products intended for sale in the Republic of South Africa states that " If a specific micro-organism is used in conjunction with the sale of any type of yoghurt with added foodstuff and/or fruit, and where the content claim is made in the labelling that refers to the presence of such specific micro-organism that has been added to supplement the yoghurt culture, it shall contain at least 10^7 colony forming units per gram (CFU/g) of that specified micro-organism." Since the AB cultures are unstable, this research can add value in obtaining novel strains that are stable and keep their viability during storage and up to consumption of the yoghurt.

Lactobacilli have been employed in the manufacture of fermented foods because they are regarded as generally safe. Food sources such as raw milk or fermented milk products contain LAB as naturally occurring microflora (Ali, 2011). Many different types of fermented foods in various geographical locations throughout the world provide numerous lactic acid bacteria which have potential probiotic characteristics (Guasch-Jané *et al.*, 2006). A specific selection criterion should be followed concerning a strain regarded to have probiotic potential which includes antibiotic resistance, production of antimicrobial substances, stability towards phenolic compounds, overcoming gastric pH, resistance to detrimental effects of bile salts, and co- aggregation (Chaudhary & Saharan, 2018). Numerous *in vivo* and *in vitro* analyses are used to screen out these properties, though there are no standardized methods to determine probiotic functionality (Lee & Salminen, 2009).

Upon consumption, the probiotic bacteria must withstand passing through the gastrointestinal tract where many challenges are encountered which include bile salts released in the upper digestive tract and the stomach low pH environment (Kailasapathy & Chin, 2000; Musikasang *et al*., 2009; Shah, 2000). There are variations in stomach pH from as low as 1- 2 experienced during fasting and 4-5 after ingesting a meal (Chou & Weimer, 1999). Acid tolerance affects the way probiotics survive in fermented foods. The most prominent vehicles for probiotics are fermented milk and yoghurt. This is because they provide an environment which is relatively low in pH necessary for probiotic survival. Hence the first attribute to evaluate for the selection of strains which have probiotic potential is acid tolerance (Tuomola *et al*., 2001). An excellent probiotic bacterium will exhibit the property of autoaggregation before it provides health benefits. This characteristic contributes to the adherence of LAB to the intestine epithelium and secreting antimicrobial substances which include organic acids, bacteriocins and hydrogen peroxide (Chaudhary & Saharan, 2018). The organic acids inhibit other bacteria from growing by creating a hostile environment with a lowered pH. Simultaneously, the organic acids have proven to be toxic to other bacteria (Jose *et al.*, 2015).

Adherence enhances the probiotic bacteria to stay in the gut for an extended period promoting host- bacteria interactions (Gueimonde & Salminen, 2006), and helping the probiotic bacteria to withstand peristalsis in the stomach (Suvarna & Boby, 2005). Probiotics should be safe; for example, they should not contribute to the breakdown of erythrocytes. Resistance to antibiotics may be vital for surviving exposure to therapeutic antibiotic drugs. The genes responsible for resistance in probiotics should be natural and non- transferrable (Jose *et al.*, 2015). Constant testing for antibiotic susceptibility is regarded as essential for cultures with probiotic potential. This will provide adequate proof that the strains are non- pathogenic, and do not participate in undesirable gene transfer. *Lactobacillus* has a natural resistance to numerous clinical antibiotics which have led to developing antibiotic/ probiotic combination therapies that can be used for the treatment of conditions such as infective endocarditis and diarrhoea (Charteris *et al*., 1998). The probiotic significance in traditional foods has made them recognized for commercial utilization. The probiotic strains can thrive when passing through the gastrointestinal passage and hence adapt to the intestine. Lindström *et al*. (2012) stated that this would enable the strains with potential probiotic effects from traditional foods to be applied in industrial processes, other than those from animals.

The primary purpose of the study was to characterize the lactobacilli isolates from the bovine and dairy origin for their probiotic attributes which include antimicrobial activity, bile and acid tolerance and safety through antibiotic susceptibility and hemolytic activity.

3.3.2 MATERIALS AND METHODS

3.3.2.1 BILE SALT TOLERANCE

The bile salt tolerance of the isolates was carried out by evaluating growth in MRS broth with 0.3% bile salt. MRS broth was prepared and supplemented with 0.3% ox- bile (Sigma- Aldrich) and then sterilised. The overnight grown *Lactobacillus* (1% v/v) strains were inoculated in MRS broth with bile salt and exposed for 0, 1, 2, and 3 hours, and the viable colony counts were measured. The broth without bile salts was considered as control. The tolerance towards bile salts was measured after incubation at 37 °C for 24 hours and expressed as viable colony counts (CFU/ mL) (Zommiti *et al.*, 2018).

3.3.2.2 ACID TOLERANCE

Lactobacillus was grown anaerobically at a temperature of 37 °C overnight and was transferred into fresh MRS broth for a further 24 hours incubation. Centrifugation at 5000 × g at 4 °C for 10 minutes was used to harvest 1 mL of overnight cultures. Phosphate buffer saline (NaCl 8%; 1M; pH 7.2) was used to wash the pellets three times before resuspension into 10^7 bacteria/ mL in MRS broth. The MRS broth was adjusted to pH 2, pH 3, and pH 5 using 0.5M hydrochloric acid. Between 0, 1, 2, and 3 hours of incubation at 37 °C aliquots of 0.1 mL were taken, and serial dilutions were prepared in 0.1% peptone water. A sample with a pH of 7.0 was used as a control. The survival of the bacteria was expressed in CFU/ mL using the spread plate method (Zommiti *et al.*, 2018).

3.3.2.3 BILE SALT HYDROLASE ACTIVITY

The *Lactobacillus* isolates were tested for bile salt hydrolase activity using Tauro- deoxycholic acid (TDCA). MRS agar plates were supplemented with 0.5% TDCA and incubated anaerobically at 37 °C for 16 hours before inoculation to ensure sterility of the medium. *Lactobacillus* cultures were grown anaerobically in MRS broth at 37 °C for 16 hours. They were streaked on MRS agar plates supplemented with a concentration of 0.5% TDCA and were incubated in anaerobic conditions at 37 °C for 48- 72 hours. Bile salt hydrolase activity was observed for the precipitation of unconjugated deoxycholic acid in the MRS agar plates with a clear halow appearance below and around the colonies (Allain *et al*., 2018).

3.3.2.4 ANTIMICROBIAL ACTIVITY USING THE DUAL AGAR METHOD

Table 3.2: Indicator pathogens used for antimicrobial activity and their sources

Indicator pathogens	Source
Enteroinvasive *E. coli* (EIEC) Enterohemorrhagic *E. coli* (EHEC) Enteroaggregative *E. coli* (EAEC) Enterotoxigenic *E. coli* (ETEC)	National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Republic of South Africa
C. albicans ATCC 10231 (C1)	American Type Culture Collection
C. albicans 1051255 (C4) *C. albicans* M0826 (C7)	(Nyanzi *et al*., 2014)

Antimicrobial activity was determined with different strains of *E. coli* and *C. albicans* using the dual overlay method. *Lactobacillus* was grown overnight and spotted on the centre of MRS agar plates and incubated for 24- 48 hours to allow the colonies to develop. The indicator *E. coli* pathogens which were enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC) were inoculated in tryptone soy broth for 16- 18 hours at 37 °C. Tryptone soy agar was prepared and around 5 mL was poured into the MRS agar plates. The indicator pathogens were spread on top of the tryptone soy agar and the plates were refrigerated for 2- 4 hours and incubated for 24 hours at 37 °C. 100 mL of sabouraud broth was prepared by mixing 1g peptone powder and 2g dextrose and sterilised for 15 minutes at 121 °C. Sabouraud broth used to inoculate *Candida albicans* ATCC 10231 (C1), *C. albicans* 1051255 (C4), and *C. albicans* M0826 (C7) and incubation were carried out at 37 °C for 18 hours. 300 mL of potato dextrose agar were prepared, and 5 mL was poured into each plate on all the MRS agar plates. The indicator *Candida* spp.

pathogens were spread on the plates and were refrigerated for 2 hours before incubation for 24 hours at 37 °C

3.3.2.5 ANTIMICROBIAL ACTIVITY USING CELL-FREE SUPERNATANT BY WELL DIFFUSION ASSAY

Lactobacilli isolates were cultured overnight in MRS broth. The *C. albicans* were cultured in sabouraud broth, and the *E. coli* pathogens were cultured in tryptone soy broth. 800 mL of sabouraud agar was prepared by mixing 12g bacteriological agar, 8g bacteriological peptone, and 32g dextrose. 1200 mL of Mueller Hinton agar was also prepared and supplemented with methylene. The *C. albicans* and *E. coli* pathogens were diluted up to 10^6 using serial dilutions (Bulgasem *et al*., 2016). 100 µL of the test pathogens were spread on the surface of sabouraud agar and Mueller Hinton Agar. Wells of 6mm size were dug using a cork borer. Cell-free supernatant was obtained by centrifuging the cultures at 5000 × g for 10 minutes at 5 °C. The pH of the cell-free- supernatant was adjusted between 7- 7.4 using 0.5M sodium hydroxide. 100 µL of the cell-free supernatant was added to the wells. The cell-free supernatant was separated into two parts. The first part was filtered only, and the second one was adjusted to pH 7 and then filtered. The control used was MRS broth. The plates were refrigerated for 2- 4 hours before incubated for 24 hours at 37 °C.

3.3.2.6 ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic resistance was examined using the agar diffusion method. MRS broth was used to grow the bacteria for 18 hours at 37 °C. Around 0.1 mL of the suspension was spread onto sterile MRS agar plates. The antibiotics discs (30µg chloramphenicol, 10µg ampicillin, 15µg erythromycin, 30µg tetracycline, 30µg kanamycin, 30µg vancomycin, 300µg streptomycin, 10µg gentamicin, and 2µg clindamycin) were placed on MRS agar plates using an antibiotic disc dispenser. Incubation was carried out for 12- 16 hours at 37 °C. The inhibition zone around the antibiotic discs was recorded. The antibiotics chosen were recommended by the European Food Safety Authority "EFSA Panel on additives and products for substances used in Animal Feed- FEEDAP, 2012". The zones of inhibitions were interpreted using the CLSI guidelines.

3.3.2.7 LIPASE PRODUCTION

Lactobacillus isolates cultured for 24 hours were inoculated on MRS agar plates. The MRS agar plates were supplemented with 1% Tween 80 as a source of fatty acids. The cultured plates were incubated for 24 hours at 35 °C. The presence of an opaque region around the colonies was confirmed as the presence of lipolytic activity (Tanasupawat *et al.*, 2015).

3.3.2.8 HAEMOLYTIC ACTIVITY

Haemolytic activity was determined by streaking cultures grown overnight on sheep blood agar plates (Selecta- Media, Thermo scientific) followed by incubation for 24 hours at 35 °C (Linaje *et al.*, 2004).

3.3.2.9 GELATINASE PRODUCTION

Lactobacillus isolates were streaked on MRS agar plates supplemented with 3% gelatine. Incubation was carried out for 24 hours at 35°C (Harrigan & McCance, 1976).

3.3.2.10 STATISTICAL ANALYSIS

The entire experimental observations were recorded as mean ± SD (standard deviation). Analysis of variance (ANOVA) was used for acid tolerance (strain × pH × time), bile tolerance (strain × time), and antimicrobial activity (strain × pathogen) to 0.05% level of significance. Duncan's multiple range test was used to calculate for significant differences between means. The software used was Stat graphics Centurion version 18 (Stat graphics Technologies 18)

3.3.3 RESULTS

3.3.3.1 BILE SALT TOLERANCE

All the strains showed an excellent resistance profile towards 0.3 % bile salts across all the time regimes. Interestingly, the reference strain ATCC 4536 also demonstrated a high resistance pattern at 0, 2, and 3 hours. Strain VLL1 at 0 and 1 hour showed higher resistance which is comparable to the reference strain. Meanwhile, the strains which showed the least resistance were V and D at the whole study periods. It is remarkable that the strains VLL1 and

LIP, which showed higher bile salt resistance was from bovine origin. Strains D and V, which showed the least resistance were from the dairy origin.

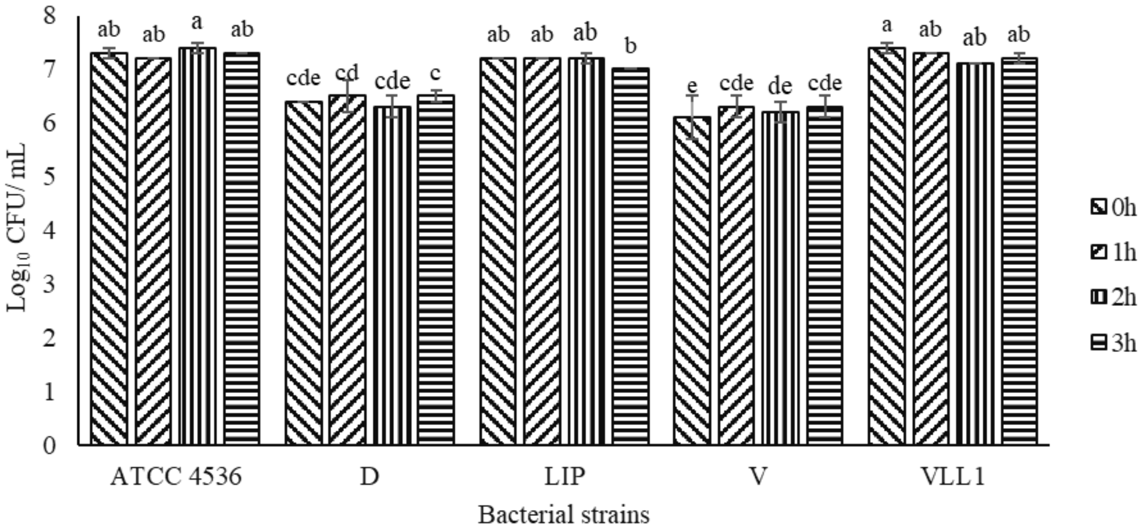

Figure 3.1: Effect of bile salts on the survival of lactic acid bacteria strains after exposure to 0.3% bile salts for 0, 1, 2 and 3 hours. The bacterial strains are *L. acidophilus*- D, *L. rhamnosus*- V from the dairy origin, *L. pentosus*- LIP, *L. plantarum*- VLL1 from the bovine origin, and *L. acidophilus* ATCC 4536 from the ATCC culture collection.

The graph shows the log of bacterial counts after exposure to 0.3% bile salts for 0, 1, 2, and 3- hours. Each bar is a mean of two independent replicates (n=2) and the corresponding standard deviation with different (sets of) letters as outcomes for their multiple range tests. Mean values showing different (set of) alphabets [a-e] for the interactions for the two factors strain and time are significantly different from each other ($p < 0.05$).

3.3.3.2 ACID TOLERANCE

Table 3.3: Analysis of variance on the effect of exposure to acidic environment on strain, pH and time

Source	*P-Value*
MAIN EFFECTS	
A: Strain	0.0000
B: Time	0.0000
C: pH	0.0000
INTERACTIONS	
AB	0.0000
AC	0.0000
BC	0.0000
ABC	0.0000

The table shows the main effects of strain (A), time (B), and pH (C) and the interactions between strain and time (AB), strain and pH (AC), time and pH (BC), and strain, time, and pH (ABC) ($P < 0.05$).

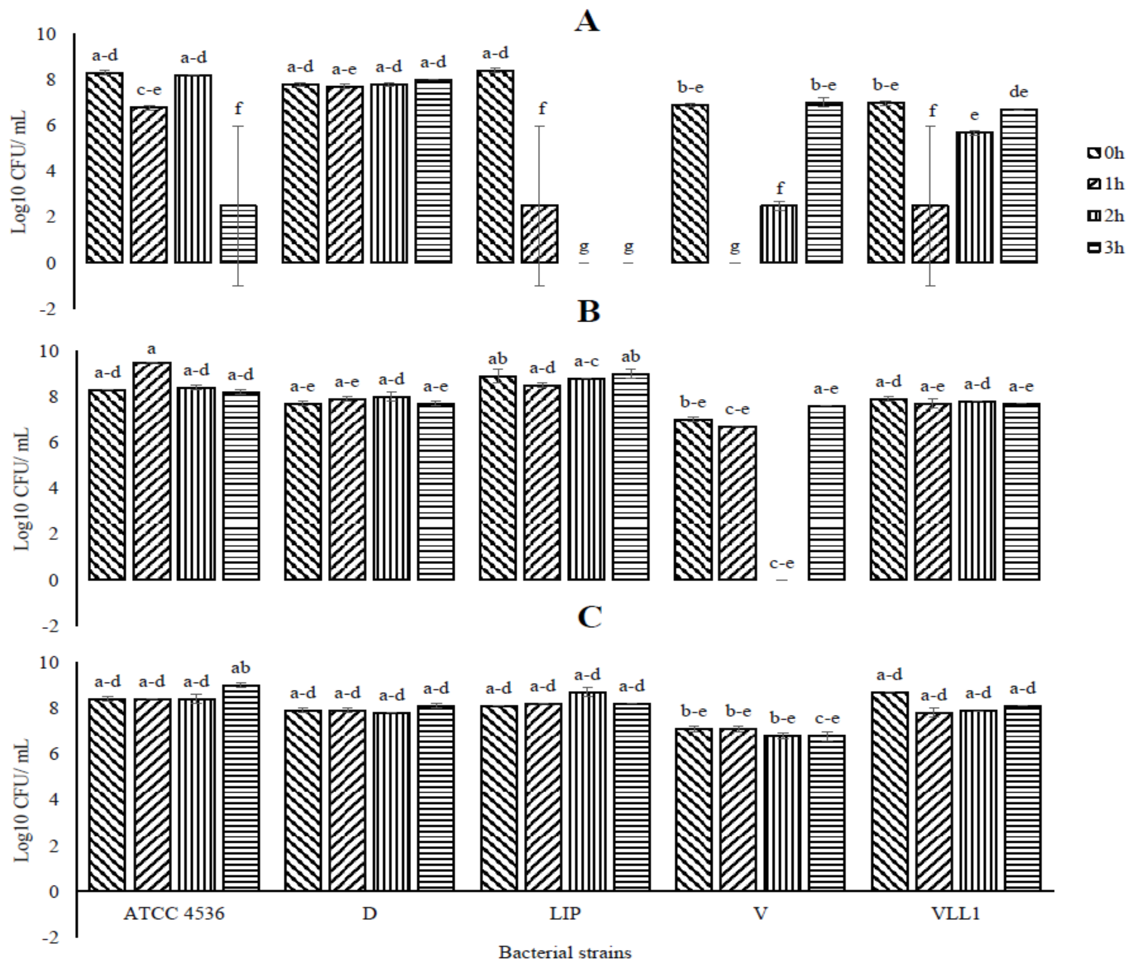

Figure 3.2: Effect of exposure to the acidic environment on the survival of lactic acid bacteria after incubation at pH 2 (A), pH 3 (B) and pH 5 (C) for 0, 1, 2 and 3 hours. The bacterial strains are *L. acidophilus*- D, *L. rhamnosus*- V from the dairy origin, *L. pentosus*- LIP, *L. plantarum*-VLL1 from the bovine origin, and *L. acidophilus* ATCC 4536 from the ATCC culture collection.

The graph shows the log of bacterial counts after exposure to the acidic environment at pH 2 (A), pH 3 (B), and pH 5 (C) for 0, 1, 2, and 3 hours. Each bar is a mean of two independent replicates (n=2) and the corresponding standard deviation with different (sets of) letters as outcomes for their multiple range tests. Mean values showing different (set of) alphabets [a-e] for the interactions for the two factors strain and time are significantly different from each other ($p < 0.05$).

There were significant interactions between strain and time ($p<0.001$) strain and pH ($p<0.001$), time and pH ($p<0.001$), and strain, time, and pH ($p<0.001$). All the strains followed a trend where they had decreased viability at pH 2 through all the time regimes. However, the viability increased at pH 3 and pH 5 across all the time regimes. The reference strain ATCC 4536 showed a high resistance profile at pH 3 time 1 hour and pH 5 time 3 hours. Strain LIP showed the same resistance pattern at pH 3 time 0 and 2 hours, and pH 5 time 2 hours. This was followed by strain VLL1 at pH 5 time 0. The strains drastically reduced their viability at pH 2. Strain LIP showed the least resistance pattern at pH 2 time 1, 2, and 3 hours. This was followed by strain V at pH 2 time 1 hour. The reference strain ATCC 4536 also showed low viability at pH 2 time 1 and 3 hours. Strain VLL1 also showed reduced viability at pH 2 time 1, 2, and 3 hours.

3.3.3.3 BILE SALT HYDROLASE ACTIVITY

All the strains had bile salt hydrolase activity because of the precipitation of unconjugated deoxycholic acid in the MRS agar plates. An iridescent halo around and below the active colonies was formed.

3.3.3.4 ANTIMICROBIAL ACTIVITY

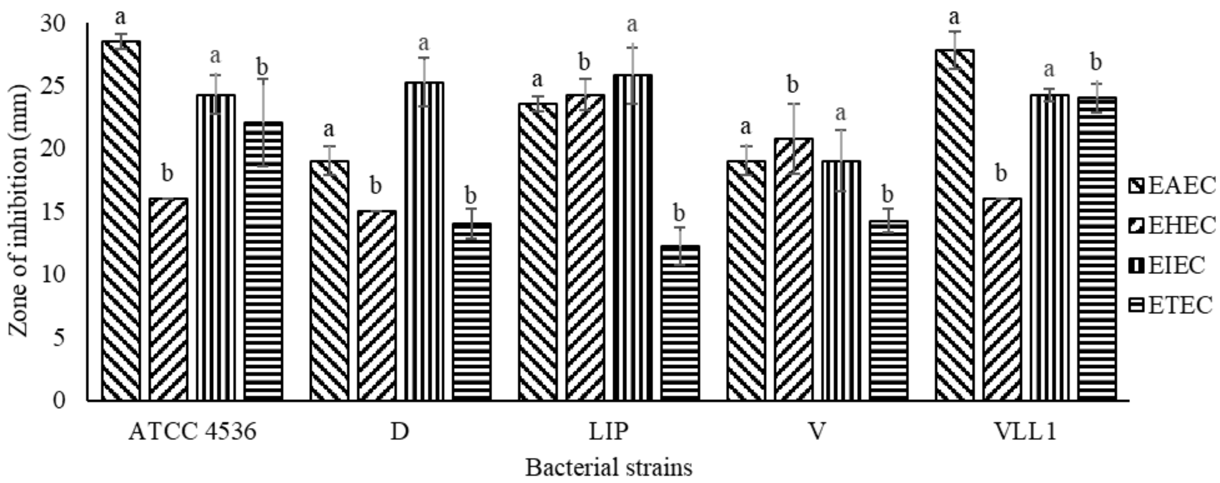

Figure 3.3: Effect of inhibitory activity of lactic acid bacteria against different strains of diarrheagenic *E. coli*. The bacterial strains are *L. acidophilus*- D, *L. rhamnosus*- V from the dairy origin, *L. pentosus*- LIP, *L. plantarum*- VLL1 from the bovine origin, and *L. acidophilus* ATCC 4536 from the ATCC culture collection. The *E.coli* pathogens are Enterohemorrhagic *E.coli* (EHEC), Enteroaggregative *E.coli* (EAEC), Enteroinvasive *E.coli* (EIEC), and

Enterotoxigenic *E.coli* (ETEC) from National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS), Republic of South Africa

The graph shows the inhibitory activity of lactic acid bacteria against diarrheagenic *E.coli* pathogens. Each bar is a mean of two independent replicates (n=2) and the corresponding standard deviation with different (sets of) letters as outcomes for their multiple range tests. Mean values with a different set of alphabets [a-b] for the inhibitory activity of the lactic acid bacteria against each *E. coli* pathogen are significantly different (p< 0.05).

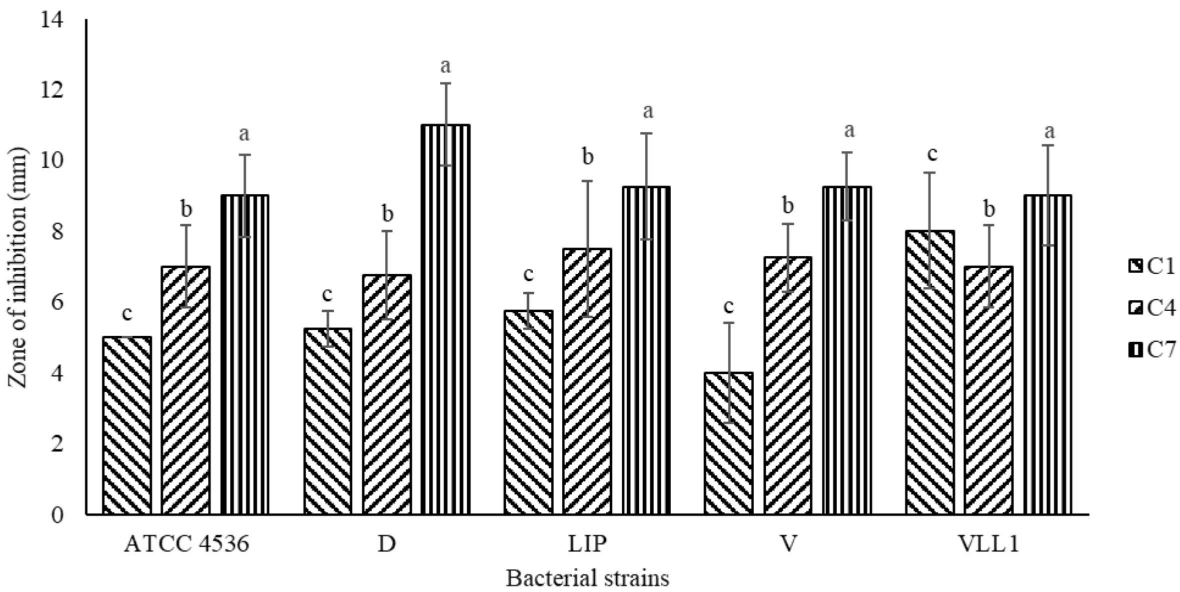

Figure 3.4: Effect of inhibitory activity of lactic acid bacteria against different strains of *C. albicans*. The bacterial strains are *L. acidophilus*- D, *L. rhamnosus*- V from the dairy origin, *L. pentosus*- LIP, *L. plantarum*- VLL1 from the bovine origin, and *L. acidophilus* ATCC 4536 from the ATCC culture collection. The *C. albicans* are *C. albicans* ATCC 10231 (C1), *C. albicans* 1051255 (C4), and *C. albicans* M0826 (C7).

The graph shows the inhibitory activity of lactic acid bacteria against *C. albicans*. Each bar is a mean of two independent replicates (n=2) and the corresponding standard deviation with different (sets of) letters as outcomes for their multiple range tests. Mean values with a different set of alphabets [a-b] for the inhibitory effect of the lactic acid bacteria against the *C. albicans* pathogens are significantly different from each other (p< 0.05).

In this test, clear zones of inhibition for *E. coli* organisms were observed. The most inhibited pathogens in respect to all the probiotic strains were EIEC (23.7 mm), EAEC (23.6 mm), EHEC (18.4 mm), and ETEC (17.3 mm) respectively. The probiotic strains showed a high level of antimicrobial activity against all *C. albicans*. Clear and large zones were observed. The antimicrobial activity throughout the probiotic strains was against C7 (9.5 mm), C4 (7.1 mm), and C1 (5.6 mm). There was no antimicrobial activity observed against pathogens induced by the supernatant. There were no zones of inhibition when filtered supernatant and filtered supernatant with pH adjusted to 7 was used. Overall, the *Lactobacillus* strains (D, V, LIP, ATCC 4536, and VLL1) had significantly good antibacterial activities ($p<0.05$) as determined by the dual agar method.

3.3.3.5 ANTIBIOTIC SUSCEPTIBILITY

Table 3.4: Effect of antibiotic susceptibility testing on lactic acid bacteria from bovine and dairy sources

Antibiotics	ATCC 4536	D	V	LIP	VLL1
Gentamicin (10µg)	S	S	S	S	S
Chloramphenicol (30µg)	S	S	S	S	S
Tetracycline (30µg)	S	S	S	MS	MS
Vancomycin (30µg)	R	R	R	R	R
Streptomycin (300µg)	S	S	S	S	S
Erythromycin (15µg)	S	S	S	S	S
Kanamycin (30µg)	R	R	R	R	R
Clindamycin (2µg)	S	S	S	S	S
Ampicillin (10µg)	S	S	S	S	S

S- Susceptible, R- Resistance, MS- Moderately Susceptible

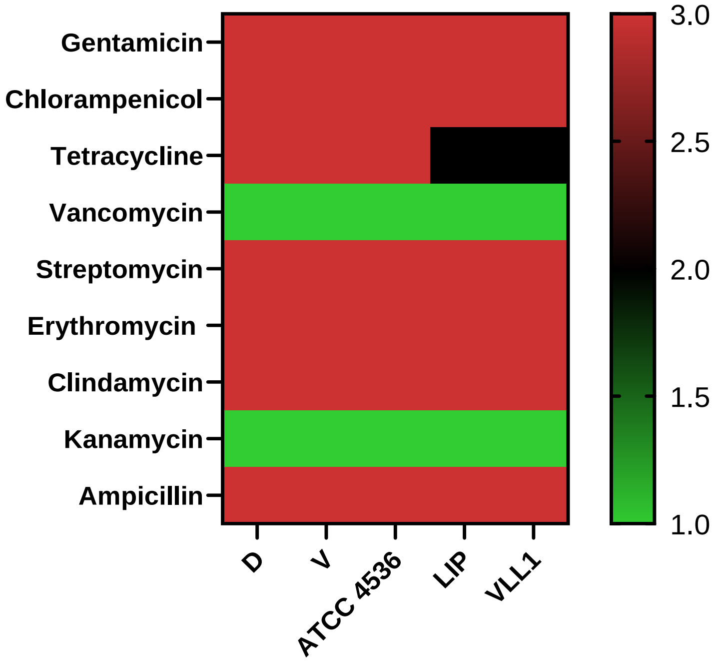

Figure 3.5: Heat map for the effect of antibiotic susceptibility testing on lactic acid bacteria from bovine and dairy sources. The bacterial strains are *L. acidophilus*- D, *L. rhamnosus*- V from the dairy origin, *L. pentosus*- LIP, *L. plantarum*- VLL1 from the bovine origin, and *L. acidophilus* ATCC 4536 from the ATCC culture collection

100% of the strains proved to be susceptible to chloramphenicol (30µg), gentamicin (10µg), erythromycin (15µg), clindamycin (2µg), and ampicillin (10µg). 60% were susceptible, and 40% moderately susceptible to tetracycline (30µg). 100% were both resistant to vancomycin (30µg) and kanamycin (30µg).

3.3.3.6 LIPASE ACTIVITY

The strains had no lipolytic activity since there was no appearance of an opaque zone around the active colonies.

3.3.3.7 HAEMOLYTIC ACTIVITY

The strains showed α- hemolysis, which was observed by the appearance of green zones. The presence of the green zones was due to the partial hydrolysis of red blood cells.

3.3.3.8 GELATINASE ACTIVITY

No gelatinase activity was observed for all the strains as the treatment and control showed no differences in colony appearance.

3.3.3.9 COMPARISON OF PROBIOTIC POTENTIALS OF THE LACTOBACILLI CULTURES

Table 3.5: Selection of dominant lactic acid bacteria as determined by probiotic properties.

Probiotic attributes	ATCC 4536	D	LIP	V	VLL1
Acid tolerance	High tolerance at pH 3 at 1 hour Low tolerance at pH 2 at 1 hour	**High tolerance at pH 5 at 3 hours** **Low tolerance at pH 3 at 3 hours**	High tolerance at pH 3 at 3 hours Low tolerance at pH 2 at 2 hours	High tolerance at pH 3 at 3 hours Low tolerance at pH 2 at 1 hour	**High tolerance at pH 5 at 3 hours** **Low tolerance at pH 2 at 1 hour**
Bile salt tolerance	High tolerance at 2 hours Low tolerance at 1 hour	**High tolerance at 3 hours** **Low tolerance at 2 hours**	High tolerance at 1 hour Low tolerance at 3 hours	High tolerance at 3 hours Low tolerance at 0 hours	**High tolerance at 1 hour** **Low tolerance at 2 hours**
Antimicrobial activity	Inhibited both *E. coli* and *C. albicans* pathogens	**Inhibited both *E. coli* and *C. albicans* pathogens**	Inhibited both *E. coli* and *C. albicans* pathogens	Inhibited both *E. coli* and *C. albicans* pathogens	**Inhibited both *E. coli* and *C. albicans* pathogens**
Antibiotic susceptibility	Resistant to vancomycin and kanamycin	**Resistant to vancomycin and kanamycin**	Resistant to vancomycin and kanamycin	Resistant to vancomycin and kanamycin	**Resistant to vancomycin and kanamycin**
Lipase activity	Negative	**Negative**	Negative	Negative	**Negative**
Gelatinase activity	Negative	**Negative**	Negative	Negative	**Negative**
Haemolytic activity	α-hemolysis	**α-hemolysis**	α-hemolysis	α-hemolysis	**α-hemolysis**

Based on comparison with the reference strain ATCC 4536 and the resistance profile on acid and bile salts, strain D and VLL1 were selected as the best strains for technological suitability evaluation. Shah (2000) stated that selecting appropriate strains depending on acid and bile tolerance can help improve the viability of the probiotic strains.

3.3.4 DISCUSSION

A concentration of 0.3% bile salts was used in this study, and its effect on the isolates was studied. 0.15- 0.3% is the typical concentration of bile salts which has been approved for selecting probiotic strains which can be used in humans (Begley *et al.*, 2006). All the isolates showed a good survival profile in 0.3% bile salts. The reference strain ATCC 4536 showed reasonable survival rate, and all the strains D, V, LIP and VLL1 showed a similar pattern. These findings are identical to those reported by Hoque *et al*. (2010), where lactobacilli isolated from yoghurt were found to multiply and grow well in 0.3% bile salt concentration. The bovine isolates were more dominant in resisting bile salts than dairy isolates. Jose *et al.* (2015) stated that bovine isolates could be able to thrive because they are subjected to the bovine intestine, where they are exposed to many stressful conditions.

The antimicrobial activity showed the lactic acid bacteria were able to inhibit different strains of diarrheagenic *E. coli*. The inhibitory activity against diarrheagenic *E. coli* by lactobacilli has been studied. This included EAEC (Lin *et al.*, 2009), EIEC (Resta-Lenert & Barrett, 2003), EHEC (Medellin-Peña *et al.*, 2007), EPEC (Parassol *et al.*, 2005), and ETEC (Wellock *et al.*, 2008). The *E. coli* pathogens were inhibited in the order EIEC (23.7 mm), EAEC (23.6 mm), EHEC (18.4 mm), and ETEC (17.3 mm). These results are similar to those reported by Davoodabadi et al. (2015) in which *L. fermentum* (S1, S8, and S17), *L. plantarum* S17, and *L. rhamnosus* S19 were found to inhibit diarrheagenic *E. coli.* The mechanism by which lactobacilli inhibited diarrheagenic *E. coli* was through secreting organic acids and hydrogen peroxide (Davoodabadi *et al.*, 2015). The strains also inhibited *C. albicans* in the order C7 (9.5 mm), C4 (7.1 mm), and C1 (5.6 mm). *L. acidophilus* and *L. plantarum* were shown to have an antagonistic effect against *Candida* spp. (Ogunshe *et al*., 2011). *L. plantarum* isolated from ogi (traditionally fermented maize) was also found to inhibit *C. albicans* (Oluwafemi & Adetunji, 2011). In this study, bovine isolates inhibited the growth of these pathogens more than dairy isolates. According to Jose *et al.* (2015), this is not likely to happen as the bovine isolates can co-exist with the pathogens or during incidences of infection by these pathogens on the host form from which the strains came from, the bovine isolates will have to compete *in vivo*.

Probiotics should be able to thrive when subjected to the gastric environment for them to access the small intestine, colonise the host and impart health benefits. *Lactobacillus* species have been considered to be inherently acid resistant (Tannock, 2004). Even though changes might be encountered among species and strains, the organisms show higher sensitivity when subjected to pH values less than 3 (Rönkä *et al.*, 2003). Henceforth, tolerance to acidity is considered when selecting potential probiotic strains. **A**ll the isolates showed they could tolerate an environment low in pH. However, there was a decline in viability when the pH was reduced. In similar studies, it was demonstrated that strains could survive exposure to MRS broth adjusted to pH 3 and a rapid decline in viability was experienced in MRS broth adjusted to pH 2 (Mishra & Prasad, 2005). This characteristic has been investigated in several probiotic bacteria, where a rapid decline in viability has been detected at pH 2 and below (Gupta *et al*., 1996). Charteris *et al*. (1997) stated that enteric lactobacilli could survive pH 3 for limited hours and several minutes at pH 2 while at pH 1 all viable lactobacilli are destroyed. All the strains tolerated pH 3 and pH 5. This is a suitable case because pH in the stomach can change from 1-2 during fasting and rise to 4- 5 after meal consumption (Chou & Weimer, 1999; Ranadheera *et al*., 2012).

The strains all managed to secrete bile salt hydrolase enzymes which facilitate the deconjugation of bile salts. The deconjugated bile salts are less soluble when the pH is low, and they precipitate because of lactic acid fermentative metabolism (Tahri *et al*., 1995). Co-precipitation with deconjugated bile salts may be linked to the elimination of cholesterol from the medium broth (Dashkevicz & Feighner, 1989). A decline in levels of cholesterol is experienced in the host cells due to microbial BSH activity. Deconjugated bile acids are less soluble, so they are absorbed less from the intestinal ileum as compared to conjugated bile acids. This leads to free bile being removed through the intestinal tract. Therefore, the level of serum cholesterol is reduced through a reduction in cholesterol absorption through the intestinal lumen. (Begley *et al*., 2006).

100% of the strains were resistant to vancomycin and kanamycin. Natural resistance to several antibiotics, especially vancomycin, is a characteristic experienced in lactobacilli (Bernardeau *et al*., 2008). Elisha and Courvalin (1995) went on further to explain that homofermentative lactobacilli are vancomycin sensitive, several lactic acid bacteria such as *L. plantarum, L. salivarius, L. acidophilus, and L. casei* are naturally resistant to this drug which is contributed by D-alanine ligase enzymes. 100% of the strains proved to be susceptible to chloramphenicol (30µg), gentamicin (10µg), erythromycin (15µg), clindamycin (2µg), and ampicillin (10µg). These results show a similar trend to Charteris et al. (1998) in which the *Lactobacillus* were susceptible to erythromycin, clindamycin, and chloramphenicol but were resistant to gentamicin and ampicillin. 60% were susceptible, and 40% moderately susceptible to tetracycline (30µg). The resistance of probiotics to certain antibiotics may result in therapeutic and preventive roles in controlling some intestinal infections (Chaudhary & Saharan, 2018). Antibiotic resistance can be vital for survival when co-administered drugs are applied (Jose *et al.*, 2015). The genes responsible for resistance should be natural and non- transferrable to other bacteria (Jose *et al.*, 2015). The strains from bovine origin were moderately susceptible to tetracycline than those of dairy origin. A typical example is a condition called postpartum metritis, which is observed in cows. When treating this condition, antibiotics have not been effective, and traces are present in milk (Abe *et al.*, 1995; Otero *et al.*, 2006). This means that bovine isolates are exposed to antibiotics most of the times and possession of non- transferrable resistance will aid in *vivo* survival.

Regarding safety concern, a potential probiotic bacterium should not cause breaking down of red blood cells. In nature, lactobacilli are usually non- haemolytic (Jose *et al.*, 2015). The strains showed α- hemolysis which is partial hydrolysis of red blood cells. This is in total disagreement with other reports of bifidobacteria and lactic acid bacteria which confirmed in nature they are non- hemolytic (Santini *et al.*, 2010). This is a significant safety concern because the presence of positive haemolytic activity destroys the underlying epithelial layer (Tanasupawat *et al.*, 2015). In selecting probiotic strains, the absence of haemolytic activity is a well-thought safety criterion (Joint, 2002). All the isolates were lipase and gelatinase negative. Positive gelatinase activity which is regarded as the ability to breakdown gelatine results in breaking down of the protective lining of the gastrointestinal tract (Marroki & Bousmaha-Marroki, 2014). A strain is non- virulent when there is an absence of lipase, gelatinase, and haemolytic activity, indicating its selection as a probiotic strain (Marroki & Bousmaha-Marroki, 2014).

3.3.5 CONCLUSION

The study showed that the isolates from bovine origin had a slightly improved tolerance to stressful conditions typical inhibitory activity against pathogens and exposure to bile salts. The probiotic characteristics evaluated from the dairy and bovine sources proved that they can be further exploited in fermentation and food industry. Valuable information has been provided on bovine and dairy isolates which will be key in identifying potential probiotic strains which can be applied in the cosmetic and pharmaceutical industry. Based on the study reports, two strains *L. acidophilus (from dairy source)* and *L. plantarum* (from bovine source) were selected for the next phase of the study.

3.4 DETERMINING THE EFFECT OF YOGHURT STARTER CULTURES ON THE SURVIVAL OF PROBIOTICS DURING AND STORAGE

ABSTRACT

Probiotic organisms in yoghurt must be viable and above the therapeutic minimum to provide health benefits. In this study, the effect of *S. thermophilus* on *L. acidophilus*, *L. plantarum*, and *L. rhamnosus* was determined over 28 days of shelf-life storage. Yoghurt samples were prepared and inoculated with *L. acidophilus*, *L. plantarum*, and *L. rhamnosus*. The yoghurt was stored for 28 days under refrigeration at 4 °C. *S. thermophilus* acted as a growth promoter which resulted in increased cell population levels among the probiotics. All of the probiotics, *L. plantarum*; *L. acidophilus* and *L. rhamnosus*, were positively affected by the yoghurt starter cultures during fermentation. They were able to keep viability above the therapeutic minimum after storage for 28 days at 4 °C.

3.4.1 INTRODUCTION

Monitoring the survival of probiotic bacteria and yoghurt cultures in yoghurt has not been taken into full consideration by the manufactures in terms of the number of viable cells present during storage which has resulted in products reaching the market containing limited amounts of viable bacteria which range from a few to several hundred per gram of product and thereby not delivering any health benefits (Viljoen & Lourens-Hattingh, 2002). The most vital parameter is to evaluate the number of viable organisms during assessing the quality of the products by counting probiotic bacteria (bifidobacteria and *L. acidophilus*) and yoghurt cultures (*L. delbrueckii* spp. *bulgaricus* and *S. thermophilus*) differentially (Dave, 1998). To gain any health benefits from the consumption of yoghurt, it should contain 10^6 cells which are viable per ml during storage till the expiry date that is after one month since the day of manufacture. The proposed concentration for probiotic bacteria to provide health benefits is 10^6 CFU/g of a product (Kemsawasd & Chaikham, 2020).

Though the information regarding the minimum concentration which is effective is still insufficient, probiotic products are generally required to have a minimum concentration of 10^6 CFU/ mL or gram, and for the probiotic effect to be delivered to the consumer a total of 10^8 to 10^9 probiotic microorganisms need to be daily consumed (Kechagia *et al*., 2013). However, in South Africa, the department of agriculture, forestry, and fisheries (DAFF) on the agricultural product standards act 1990, (ACT No, 119 OF 1990) states that "yoghurt with added foodstuff and or fruit shall and contain at least 10^7 colony forming units per gram (CFU/g) of viable yoghurt culture micro-organism per gram of a product" and " if a specific micro-organism is used in conjunction with the sale of any type of yoghurt with added foodstuff and fruit, and where the content claim is made in the labelling that refers to the presence of such specific micro-organism that has been added to supplement the yoghurt culture, it shall contain at least 10^7 colony forming units per gram (CFU/g) of that specified micro-organism."

The objective of this study was to determine the effect of yoghurt starter cultures on the survival of probiotics with the aim of producing a yoghurt that has therapeutic benefits.

3.4.2 MATERIALS AND METHODS

3.4.2.1 ACTIVATION OF PROBIOTICS AND STARTER CULTURES

L. plantarum (strain VLL1) from bovine and *L. rhamnosus* (strain V), and *L. acidophilus* (strain D) from dairy origin (controls) were cultured in 9 mL MRS broth (De Man *et al*., 1960). Incubation was carried out at 37 °C for 16- 18 hours. The cells were harvested by centrifugation at 5000 × g for 10 minutes at 4 °C. The cell-free supernatant was discarded and was washed once with sterilised phosphate buffer saline (0.1M NaOH; pH 7). The culture biomass was used for inoculation. A lyophilised yoghurt starter (YO- MIX, Danisco, Madison, WI, USA), which contained a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* was used to prepare the yoghurt samples (Mani-López *et al*., 2014). 1000 mL of UHT milk was distributed into four 250 mL Schott bottles and sterilised at 121 °C for 5 minutes and cooled to 37- 40 °C in a water bath. The sterilised milk was inoculated respectively with the three probiotics, and one bottle was inoculated with 1 g of YO- MIX culture (Danisco, Madison, WI, USA). The bottles were incubated at 37- 40 °C in a water bath for 24 hours. The experimented was repeated once.

3.4.2.2 PREPARATION OF FERMENTED MILK (CONTROLS)

Fermented milk, that served as controls, were prepared using 400 mL of UHT milk, 4% skim milk powder, 3% sucrose, 0.4% pectin, 0.09% xanthan gum, and 0.6% gelatine (Aryana & Olson, 2017). The ingredients were mixed and autoclaved at 121 °C for 5 minutes and cooled to 37- 40 °C in a water bath A total of four 500 mL Schott bottles were prepared and inoculated respectively, with the three probiotics in a 2% ratio, and one bottle was inoculated with 2% yoghurt culture. The bottles were left to ferment in a water bath at 37 °C for 6- 8 hours until the pH ranged from 4.5- 4.6.

3.4.2.3 PREPARATION OF YOGHURT

Yoghurt was prepared using 400 ml UHT milk**,** 4% skim milk powder, 3% sucrose, 0.4% pectin, 0.09% xanthan gum, and 0.6% gelatine (Aryana & Olson, 2017). The ingredients were mixed and autoclaved at 121°C for 5 minutes and cooled to 37- 40 °C in a water bath. A total of six 500 mL Schott bottles were prepared. Upon cooling, two bottles were inoculated with 2 % *L. rhamnosus* and 2% yoghurt culture. The other two bottles were inoculated with 2 % *L.*

acidophilus and 2% yoghurt culture, and the remaining two were inoculated with 2% *L. plantarum* and 2% yoghurt culture. The bottles were left to ferment in a water bath at 37 °C for 6- 8 hours until the pH ranged from 4.5- 4.6.

3.4.2.4 STORAGE

The yoghurt samples, together with fermented milk (controls), were kept in a refrigerator for 28 days at 4 °C. Enumeration of viable cells of *L. acidophilus*, *L. plantarum*, *L. rhamnosus* and *S. thermophilus* was done at 7, 14, 21, and 28 days. *L. delbrueckii* ssp. *bulgaricus* could not be enumerated because it could not be differentiated from other lactic acid bacteria.

3.4.2.5 ENUMERATION OF VIABLE CELLS OF *S. THERMOPHILUS*, *L. PLANTARUM*, *L, RHAMNOSUS*, AND *L. ACIDOPHILUS*

The viable cells of *S. thermophilus*, *L. plantarum*, *L, rhamnosus*, and *L. acidophilus* were enumerated at day 7, 14, 21 and 28 of storage at 4 °C. The cells were enumerated by taking 10 mL of yoghurt or control fermented milk per each sample and suspended in 90 mL buffered peptone water. M17 agar (Sigma, Modderfontein, South Africa) was prepared and supplemented with 0.15% lactose (Birollo *et al.*, 2000). MRS agar was also prepared and supplemented with 0.15% bile salts (Mortazavian *et al.*, 2007). Pour plate method was used, and 0.1 mL aliquot dilution was plated in duplicates over the different media. The plates were incubated for 48- 72 hours at 37 °C. The M17 agar was used to enumerate *S. thermophilus* and MRS bile agar to selectively enumerate mixed probiotic bacteria in the presence of yoghurt cultures (Sohrabvandi *et al.*, 2012). In the commercial production of fermented milk products yoghurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) are added to probiotic cultures as adjunct cultures. Therefore, they can affect the selective enumeration of the probiotic strains in the final products. Adding bile salts (0.15% w/w) in the culture media avoids the growth of the yoghurt bacteria after incubation for at least 72 hours at 37 °C (Mortazavian *et al.*, 2007; Vinderola & Reinheimer, 1999). The cell walls of yoghurt cultures are hydrolysed in the presence of bile salts, while probiotics are resistant to bile (Mortazavian *et al.*, 2007).

3.4.2.6 STATISTICAL ANALYSIS

The experimental observations were recorded as mean ± SD (standard deviation). Analysis of variance (ANOVA) was used to determine the effect of storage on *S. thermophilus*, *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* over 28 days at 4 °C at 0.05% level of significance. Duncan's multiple range was used to calculate for significant differences between means for duplicates and repetitions. The software used was Stat graphics centurion version 18 (Stat graphics Technologies 18).

3.4.3 RESULTS

3.4.3.1 EFFECT OF *S. THERMOPHILUS* AND STORAGE ON *L. PLANTARUM* IN YOGHURT

Table 3.6: Analysis of variance on the effect of storage on *S. thermophilus*.

Source	P-Value
MAIN EFFECTS	
A: *S. thermophilus*	0.0000
B: Storage time	0.0001
INTERACTIONS	
AB	0.0106

The table below shows the main effects and interactions between *S. thermophilus* (A) and storage time (B) (P <0.05).

Table 3.7: Effect of storage on *S. thermophilus* in yoghurt at 4 °C (n=4)

Storage time (days)	*S. thermophilus* (control)	*S. thermophilus* (treatment)
7	8.0 ± 0.1^{b}	8.2 ± 0.1^{a}
14	8.0 ± 0.0^{b}	8.0 ± 0.0^{b}
21	8.0 ± 0.0^{b}	8.2 ± 0.1^{a}
28	7.9 ± 0.0^{c}	8.1 ± 0.0^{b}

Values are mean ± SD of two duplicates and repetitions (n= 4)

$^{a-c}$ Means within a column with different superscripts are significantly different (P< 0.05)

Although the statistical analyses indicated a significant difference in the growth of *S. thermophilus* during storage, this is not of practical significance (Table 3.6). The levels decreased for both treatments over 28 days at 4 °C with only 0.1 log. The *S. thermophilus* counts remained comparable between the two treatments during storage (Table 3.7).

Table 3.8: Analysis of variance on the effect of storage on *L. plantarum*.

Source	P-Value
MAIN EFFECTS	
A: *L. plantarum*	0.0000
B: Storage time	0.2051
INTERACTIONS	
AB	0.0153

The table below shows the main effects and interactions between *L. plantarum* (A) and storage time (B) (P <0.05).

Table 3.9: Effect of storage on *L. plantarum* over in yoghurt at 4 °C (n=4)

Storage time (days)	*L. plantarum* (control)	*L. plantarum* (treatment)
7	6.9 ± 0.2^{c}	8.1 ± 0.2^{a}
14	6.8 ± 0.0^{c}	8.2 ± 0.3^{a}
21	6.8 ± 0.1^{c}	8.1 ± 0.1^{a}
28	7.1 ± 0.1^{b}	8.1 ± 0.1^{a}

Values are mean ± SD of two duplicates and repetitions (n= 4)

$^{a\text{-}c}$ Means within a column with different superscripts are significantly different (P< 0.05)

There was a significant difference in the growth between the two treatments over time (p<0.05) (Table 3.8). The *L. plantarum* levels in the control were lower than those of the *L. plantarum* combined with the yoghurt cultures after 7 days of storage at 4 °C. This trend continued throughout the storage period, with significantly (p<0.05) higher levels of *L. plantarum* on day 28 of storage (Table 3.9). This contrasts with the *S. thermophilus* that was not affected at all by the inclusion of *L. plantarum* during fermentation and storage but seemed to affect the growth of *L. plantarum*

3.4.3.2 EFFECT OF *S. THERMOPHILUS* AND STORAGE ON *L. ACIDOPHILUS* IN YOGHURHT

Table 3.10: Analysis of variance on the effect storage on *S. thermophilus*.

Source	P-Value
MAIN EFFECTS	
A: *S. thermophilus*	0.0005
B: Storage time	0.0217
INTERACTIONS	
AB	0.0347

The table below shows the main effects and interactions between *S. thermophilus* (A) and storage time (B) (P <0.05)

Table 3.11: Effect of storage on *S. thermophilus* in yoghurt at 4 °C (n=4)

Storage time (days)	*S. thermophilus* (control)	*S. thermophilus* (treatment)
7	8.0±0.1^{a}	8.0±0.0^{bc}
14	8.0±0.0^{ab}	7.9±0.1^{c}
21	8.0±0.0^{ab}	7.9±0.0^{c}
28	7.9±0.0^{c}	7.9±0.1^{c}

Values are mean ± SD of two duplicates and repetitions (n= 4)

$^{a-c}$ Means within a column with different superscripts are significantly different (P< 0.05)

Similar to the yoghurt treatments with *L. plantarum* the statistical analyses indicated a significant difference in the growth of *S. thermophilus* during storage in combination with *L. acidophilus*, this is not of practical significance (Table 3.10). The levels decreased for both treatments over 28 days at 4 °C with only 0.1 log. The *S. thermophilus* counts remained comparable between the two treatments during storage (Table 3.11).

Table 3.12: Analysis of variance on the effect of storage on *L. acidophilus*

Source	P-Value
MAIN EFFECTS	
A: *L. acidophilus*	0.0000
B: Storage time	0.0081
INTERACTIONS	
AB	0.0023

The table below shows the main effects and interactions between *L. acidophilus* (A) and storage time (B) (P <0.05)

Table 3.13: Effect of storage on *L. acidophilus* in yoghurt (n=4)

Storage (days)	*L. acidophilus* (control)	*L. acidophilus* (treatment)
7	6.8 ± 0.0^{d}	7.8 ± 0.1^{bc}
14	6.8 ± 0.0^{d}	7.9 ± 0.1^{b}
21	6.9 ± 0.1^{d}	7.8 ± 0.1^{c}
28	6.8 ± 0.1^{d}	8.1 ± 0.0^{a}

Values are mean ± SD of two duplicates and repetitions (n= 4)

$^{a-c}$ Means within a column with different superscripts are significantly different (P< 0.05)

There was a significant difference in the growth between the two treatments over time (p<0.05) (Table 3.12). The *L. acidophilus* levels in the control were lower than those of the *L. acidophilus* combined with the yoghurt cultures after 7 days of storage at 4 °C. This trend continued throughout the storage period, with significantly (p<0.05) higher levels of *L. acidophilus* on day 28 of storage (Table 3.13). However, the *L. acidophilus* levels were slightly lower than the counts of *L. plantarum* in the yoghurt but reached similar levels at day 28 of storage. Although *S. thermophilus* was not affected at all by the inclusion of *L. acidophilus* during fermentation and storage, as with *L. plantarum* it seemed that the growth and survival of *L. acidophilus* was enhanced by the yoghurt cultures in comparison to the control.

3.4.3.3 EFFECT OF *S. THERMOPHILUS* AND STORAGE ON *L. RHAMNOSUS* IN YOGHURT

Table 3.14: Analysis of variance on the effect of storage on *S. thermophilus*

Source	P-Value
MAIN EFFECTS	
A: *S. thermophilus*	0.0107
B: Storage time	0.0021
INTERACTIONS	
AB	0.0002

The table below shows the main effects and interactions between *S. thermophilus* (A) and storage time (B) ($P < 0.05$)

Table 3.15: Effect of storage on *S. thermophilus* in yoghurt (n=4)

Storage (days)	*S. thermophilus* (control)	*S. thermophilus* (treatment)
7	8.0 ± 0.1^{b}	8.1 ± 0.1^{a}
14	8.0 ± 0.0^{b}	7.9 ± 0.1^{c}
21	8.0 ± 0.0^{b}	8.1 ± 0.0^{b}
28	7.9 ± 0.0^{c}	8.1 ± 0.0^{ab}

Values are mean ± SD of two duplicates and repetitions (n= 4)

$^{a-c}$ Means within a column with different superscripts are significantly different ($P < 0.05$)

Similar to the yoghurt treatments with *L. plantarum* as well as *L. acidophilus* the statistical analyses indicated a significant difference in the growth of *S. thermophilus* during storage in combination with *L. rhamnosus*, this is not of practical significance (Table 3.14). The *S. thermophilus* counts remained comparable between the two treatments during storage and stable during the 28 days of storage at 4 °C (Table 3.15).

Table 3.16: Analysis of variance on the effect of storage on *L. rhamnosus*

Source	P-Value
MAIN EFFECTS	
A: *L. rhamnosus*	0.0000
B: Storage time	0.7090
INTERACTIONS	
AB	0.0873

The table below shows the main effects and interactions between *L. rhamnosus* (A) and storage time (B) (P <0.05)

Table 3.17: Effect of storage on *L. rhamnosus* in yoghurt (n=4)

Storage (days)	*L. rhamnosus* (control)	*L. rhamnosus* (treatment)
7	6.6 ± 0.1^{b}	8.1 ± 0.0^{a}
14	6.7 ± 0.1^{b}	8.1 ± 0.1^{a}
21	6.6 ± 0.1^{b}	8.1 ± 0.1^{a}
28	6.6 ± 0.1^{b}	8.1 ± 0.1^{a}

Values are mean ± SD of two duplicates and repetitions (n= 4)

$^{a-c}$ Means within a column with different superscripts are significantly different (P< 0.05)

There was no difference in the growth between the two treatments over time as both maintained similar levels as determined on day 7 of storage throughout, up to 28 days (Table 3.16). The *L. rhamnosus* levels in the control were significantly lower (p<0.05) than those of the *L. rhamnosus* combined with the yoghurt cultures. This trend continued throughout the storage period. In contrast to *L. plantarum* and *L. acidophilus*, *L. rhamnosus* did not grow during storage, but the levels remained stable (Table 3.17). As indicated with the yoghurt-*L. acidophilus* and yoghurt-*L. plantarum* treatments the *S. thermophilus* was not affected at all by the inclusion of *L. rhamnosus* during fermentation and storage. Similarly, to previously stated, it seemed that the growth and survival of *L. rhamnosus* was enhanced by the yoghurt cultures in comparison to the control.

3.4.4 DISCUSSION

We showed during our study that all 3 of the probiotics, *L. plantarum*; *L. acidophilus* and *L. rhamnosus,* were positively affected by the yoghurt starter cultures during fermentation. This is in contrast to their growth in the control fermented milk. Tharmaraj and Shah (2003) explained that several probiotic organisms are added into dairy-based foods, but they grow very slowly during fermentation in milk. This has been solved by adding yoghurt cultures (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) together with probiotics. The yoghurt cultures are added to the fermentation process because they grow rapidly. (Shah & Jelen, 1990). Gopal (2011) explained that in terms of growth rate *S. thermophilus* grows quickly than *L. acidophilus.* This is important because of the metabolites formed by *S. thermophilus,* which act as growth promoters to *L. acidophilus.* The growth promoters include substances such as carbon dioxide and formic acid.

The cell population levels of *L. rhamnosus* and *L. plantarum* treatment remained constant during the storage time. However, *L. acidophilus* showed a slight increase and according to Gopal (2011) the stability of the *L. acidophilus* cells added to yoghurt are affected by hydrogen peroxide that is produced by lactobacilli found in the yoghurt cultures. *L. bulgaricus* and *S. thermophilus* produce little amounts of lactic acid from lactose fermentation since they are active at refrigeration temperatures (Laroia & Martin, 1991). The cell viability of *L. acidophilus* is affected by this acid production after incubation (Ishibashi & Shimamura, 1993). The *L. plantarum* isolated from bovine sourcces perfomed simillar to well known probiotics like *L. acidophilus* and *L. rhamnosus.* However, there is no information of the use of *L. plantarum* from bovine sources together with starter cultures.

The growth of *S. thermophilus* was not affected by storage time or by the addition of any of the probiotics. This may be due to the rapid onset of growth by *S. thermophilus* (Tharmaraj & Shah, 2003). It seems as though *S. thermophilus* acted as a growth promoter to *L. acidophilus,* *L. rhamnosus,* and *L. plantarum* increasing the cell population levels above the therapeutic minimum. In South Africa, the department of agriculture, forestry, and fisheries (DAFF) on the agricultural product standards act 1990, (ACT No, 119 OF 1990) states that "yoghurt with added foodstuff and or fruit shall and contain at least 10^7 colony forming units per gram

(CFU/g). The probiotics included in our study were able to attain the levels indicated in the agricultural products standard act.

3.4.5 CONCLUSION

Bovine sources could potentially be a source of probiotics. Probiotic lactobacilli are not affected by the initial fermentation of yoghurt. Yoghurt is a good vehicle for delivering therapeutic probiotics at required minimum.

CHAPTER 4: GENERAL DISCUSSION

4.1 CRITICAL REVIEW OF METHODOLOGY

The preliminary identification of the lactic acid bacteria isolates and the confirmation of identities of the strains was done using phenotypic methods which included Gram staining, catalase slide test, oxidase test, and carbon dioxide production from glucose. There are limitations to phenotypic methods which include poor reproducibility, ambiguity experienced in the techniques contributed by enormous plasticity in bacterial growth, and large scale reduced discriminatory power. Also, the whole information partnering to the potential of the genome is never expressed, that is gene expression is directly affected by environmental conditions for example growth conditions experienced in the laboratory (Mohania *et al.*, 2008). Tannock *et al*. (1999) stated that it is challenging to identify and differentiate lactic acid bacteria using phenotypic methods because in most cases it requires the evaluation of bacterial characteristics outside of those reported in common fermentation assessments. Schleifer *et al*. (1995) explained that considerable variations in biochemical characteristics experienced among strains which are believed to belong to the same species makes it difficult to characterise *Lactobacillus* at species level using phenotypic methods. The identification of the lactic acid bacteria could have employed molecular techniques which have shown to be more reliable. Some of the methods include DNA- DNA hybridisation, amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PEGE) of rare- cutting restriction enzyme fragments, 16S rDNA sequencing, species-specific PCR (Gopal, 2011), and matrix-assisted laser desorption/ ionisation time- of- flight- mass spectrometry (MALDI- TOF- MS) (Randazzo *et al.*, 2009).

Carbon dioxide gas production from glucose classifies the lactic acid bacteria as either homofermentative or hetero-fermentative. Homofermentative lactic acid bacteria produce lactic bacteria as the only by-product of fermentation. Hetero-fermentative lactic acid bacteria produce carbon dioxide, ethanol, and lactic acid as the byproducts of fermentation. This classification plays a significant role in choosing the food matrix where the potential probiotic microorganisms may be incorporated (da Silva *et al.*, 2019), and also their functional role in the food and pharmaceutical industry. Hetero-fermentative lactic acid bacteria are of technological importance in products such as cheese and kefir where the production of gas is a critical factor (Leite *et al*., 2013; Pedersen *et al*., 2013). This classification has also led to some homofermentative species such as *L. gasseri, L. johnsonii, L. gallinarium., L. amylovarius, L. crispatus, L. acidophilus* to be tested for use as probiotics in humans (Ferreira, 2012).

The acid resistance profile of the probiotic strains was evaluated at pH 2, pH 3 and pH 5. This helped to conclude that the probiotic bacteria can be able to pass through the stomach where the pH can be very low from 1.5 to 2 (Dunne *et al*., 2001) before they reach the intestinal tract. They must survive the lowest pH conditions for them to be used in industrial food preparations. The pH was also selected based on the reason that the pH in the gut ranges from 2- 5 under normal conditions and can reach up to pH 1 when fasting (Jose *et al*., 2015). A concentration of 0.3% bile salts was used in the study because it has been approved for selecting probiotic strains which can be used in humans (Begley *et al*., 2006). It is of great importance to evaluate how probiotic organisms tolerate bile salts because they act as antimicrobial molecules which have a significant influence on the intestinal flora. The bile is produced by the hepatocytes in the liver.

Antibiotic resistance was established using a phenotypic technique, and it is difficult to establish if the observed antibiotic resistance is because of transferrable resistance genes. Molecular biology approaches may be used to confirm the decision relating to transferability of antibiotic resistance if they are any present. The antibiotics were chosen according to the regulations of the European Food Safety Authority (EFSA, 2012). According to Additives and Feed (2012), the minimum inhibitory concentration (MIC) of the antimicrobial denoted as ug/ mL mg/ L should be evaluated for the following substances chloramphenicol, tetracycline, clindamycin, erythromycin, streptomycin, kanamycin, gentamicin, vancomycin, ampicillin, and in specific scenarios trimethoprim, sulphonamide, nalidixic acid, apramycin, and tylosin. These antimicrobials are selected to maximise the detection of genotypes which are resistant to the frequently used antimicrobials by assessing the resistant phenotypes.

Antimicrobial activity was carried out to evaluate the extent to which the lactic acid bacteria inhibit pathogens in the gut (Zommiti *et al*., 2018). The inhibition effect was carried out using probiotic cells and cell-free supernatant. The cell-free supernatant measured the bacteriocin activity of the isolates (Chaudhary & Saharan, 2018). The probiotic strains can be exploited in controlling foodborne pathogenicity and the safe biopreservation of foodstuffs using natural antimicrobial agents such as bacteriocin. The test pathogens included diarrheagenic *E.coli* and *C. albicans*. Pathogens such as *S. aureus*, *B. subtilis*, *S. pyogenes*, *P. aeruginosa*, and *L. monocytogenes* (Chaudhary & Saharan, 2018), should have been included since they participate in food spoilage. The ability of the isolates to reduce blood cholesterol levels was done using bile salt hydrolase activity (Begley *et al*., 2006). Probiotic properties such as survival in simulated gastric juice, autoaggregation, co-aggregation, and adhesion properties could have also been evaluated since they play a role in aiding beneficial microbes to colonise the human gut.

The safety assessment also included haemolytic activity as well as gelatinase activity. The haemolytic activity ensures that the probiotic cells do not breakdown red blood cells (Jose *et al.*, 2015). The lipolytic and gelatinase activity detect the enzymes lipase and gelatinase, which can degrade the epithelial layer lining, which is made up of lipids (Marroki & Bousmaha-Marroki, 2014). The fate of the probiotic strains, when used with regular fermenting bacteria during fermentation as well as storage, was determined by analysing the cell population levels of viable probiotic organisms and yoghurt cultures to ensure they remained above the therapeutic minimum during refrigerated storage. This ensures the viability remains above the therapeutic minimum for health benefits to be delivered. This was done by differentially enumerating and counting viable cells of the probiotic organisms and yoghurt cultures using MRS bile agar and M17 agar (Sohrabvandi *et al.*, 2012). However, analysis such as determining titratable acidity, proximate analysis, and sensory evaluation could have also been carried out. This could have provided information about the nutritional quality and consumer perception of the yoghurt. The location and growth of probiotic bacterial colonies within the yoghurt could also be evaluated using microscopy techniques to show how they would be embedded in the food matrix. Hickey *et al*. (2015) explained that assessing the location of bacteria in the food matrices of dairy products such as cheese and yoghurts is valuable because of the possible effects exhibited by these bacteria and their enzymes on the ripening of these products, especially at the microscopic level.

4.2 MAIN RESEARCH FINDINGS

Based on the identification using phenotypic methods, the isolates were confirmed to be lactic acid bacteria since they were gram-positive, catalase and oxidase negative. Holzapfel *et al.*

(2001) explained that lactic acid bacteria are non- spore-forming, catalase-negative, gram-positive, and strictly fermentative organisms which produce lactic acid as the standard product of carbohydrate metabolism. The microorganisms were able to produce carbon dioxide from glucose metabolism, indicating heterofermentative metabolism. Hetero-fermentative lactic acid bacteria produce carbon dioxide, ethanol, and lactic acid as the byproducts of fermentation. The acid resistance profile of the isolates showed their viability was reduced at pH 2 at time 0 up to 3 hours, but they were able to tolerate pH 3 and pH 5. The isolates showed an excellent resistance profile towards 0.3% bile salt concentration. The typical strains from bovine origin were good at resisting bile acids than those of dairy origin. The probiotic live cells were able to inhibit *C. albicans* and diarrheagenic *E. coli.* Still, the cell-free supernatant failed to inhibit the pathogens as there were no zones of inhibition recorded. On antibiotic susceptibility, the isolates were resistant to vancomycin and kanamycin and susceptible to gentamicin, chloramphenicol, streptomycin, erythromycin, clindamycin, and ampicillin. Lactobacilli are naturally resistant to vancomycin and kanamycin (Danielsen & Wind, 2003); the resistance is due to the presence of D-alanine ligase-related group of enzymes (Elisha & Courvalin, 1995). The diary isolates were susceptible to tetracycline while the bovine strains were moderately susceptible.

The isolates showed the ability to secrete bile salt hydrolase enzymes which help in regulating blood cholesterol levels. They were not able to secrete lipase and gelatinase enzymes which are known to degrade the epithelial lining. The lactic acid bacteria also showed α- haemolysis that is they partially break down red blood cells which might affect their capacity to carry oxygen around the body The growth rate of *L. acidophilus*, *L. rhamnosus*, and *L. plantarum* was increased because of *S. thermophilus*, which acted as a growth promoter. The probiotics showed an increase in the number of cell population levels from day 7 to day 28 under storage at 4 °C.

CHAPTER 5 RECOMMENDATIONS AND CONCLUSIONS

The main aim of this study was to evaluate the safety and characterise the probiotic properties of lactic acid bacteria from the bovine and dairy origin for application in yoghurt. The first objective was to identify and confirm if the isolates were lactic acid bacteria by using phenotypic methods. The organisms were found to be gram-positive, catalase-negative, oxidase negative and produced carbon dioxide gas from glucose. They were confirmed to be of the genus *Lactobacillus*, but no species was confirmed. The phenotypic methods can not characterise lactic acid bacteria which are believed to belong to the same species at the species level because of considerable variations in biochemical properties. Therefore, molecular techniques are recommended for identification at the species level.

The second objective was to evaluate and compare the probiotic properties of the lactic acid bacteria. The probiotic bacteria properties evaluated play a role in aiding the strains to colonise the gastrointestinal tract of the host. The lactic acid bacteria strains had the ability to tolerate acidic environments of pH 3 and pH 5 and bile salt concentration of 0.3%. Therefore, the strains can tolerate the harsh conditions of the gastrointestinal tract. The strains from bovine origin were able to survive bile salts with more population levels than that of dairy origin because they are always exposed to the bovine gastrointestinal tract where they are subjected to harsh conditions. The bacterial strains showed antimicrobial activity against the diarrheagenic *E. coli* and *C. albicans* pathogens. However, some pathogens such as *S. aureus*, *B. cereus*, *P. aeruginosa*, *S. pyogenes, and L. monocytogenes* could have been investigated since they are involved in food spoilage.

The bacterial isolates also produced bile salt hydrolase enzymes which facilitate the deconjugation of bile salts. The deconjugation of the bile salts is linked to the elimination of cholesterol. The lactic acid bacteria isolates were susceptible to gentamicin, chloramphenicol, streptomycin, erythromycin, clindamycin, and ampicillin. There were resistant to kanamycin and vancomycin. The bovine isolates were moderately susceptible to tetracycline. The resistance pattern was established using a phenotypic technique and is difficult to establish if the observed antibiotic resistance is because of transferrable resistance genes. Molecular biology approaches are recommended to confirm the transferability of resistance genes if they are present.

Furthermore, the isolates were not able to produce the enzymes lipase and gelatinase, which can destroy the epithelial lining of the gastrointestinal tract. They also showed α- haemolysis which is the partial breakdown of red blood cells. However, in nature, lactic acid bacteria are non- haemolytic. Other probiotic properties such as autoaggregation, co-aggregation, and adhesion property which deal with the ability of the probiotic bacteria to adhere to the enterocytic cellular lines of the GIT in order to show their health benefits are recommended for further study.

The third objective was to determine the effect of starter cultures on the survival of probiotics during fermentation and storage. The probiotic organism's population levels increased during storage in the presence of *S. thermophilus*. The *S. thermophilus* is known to produce substances such as formic acid and carbon dioxide, which act as growth promoters to the probiotic organisms. However, analysis such as determining titratable acidity, proximate analysis, and sensory evaluation is recommended for further study. This could provide information about the nutritional quality and consumer perception of the yoghurt.

The results of this study demonstrated that the safety assessment of lactic acid bacteria and evaluating their probiotic attributes is a valuable procedure for bacterial strains intended to be used in the food industry. In South Africa consumers are becoming more health-conscious, which has put the functional dairy-based industry under pressure of finding new bacterial strains which are safe and at the same time deliver health benefits. The market is dominated by yoghurts which contain strains *L. acidophilus* (A) and *Bifidobacterium* (B) mainly known as A-B cultures which are unstable. The dairy industry can find new bacterial strains evaluate their safety and probiotic attributes to make sure they are stable from shelf life up to consumption to ensure delivering health benefits to the consumers as recommended by the agricultural product standards act 1990.

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