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Isolation, identification and screening of potential probiotic bacteria in milk from South African Saanen goats

by

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Submitted in partial fulfilment of the requirements for the degree

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In the Faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa

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DECLARATION

I declare that the dissertation "Isolation, identification and screening of potential probiotic bacteria in milk from South African Saanen goats", which I hereby submit for the degree of Magister Scientiae at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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LIST OF ABBREVIATIONS

GIT:	Gastrointestinal tract
LAB:	Lactic acid bacteria
GRAS:	Generally Regarded as Safe
DFM:	Direct fed microbials
cfu:	Colony forming units
FDA:	Food and Drug Administration
CHD:	Coronary heart disease
BHT:	Breath hydrogen test
CRC:	Colorectal cancer
Bp:	Base pair
Kb:	Kilo base
TVBC:	Total viable bacteria count
DNA:	Deoxyribonucleic acid
rDNA:	Ribosomal Deoxyribonucleic acid
RNA:	Ribonucleic acid
H_2O_2:	Hydrogen peroxide
CO_2:	Carbon dioxide
NaCl:	Sodium chloride
PCR:	Polymerase chain reaction
HCL:	Hydrogen chloride
ARC:	Agricultural Research Council

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MALDI-TOF:	Matrix assisted laser desorption ionization-time of flight
MRS:	De Man, Rogosa and Sharpe
MRS-cysHCL:	De Man, Rogosa and Sharpe supplemented with cysteine-hydrochloride
ATCC:	American type culture collection
BSH:	Bile salt hydrolytic
NaOH:	Sodium hydroxide
MDR:	Multidrug resistant
r. p. m:	Revolutions per minute
PW:	Post- weaning
AGP:	Antibiotic Growth promoter
ml:	Millilitre
µl:	Microlitre
NCBI:	National Center for Biotechnology Information

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SUMMARY

Isolation, identification and screening of potential probiotic bacteria in milk from South African Saanen goats

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In order to meet the increasing demand for food quality and safety, the control of pathogenic microorganisms from farms to consumers remains a continuous challenge. Disease has always been a critical issue in animal production, affecting animal health and wellbeing. For several decades, antibiotics and chemotherapeutic agents have been used in animal feed to treat and prevent infectious diseases or to promote growth. However, there are concerns about the risk of development of cross-resistance and multiple antibiotic resistance in pathogenic bacteria in both human and livestock. To slow the development of resistance, some countries have restricted or banned use of antibiotics in feeds. Therefore, the need to find alternatives to growth-promoting and prophylactic uses of antibiotics is of outmost importance in agriculture. Beneficial bacteria, mainly lactic acid bacteria have been effectively used previously as feed additives in livestock to manipulate the gut microbiota in order to support animal health.

Therefore, the current study focused on isolation and characterisation of probiotic bacteria from raw goats' milk. The first part of the study aimed at isolating and identifying potential probiotic bacteria. Bacteria from raw milk were cultured onto selective media including, M17 agar and MRS agar supplemented with 0.05 g/L cysteine-hydrochloride. A total of seventeen lactic acid bacteria were isolated, and were then identified using phenotypic assays, 16S rDNA gene sequencing and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). *Lactobacillus plantarum* strains (KJ026587.1, KM207826.1, KC83663.1, and KJ958428.1) and *Pediococcus acidalactici* were obtained. Potential probiotic bacteria were identified based

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on their ability to survive in the gastrointestinal conditions that include growth at low pH and bile tolerance, production of antimicrobial compounds and adhesion to the intestinal mucosa. The second part of the study focused on *in vitro* screening of probiotic attributes in the isolates. Production of antimicrobial activity, ability to adhere to intestinal cells and survive in the gastrointestinal tract, as well as antibiotic susceptibility, were among the main probiotic properties that were analysed to assess functionality and safety of the isolates.

The *in vitro* studies revealed that the five isolates were tolerant to acidic pH and high concentrations of bile salts, which are characteristics necessary for the probiotics to survive in the gastrointestinal tract. These isolates were also found to exhibit antimicrobial activity against some of the pathogens affecting the goats' industry. The five selected LAB strains displayed resistance to vancomycin, gentamycin and nalidixic acid, but were susceptible to a broad range of other antibiotics. However, the antibiotics to which the isolates were resistant might not pose problems as it is unlikely to be transmissible between bacteria cells. Fluorescent microscopy analysis, revealed strong adhesion of the isolates to the ileum mucus following their staining with BacLight viability probe.

This study is the first in South Africa that isolated and characterised probiotic bacteria from raw goats' milk. Most studies on application of probiotics in ruminants have been performed in cows and calves, and there is very little information for lambs and goats. The results of this study suggest that the five selected bacteria could be used as potential candidates for the development of direct fed microbials and growth promoters that can be used in improving the overall health status of the goats. Formulation of the best cocktail and its use as direct fed microbials for goats will result in improved nutrition and productivity.

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INTRODUCTION

The name probiotic comes from the Greek words 'pro bios' which means 'for life'. Probiotic microorganisms are associated with the beneficial effects for humans and animals. These microorganisms contribute to intestinal microbial balance and play a role in maintaining health. According to Shah (2007) and Chow (2002), the most popular probiotic strains are represented by the following genera: Lactobacillus, Streptococcus and Bifidobacterium, but other organisms including *Enterococci* and yeasts have been used as well. Some of these strains have been chosen based on selection criteria that are believed to be important for their efficacy such as origin of the strain, *in vitro* adherence to intestinal cells, and survival during passage through the gastro-intestinal tract (GIT) (Soccol et al., 2010). Although probiotic strains can be isolated from many sources, there is evidence in literature indicating that many of these bacteria exhibit host specificity (McCoy and Gilliland, 2007).

Probiotics can be found in dairy and non-dairy products. The primary probiotic bacteria associated with dairy products have been *Lactobacillus acidophilus, Lactobacillus casei* and *bifidobacteria*. According to Cheriguene et al. (2007), raw goats' milk may be a suitable source of potential probiotic microorganisms. Goats' milk and its products are widely consumed in developing countries, and are attractive to many consumers due to their therapeutic and nutritional values (Yangilar, 2013). At present, cheese and yoghurt are the only widely available goats' milk products in the South African market. The development of different types of goats' milk products incorporating novel probiotic bacteria with satisfactory viability and functionality may prove valuable in expanding the market potential of goats' milk and fulfilling consumer needs.

Over the last three decades there has been concern over the problem of antibiotic resistance among human pathogens (Amenu, 2014). Much concern has been directed against the indiscriminate use of antibiotics in animals, with particular focus on antibiotic growth promoters. This concern has led to the publication of a number of reports from committees and groups in Europe, UK, USA and Australia. These reports emphasised the need for greater control over the use of antibiotics in veterinary medicine and animal husbandry (Barton, 2000). In 2006, Europe implemented a complete ban on the use of growth-promoting antibiotics in animal feed (Anandón et al., 2006). In agriculture, direct fed microbials also known as probiotics that are used in animal feeds are becoming accepted as potential alternatives to

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antibiotic for enhancing growth, and in selected cases, for controlling specific enteric pathogens. For these reasons the development of new and more effective products that can be licensed for animal use continues to receive considerable interest. During the last 15 years, many laboratories have worked towards the identification of probiotic candidates which can be effective against enteric pathogens. Currently, there is no universal class of probiotic bacterium. However, the most common types that have been reported to be effective involve lactic acid bacteria (LAB) (Tellez et al., 2012).

LAB produce a variety of metabolic end products with antagonistic properties against pathogens. These products include bactericidal proteins and antibiotic-like substances termed bacteriocins. Bacteriocins are considered as safe natural preservatives or biopreservatives, because it is assumed that they are degraded by proteases in the GIT (Cleveland et al., 2001). The inhibitory spectrum of several bacteriocins includes food spoilage microorganisms and/or foodborne pathogens (Schillinger et al., 1996). The discovery of nisin, the first bacteriocin utilized on a commercial scale as a food preservative dates back to the first half of last century but research on bacteriocins associated with LAB has expanded and this has resulted in the constant search for novel bacteriocins producing strains from dairy, meat, and traditional fermented food products (Chauhan, 2012).

The continuous search for novel probiotics of importance in medical, industrial and agricultural environments has spurred our interest into this study. To our knowledge, there are surprisingly no studies done on the isolation and analysis of potential probiotic bacteria from raw goats' milk in South Africa. Most studies on the microbiology of goats' milk are restricted to the identification of potential pathogenic bacteria (Little and de Louvois, 1999). This study therefore aimed at providing knowledge base as well as bridge the existing gap in knowledge with regards to probiotics from goats' milk, which could be beneficial for agriculture. This may include the use of probiotics in goats' farming as direct fed microbials and growth promoters, and possible for use in infant health due to inherent properties associated with goats' milk. The potential probiotic bacteria must be able to bestow some of the probiotic properties, such as survival in the gastrointestinal conditions particularly low pH and bile tolerance, production of antimicrobial compounds and adhesion to the intestinal mucosa.

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The specific objectives of the study were therefore:

- To isolate potential probiotic bacteria from raw goats' milk
- To identify the isolates using biochemical tests and molecular techniques
- To screen the identified bacteria for selected probiotic properties

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CHAPTER 1

Literature review

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1.1. History and definition of probiotics

The word 'probiotic' comes from Greek language 'pro bios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The concept evolved from a theory proposed by Nobel Prize winner Ellie Metchnikoff, who suggested that the long life of Bulgarians resulted from their consumption of fermented milk products which consists of rod shaped bacteria. The term probiotic was first coined by Lilly and Stillwell (1965) and it was used to describe 'substances secreted by one microorganism which stimulate the growth of another. Thus explains why it was contrasted with the term antibiotic.

The definition of probiotics has evolved over the years. The word 'probiotic' has also been used to describe "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974). This general definition was, however, unsatisfactory because of the use of an imprecise word such as 'substances' which might include a variety of supplements, including antibiotics. In 1989, Fuller attempted to improve Parker's definition of probiotics with the following description: "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". This revised definition emphasizes the requirement of viability for probiotics and introduces the aspect of a beneficial effect on the host, which was, according to his definition, an animal. Guarner and Schaafsma (1998) defined probiotic as living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition. Another definition is microbial cells that have a beneficial effect on the health and well-being of the host (Salminen et al., 1999). Sanders (2003) later defined probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host. Today the universal meaning of the term 'probiotic' was established by the World Health Organization and Food and Agriculture Organization of the United States. These two organizations defined probiotics as "live microorganisms which when administered in adequate amounts, have a beneficial effect on the health of the host organism" (Corcionivoschi et al., 2010). Such microorganisms may not necessarily be constant inhabitants of the GIT, but they should have a beneficial effect on the general and health status of humans and animals. In relation to food, probiotics are considered as "viable preparations in foods or dietary supplements to improve the health of humans and animals" (Holzapfel et al., 2001).
According to these definitions, an impressive number of microbial species and genera are considered as probiotics. Among them, those that are expected to beneficially affect the host by improving the intestinal microbial balance, and hence are selected as probiotics, include

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species of the genera *Lactobacillus, Bifidobacterium, Bacillus, Saccharomyces* and *Enterococcus* (Soccol et al., 2010). The representative species include *Lactobacillus acidophilus, Lactobacillus johnsonii, Lactobacillus gasseri, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium bifidum, Bifidobacterium infantis, Enterococcus faecalis,* and *Enterocuccus faecium.* In particular lactobacilli are generally used as probiotics. This may have historical reasons since Metchnikoff proposed that the lactobacilli present in yoghurt would have a health promoting effect. However, other microbes and even yeasts have been developed as potential probiotics during recent years (Ouwehand et al., 2002). Some bacteria that do not normally inhabit the intestinal tract may also come under the category of probiotics. They are used as starters in dairy products and include mainly *Lactobacillus bulgaricus, Streptococcus thermophiles, Leuconostoc* and *Lactococcus* species (Ishibashi and Yamazaki, 2001).

Microorganisms from the genera *Lactobacillus* and *Bifidobacterium* mainly, and some other species such as *Streptococcus* have been used as probiotics for hundreds of years in food manufacturing and therapeutic applications. Many reports have shown that selected strains of lactobacilli and bifidobacteria are increasingly being introduced into various food products because they are considered to be non-pathogenic and safe (Tham et al., 2011).

1.2. Classes of microorganisms used as probiotics

Microbes from many different genera are being used as probiotics (Table 1.1). The most commonly used strains are members of the heterogeneous group of lactic acid bacteria: lactobacilli, enterococci and bifidobacteria (Ouwehand et al., 2002). However, some *Bacillus* spp. and fungi such as *Saccharomyces* spp. and *Asperigillus* spp. are also regarded as probiotics (Gibson, 2004). *Lactobacillus* and *Bifidobacterium* species have achieved popularity in the manufacture of probiotic products because of their convincing beneficial effects on human health and their possession of Generally Regarded as Safe (GRAS) status. It is generally accepted that with the exception of some streptococci and enterococci, LAB are rarely pathogenic to humans and animals (Collins et al., 1998).

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Table 1.1: Microorganisms used as probiotic cultures

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	***Other* spp.**
L. acidophilus	*B. bifidum*	*Escherichia coli Nissle*
L. casei	*B. breve*	*Saccharomyces boulardii*
L. crispatus	*B. infantis*	*Streptococcus thermophilus*
L. delbrueckii subsp. bulgaricus	*B. longum*	*Enterococcus francium*
L. fermentum	*B. lactis*	*Propionibacterium*
L. gasseri	*B. animalis*	*Pediococcus*
L. johnsonii	*B. adolescentis*	*Leuconostoc*
L. paracasei	*B. essensis*	
L. plantarum	*B. laterosporus*	
L. reuteri		
L. rhamnosus		
L. helveticus		
L. lactis		
L. sporogenes		

Adopted from Senok et al. (2005); Shah (2007); Sari et al (2011); Caplan and Frost (2011)

1.2.1. *Lactobacillus*

The genus *Lactobacillus* is wide and heterogeneous taxonomic unit, comprising more than 100 different species, belonging to the group of lactic acid producing bacteria (LAB). They are characterised by the formation of lactic acid as a sole or main end product of carbohydrate metabolism. The lactobacilli are Gram-positive, non- spore forming rods or coccobacilli with a G+C content usually below 50 mol %. They are strictly fermentative, aerotolerant or anaerobic, acidoduric or acidophilic, and have complex nutritional requirements (carbohydrates, amino acids, peptides, fatty acids esters, salts, nucleic acid derivatives, vitamins) (Tannock, 2004). Many species are significant constituents of the normal gut microbiota of humans and animals, and their occurrence and number are host dependent (Gaggia et al., 2010). Several *Lactobacillus* species have a long history of safe use in human food and nutrition (D'Aimmo et al., 2007). Their main application in the food industry has been in the manufacture of dairy products such as yogurts. Presently, 56 species within the genus *Lactobacillus* have been identified, with *L. acidophilus* being the most commonly recognized species. The optimal growth temperature for lactobacilli is in the range of 35°C-40°C and pH range of 6.4-4.5 (Shah, 2007).

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1.2.2. *Bifidobacterium*

Bifidobacteria were first isolated and described in 1899-1900 by Tissier, who described them as rod-shaped, non-gas producing anaerobic microorganisms with bifido-bacterial morphology that are usually present in the faeces of breast-fed infants, which he termed *Bacillus bifidus*. They are generally characterised as Gram-positive, non- spore-forming, non-motile, catalase positive and rod-shaped anaerobes that produce acetic and lactic acids from carbohydrates without the generation of CO_2 (Zindine and Faid, 2007). The optimal growth temperature for *Bifidobacterium* is in the range of 37°C-41°C and pH of 6.0-7.0 (Delcenserie et al., 2007; Shah, 2007). Presently, 30 species are included in the genus *Bifidobacterium,* 10 of which are from human sources (dental caries, faeces and vagina), 17 from animal intestinal tracts or rumen, 2 from wastewater and 1 from fermented milk (Soccol et al., 2010).

Bifidobacteria are microorganisms of paramount importance in the active and complex ecosystems of the intestinal tract of humans and other warm-blooded animals, as well as honeybees. Generally, they are non-pathogenic and are part of the normal intestinal microflora of humans and animals. Several species are host specific. Some *Bifidobacterium* strains are considered as important probiotics and are used in the food industry. Different species and /or strains of bifidobacteria may exert a range of beneficial health effects, including regulation of intestinal microbial homeostasis, inhibition of pathogens and harmful bacteria that colonize and /or infect the gut mucosa, modulation of local and systemic immune responses, repression of pro-carcinogenic enzymatic activities within the microbiota, production of vitamins and bioconversion of a number of dietary compounds into bioactive molecules (Mayo et al., 2010). Despite the fact that probiotic properties are species and/or strain specific, bifidobacteria are very promising probiotics (Gaggia et al., 2010)

1.2.3. *Bacillus*

Bacillus species are Gram-positive, spores-forming, catalase-positive, and aerobic microorganisms, commonly associated with soil, water and air. *Bacillus* species are normally allochthonous microbes to the human intestinal tract. Contact with these organisms usually result from the inadvertent ingestion of contaminated food or ingestion of fermented foods (Sanders et al., 2006). Currently there are 77 recognized species of genus *Bacillus.* This group of bacteria is quite diverse and the G+C content of individual organisms range from 32 to 69

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%. The scientific interest in *Bacillus* species as probiotics has really grown only in the last 15 years. Of these 77 species, the following have been evaluated for probiotic functionality with several currently being sold worldwide as components of products for human and animal use: coagulans, subtilis, clausii, and cereus. *Bacillus* species are commonly associated with soil, and as such are isolated almost ubiquitously from soil, water, dust, and air. They are not normal colonizing inhabitants of the human intestinal tract. Generally, when a *Bacillus* is used as a probiotic, it is used in the spore form and thus can be resistant to unfavourable conditions encountered during its transit through the gastrointestinal tract of animals (Guo et al., 2006).

Spores that are heat-stable have a number of advantages over the non-spore-formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier which is not the case of all species of *Lactobacillus* so in principle a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact (Permpoonpattana et al., 2012).

1.2.4. *Enterococcus*

Enterococci belong to the lactic acid bacteria (LAB) and they are of importance to foods due to their involvement in food spoilage and fermentation. They are Gram-positive, catalase negative and have the ability to convert glucose into lactic acid as the main product of primary metabolism. They do not produce spores, are oxidase negative and facultative anaerobes. They are regular commensals of the gastrointestinal tract, the oral cavity, and the vagina in humans (Fritzenwanker et al., 2013). Currently, 37 species of *Enterococcus* are validly described (Holzapfel, 2006), which fall into seven species grouped on the basis of 16 rRNA gene similarity. Moreover, organisms in this genus grow at an optimum temperature of 35°C, although some species grow at temperatures ranging from 10°C to 45°C. Most of them grow at high NaCl concentrations (up 6.5 %), pH of 9.6, survive at 60°C for 30 minutes, hence they are considered thermoduric.

Strains of *Enterococcus* species are mainly used in pigs and poultry nutrition. The genus *Enterococcus* comprises of many different species, but only two of them *E. faecalis* and *E. faecium* are most often associated with probiotics (Franz et al., 2002). *Enterococcus faecalis* is

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mainly used as a human probiotic while *E. faecium* is primarily used as an animal probiotic but also for human application (Klein et al., 1998) *Enterococcus faecium* SF68 has been used to treat diarrhoea and it is considered an alternative to antibiotic treatment. These microorganisms are used as starter cultures in food products, such as cheese, as probiotic cultures for humans and animals and as silage additives (Gaggia et al., 2010). They have some desirable characteristics for this purpose such as resistance to gastric juice and bile salts (Rossi et al., 2003), and production of antimicrobial compounds such as enterocin (Saarela et al., 2000).

1.2.5. *Saccharomyces*

Saccharomyces is a genus of budding yeast. The genus *Saccharomyces* has 16 species, including *S. cerevisiae* and *S. boulardii*, which are described in the literature as possessing biotherapeutic agents. *Saccharomyces cerevisiae* tolerates a wide range of pH, with optimum growth at acidic pH and temperatures ranging from 30ºC-35ºC. Yeast from *Saccharomyces* genus has been used in human and animal nutrition for many centuries and new applications in agro-industries are being developed (Suharja et al., 2012). Live yeasts (*S. cerevisiae*) are used as probiotic feed additives for ruminants, and their modes of action depend on the viability and stability in the rumen ecosystem. *Saccharomyces cerevisiae* is included in foods and beverages for its key role in fermentation processes and improving the health status of foods. Live yeast cells have been found to enhance digestion through the secretion of selected enzymes in the gastrointestinal tract. This yeast is widespread in nature and can be found in plants, fruit and soil (Gaggia et al., 2010).

1.3. Criteria for the selection of probiotics

To help ensure that the probiotic culture being used has a positive effect, certain requirements are needed. Ibnou-Zekri et al. (2003) have reported that probiotic effects are strain specific. Thus, a beneficial effect attributed to one strain cannot necessarily be expected from another strain, even among the same species. A potentially successful probiotic strain is expected to have several desirable properties as outlined in Table 1.2, and these should be assessed during the development of new strains and novel probiotic products. However, no one strain should be expected to produce all potential benefits. The source of origin is one of the important factors to consider since bacterial species that are present in the intestinal flora could have a better chance of survival in their native environment tolerating harsh gastrointestinal

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conditions (Vasiljevic and Shah, 2008). It is commonly noted that probiotics are host specific, and therefore micro-organisms of human origin may be desirable if they are intended for human use (Ouwehand et al., 1999). This is based on the observation that only human strains can be adhesive and colonize the human GI tract, which is the first step in promoting colonisation resistance. It is proposed that species specificity does occur and for strains to be beneficial to a particular host they should be isolated from that species (Collins et al., 1998). The ability of microorganisms to colonize is often considered as one of the main selection criteria for potential probiotics.

Probiotic bacteria, which have high ability to adhere to the intestinal surface, are expected to strongly interfere with the adhesion of pathogenic bacteria (Fuller, 1991). Furthermore, the adherence of probiotic bacteria is associated with their immunological effects (Ouwehand et al., 2000). Adhesion can be non-specific, based on physico-chemical factors, or specific, involving adhesion molecules on the surfaces of adherent bacteria and receptors on the epithelial cells. One of the most important characteristics of a probiotic strain is that it must be non-pathogenic and must not have adverse effects of any sort, and furthermore, it should possess GRAS status. It should have a beneficial effect in the form of growth promotion or increased resistance to disease (Fuller, 1998).

The bacterial strain must tolerate and survive gastric and bile secretions during transit through the upper gastrointestinal tract, and then proliferate and/or colonize in the intestine. To have an impact on the colonic flora, it is desirable for probiotic strains to have antagonistic effects towards enteric pathogens. This can happen via antimicrobial substance production or competitive exclusion (Saarela et al., 2000). Due to the indiscriminate use of antibiotics in human and veterinary medicine and as animal growth promoters, antibiotic resistance has become an increasingly common characteristic in microorganisms (Austin et al., 1999). Checking the ability of a proposed probiotic strain to act as a donor of conjugative antibiotic resistance genes may be a prudent precaution especially when probiotics are administered during antibiotic therapy, and in the case of animal feeding (Saarela et al., 2000). The probiotic strain should survive during food processing and storage, and should also have good technological properties, such as the ability to withstand freezing temperatures and maintain an adequate level of viability at the time of consumption. Furthermore, the potential probiotic should not have negative effects on organoleptic properties when applied to food (Lee and

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Salminen, 1995; Saarela et al., 2002; Vasiljevic and Shah, 2008) and the health effects should be clinically validated in order to be considered as a suitable probiotic.

Table 1.2: **Desirable properties of probiotic bacteria**

Probiotic strain properties	**Functional properties**
Non-toxic and non-pathogenic	Most probiotics are marketed as foodstuffs or drugs. Consideration of the safety of probiotics is therefore of outmost important. One of the most important characteristics to establish regarding a probiotic strain is that it must be non-pathogenic and, furthermore, should possess GRAS status
Normal inhabitant of the targeted species	It is proposed that species specificity does not occur and for strains to be beneficial to a particular host they should be isolated from that species
Resistance to pancreatic enzymes, acid and bile	In order to survive passage through the gastrointestinal tract, resistance to low pH, bile and pancreatic enzyme are important
Adhesion to epithelium or mucus	Adhesion of lactic acid bacteria to intestinal cells is the first step of colonization. Adhesion can be non-specific, based on physico-chemical factors or specific, involving adhesion molecules on the surfaces of adherent bacteria and receptor molecules on epithelial cells
Stable and capable of remaining viable for long periods under storage and field conditions	Strain viability and maintenance of desirable characteristics during product manufacture and storage is also a necessity for probiotic strains. Potential probiotics need to have good technological properties so that they can be cultured on large scale, and have an acceptable shelf life
Capable of exerting a beneficial effect on host animal, e.g. increased growth or resistance to disease	Ingestion of LAB has been suggested to confer a range of health benefits including immune system modulation, increased resistance to malignancy and infectious illness

1.4. Health benefits and therapeutic effects of probiotics

There are a variety of proposed beneficial health effects of probiotics, although only a few have significant research to back up the claims. It may be more reasonable to consider probiotics as

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prophylactic rather than a therapeutic agents. A brief discussion of some of these health effects is provided in the following subsections.

1.4.1. Control of intestinal infection

The belief that probiotics have beneficial effects is based on the knowledge that the intestinal flora can protect humans against infections and that disturbance of this flora can increase susceptibility to infections. Numerous *in vivo* and *in vitro* studies have shown that the normal intestinal flora is an extremely effective barrier against pathogenic and opportunistic microorganisms (Fuller, 1991). Several reviews have documented the use of probiotic bacteria to treat intestinal disorders, e.g. acute rotavirus diarrhoea in children, as well as food allergy and colonic disorders driven by pelvic radiotherapy and sometimes associated with the development of colon cancer (Gomes and Malcata, 1999). It is suggested that probiotic bacteria interfere with the colonisation and subsequent proliferation of pathogens, thus preventing the manifestation of infections (Panesar, 2011). Probiotic bacteria enhance resistance against intestinal pathogens via antimicrobial mechanisms. These include competitive colonisation and production of organic acids, such as lactic and acetic acids, antimicrobial peptides such as bacteriocins and other primary metabolites, such as hydrogen peroxide, carbon dioxide and diacetyl (Kailasapathy and Chin, 2000). By competitive colonisation, probiotic bacteria inhibit adhesion of gastrointestinal pathogens to the intestinal mucosa. Production of organic acids, by probiotic bacteria lowers intestinal pH and thereby inhibits the growth of pathogens. These organic acids also increase peristalsis, thereby indirectly removing pathogens by accelerating their rate of transit through the intestine (Laroia and Martin, 1990).

1.4.2. Stimulation or modulation of the immune system

The immune system provides the primary defence against microbial pathogens that have entered the human body. The epithelial lining of the gastrointestinal tract offers a vast surface area for the absorption of molecules and presents a barrier to the variety of extraneous antigens that may pass through the gut. There is strong evidence to suggest that when certain quantities of lactic acid bacteria are consumed, they are able to modulate aspects of both natural and acquired immune responses. Gill and Guarner (2004) has reported that there is sufficient evidence to suggest that lactic acid bacteria exert their immunity enhancing effects by

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augmenting both non-specific (e.g. phagocyte function, NK cell activity) and specific (e.g. antibody production, cytokine production, lymphocyte proliferation, delayed-type hypersensitivity) host immune responses (Kailasapathy and Chin, 2000). Schiffrin et al. (1995) reported that there was enhanced phagocytic capacity of peripheral blood leucocytes in healthy human adults administered with fermented milk supplemented with specific strains of probiotics (*Lactobacillus johnsonii* La1 or *Bifidobacterium lactis* Bb12). In animals, lactic acid bacteria also exhibit immunostimulating capacity. Perdigon et al. (1995) also reported that different strains of *Lactobacillus* and *Streptococcus thermophilus* were capable of stimulating non-specific (macrophages) and specific (lymphocytes B and T) immunity in mice. The improvements in phagocytic activity were sustained for several weeks after cessation of probiotic consumption and granulocytes showed higher increases in phagocytic cell function compared with monocytes (Schiffrin, 1995). Phagocytosis is responsible for early activation of the inflammatory response before antibody production.

The effect of lactic acid bacteria on the secretory immune system has also been described. The entry of the antigens by the oral route is essential to induce a mucosal immune response. This fact was determined in germfree mice receiving a diet free of antigen, where it was demonstrated that the presence of microflora increased the number of IgA secreting cells in the lamina propria of the intestine, mesenteric node or in the bone marrow (Perdigon et al., 2001). Oral introduction of *Bifidobacterium bifidum* was shown to enhance antibody response to ovalbumin while *Bifidobacterium breve* was shown to stimulate IgA response to cholera toxin in mice (Isolaui et al., 2001).

1.4.3. Improvement of lactose utilization

Lactose intolerance, or more specifically lactose maldigestion, is a congenital deficiency of the enzyme β-galactosidase. This deficiency results in an inability to digest and absorb lactose (Rolfe, 2000). The consumption of lactose by certain people can result in intestinal discomfort due to the colonic fermentation of lactose that passes through the small intestine undigested. The origin of the abdominal pain that is associated with the consumption of lactose by lactose maldigesting subjects is not well understood though it does not appear to relate to the production of gasses from the fermentation of lactose by the intestinal microflora (Ouwehand

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et al., 2002). Fermented dairy products have been repeatedly shown to enhance tolerance to lactose (Shah, 1993).

The well-recognized beneficial effect of fermented products on lactose absorption in cases of lactase deficiency could be partly explained by the presence of bacterial lactase (β-galactosidase) in yoghurt or fermented products, that help with lactose cleavage and its subsequent absorption in the form of monosaccharides (Heyman, 2000). This observation has been attributed in part to the ability of *Streptococcus thermophiles, Lactobacillus bulgaricus* and other lactobacilli to serve as a source of lactase in the small intestine, contributing to the digestion of lactose in lactase deficient individuals (Sanders and Klaenhammer, 2001). This was confirmed when pasteurization and freezing of yoghurt destroyed the β-galactosidase activity. Savaiano et al. (1984) have demonstrated that yoghurt is superior to cultured buttermilk or pasteurized yoghurt in enhancing the digestion of lactose. Upon ingestion, the bacteria (starter cultures in yoghurt) are lysed by bile in the small intestine, the enzyme is released and degrades lactose. In addition to this, the more viscous properties of fermented milk products, compared to plain milk, gives them a longer gastro-caecal transit time, thus further aiding the digestion of lactose (Vesa et al., 2000).

The normal yoghurt culture, *Lactobacillus delbrueckii* spp. *bulgaricus* and *Sreptococcus thermophiles*, produce β-galactosidase in yoghurt, but these bacteria cannot survive and grow in the intestinal tract due to their low bile salt tolerance. In contrast, probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* resistance to bile acids or digestion helps them to survive intestinal passage but at the same time prevents β-galactosidase release into the small intestine. In the study by (Kim and Gilliland, 1984) lactose maldigestors were subjected to breath hydrogen test (BHT) at seven day intervals using control milk or milk containing cells of *L. acidophilus* at a test dose of 5 ml/kg body weight followed 12 hour fasts. The results revealed significantly lower levels of breath hydrogen when milk containing the lactobacilli was used. The benefits was due to the presence of β-galactosidase in the *L. acidophilus.* Jiang et al. (1996) studied the effect on lactose digestion of the consumption of milk together with 2 strains of *B. longum* grown in a medium containing either lactose or lactose plus glucose. Growth of *B. longum* B6 in the lactose- containing but glucose free de Man, Rogosa and Sharpe (MRS) broth increased lactase activity, improved lactose digestion, and decreased hydrogen exhalation.

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1.4.4. Control of some cancers

Colorectal cancer (CRC) represents a major public health problem accounting for 1 million cases and about half a million deaths worldwide. Although chemotherapy and radiotherapy have been applied as the surgical adjuvant treatments of colon cancer, they vary in success rates for local recurrence disease free survival and overall survival. Evidence from a wide range of sources supports the assumption that the link between diet and CRC may be due to an imbalance of the intestinal microflora (Uccello et al., 2012). The metabolic activities of the gut microflora can have wide-ranging implications for the health of the host, resulting in both beneficial and detrimental effects (Burns and Rowland, 2000). Evidence from a wide range of sources supports the view that colonic microflora are involved in the aetiology of cancer (Rafter, 2004).

A number of studies in animal models and in human populations have demonstrated that consumption of probiotics is effective in various medical conditions such as lactose intolerance, antibiotic-induced diarrhoea, gastroenteritis, constipation, and genitourinary tract infections (Iannitti and Palmieri, 2010). Moreover, accumulating evidence suggests that the ingestion of probiotics may be able to play a preventative role in the onset of CRC. In general, species of *Bifidobacterium* and *Lactobacillus*, have low activities of the enzymes involved in carcinogen formation and metabolism by comparison to other major anaerobes in the gut such as bacteroides, eubacteria and clostridia (Burns and Rowland, 2000). This suggests that increasing the proportion of LAB in the gut could modify the levels of xenobiotic metabolising enzymes. The reported anti-CRC mechanisms of probiotics encompass intraluminal, systemic, and direct effects on intestinal mucosa. The intraluminal effects include competitive exclusion of pathogenic intestinal flora, alteration of intestinal microflora enzyme activity, reduction of carcinogenic secondary bile acids, binding of carcinogens and mutagens, and increasing short chain fatty acids production. Reduction of DNA damage and suppression of aberrant crypt foci formation have been well demonstrated as direct anti-CRC effects of probiotics on intestinal mucosa (Chong, 2014).

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1.4.5. Control of serum cholesterol levels

Coronary heart disease (CHD) is one of the major causes of death worldwide (Pereira and Gibson, 2002). Although cholesterol is an important basic block for body tissues, elevated blood cholesterol is a well- known major risk factor for CHD (Aloğlu and Öner, 2006). Current dietary strategies for the prevention of cardiovascular disease advocate adherence to low-fat/low-saturated-fat diets. Due to low consumer compliance, attempts have been made to identify other dietary components that can reduce blood cholesterol levels. However, many patients prefer nondrug treatments for many reasons, including the adverse effects of the drugs, contraindications to drugs or personal preference for natural or alternative therapies (Zhuang et al., 2012). Supplementation of diet with fermented dairy products or lactic acid bacteria has been shown to potentially reduce serum cholesterol levels. Various approaches have been used to alleviate this issue, including the use of probiotics, especially *Bifidobacterium* spp. and *Lactobacillus* spp. (Kumar et al., 2012).

Studies examining the efficacy of probiotics in reducing cholesterol often do not sufficiently address the mechanisms by which probiotics modulate hypocholesrerolemic effects and the optimum dose, frequency, and duration of treatment for different probiotic strains (Ooi and Liong, 2010). Several mechanisms have been hypothesized, which include enzymatic deconjugation of bile acids by bile-salt hydrolase of probiotics (Lambert et al., 2008), assimilation of cholesterol by probiotics (Pereira and Gibson, 2002), co-precipitation of cholesterol with deconjugated bile (Liong and Shah, 2006), cholesterol binding to cell walls of probiotics (Liong and Shah, 2005), incorporation of cholesterol into the cellular membranes of probiotics during growth (Lye et al., 2010), conversion of cholesterol into coprostanol (Lye et al., 2010) and production of short-chain fatty acids upon fermentation by probiotics in the presence of prebiotics (De Preter et al., 2007).

1.5. Applications and relevance of probiotics

1.5.1. Human health

Humans live in close association with a vast majority of microorganisms that are present on the skin, in the mouth, and in the GIT. These commensal microbes have coevolved with humans and demonstrate a high degree of interdependence with them (Alvarez-Olmos and

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Oberhelman, 2001). The GIT harbours a rich flora of > 500 different bacterial species, some of which have important health functions, including the ability to stimulate the immune system, protect the host from invading bacteria and viruses, and aid in digestion (Isolauri, 2001). Several factors may decrease host resistance to disease and may predispose them to infections, inflammatory, degenerative, and neoplasic conditions. The use of antibiotics, immunosuppressive therapy, and irradiation, among others, may cause alteration in the composition and effect of the gut flora. Therefore, the introduction of beneficial bacterial species into the GIT has recently become a very attractive option to establish the microbial equilibrium and prevent disease (Bengmark, 1998).

Nowadays, consumers are aware of the link between lifestyle, diet and good health, which explains the emerging demand for products that are able to enhance health beyond basic nutrition. The list of health benefits accredited to functional foods continues to increase and probiotics are one of the fastest growing categories within food for which scientific researchers have demonstrated therapeutic evidence (Soccol et al., 2010). The original idea with probiotics has always been to change the composition of the normal intestinal microflora from a potentially harmful composition towards a microflora that is beneficial for the host. There is increasing evidence in favour of the claims of beneficial effects attributed to probiotics, including improvement of intestinal health, enhancement of the immune response, reduction of serum cholesterol, and cancer prevention. These health properties are strain specific (Kechagia et al., 2012). Several studies have supplied clinical evidences of the benefits generated by probiotics, as for example in diarrhoeal treatment (De Vrese and Marteau, 2007), lactose intolerance (He et al., 2008), irritable bowel syndrome (De Vrese et al., 2001), allergies (Jain et al., 2010), cancer (Chen et al., 2009) and hypercholesterolemia (Baroutkoub, 2010). According to recent meta-analysis based on well conducted clinical trials with probiotics, a clear protective effect was evident, which did not vary significantly between products containing *Lactobacillus casei, L. rhamnosus GG, L. acidophilus, L. bulgaricus, L. Bifidobacterium longum, B. bifidum var. infantis* and *B. animalis var. lactis* (Sazawal et al., 2006).

There are a number of studies that have been conducted to search for the health benefits of fermented foods and probiotics. Many reports of their positive health benefits have been published with little scientific back up. If health claims regarding probiotic bacteria are to be substantiated, it is imperative to establish which strains have been used and from which source

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they have been obtained (Collins et al., 1998). Preferably, strains used for humans should be of human origin. This is based on the observation that only human strains can be adhesive and colonize the human GIT, which is the first step in promoting colonization resistance (Huis in't Veld et al., 1994). The ingested bacteria are selected to survive gastrointestinal transit and arrive viable to contribute positively to the activity of the intestinal microbiota, and thus, the health of the host.

1.5.2. Agriculture

Probiotic applications have been extended from human applications to diversity of agricultural usage. Agricultural applications include both animals and plants. Probiotics, with regard to animal applications, is defined as live microbial feed supplements that beneficially improve the intestinal microbial balance in a host animal (Ibrahim et al., 2010). Probiotics are used as animal feed to improve the animal health and also to improve food safety and these have been applied in poultry, ruminants and pig farming as well as in aquaculture (Song et al., 2012).

Recent outbreaks of food-borne diseases highlight the need for reducing bacteria pathogens of animal origin in foods. It is well recognized that pathogens, such as *Campylobacter* and *Salmonella* can be transmitted along the food chain and can be the source of human illness. In the past, antibiotics have been included in animal feed at sub-therapeutic levels, acting as growth promoters (Dibner and Richards, 2005). However, worldwide concern about the development of antimicrobial resistance and the transmission of antibiotic resistance genes from animal to human microbiota, led to the ban of the use of antibiotics as growth promoters (Mathur and Singh, 2005). The ban of antibiotics as growth promoters (AGPs) has been a huge challenge for animal nutrition increasing the need to search for alternative methods to control and prevent pathogenic bacterial colonization (Gaggia et al., 2010). For these reasons, continued research on sustainable alternatives to antibiotic growth-promoters for animal production such as probiotics or direct fed microbials (DFM) consisting of live or dead organisms and spores, non-traditional chemicals, bacteriophages, organic acids, plant extracts and essential oils is increasingly becoming more important. These potential solutions have emerged in the last decades as some of the tools that could be potentially useful in the near future for pathogen control.

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Probiosis, although not a new concept, has only recently begun to receive an increasing level of scientific interest. In agriculture, probiotics/DFM used in animal feed have become accepted as potential alternatives to antibiotics for use as growth-promoters, and in selected cases, for control of specific enteric pathogens (Boyle et al., 2007; Villa et al., 2009). Farm animals are often subjected to environmental stresses, which can cause imbalance in the intestinal ecosystem and this could be a risk factor for pathogenic infections. In commercial swine production, for example, the most common stresses are related to the weaning and post-weaning (PW) periods (separation from the sow, end of the lactation immunity, early and critical transition from milk to a diet based on plant polysaccharides, as well as transport to a production farm). These periods are characterized by an immediate but transient drop in feed intake impairing growth performance of the animals. All these factors can negatively disturb the immune function and the intestinal microbiota equilibrium of the pigs (Modesto et al., 200), leading to increased susceptibility to gut disorders, infections and diarrhoea (Gaggia et al., 2010). The most common probiotics for monogastric animals are yeasts (*Saccharomyces boulardii*) and bacteria (*Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Bacillus* spp.) targeting the hindgut (caecum and colon), which harbours an abundant and very diverse microbial population mainly composed of bacteria and archaea (Chaucheyras-Durand and Durand, 2010).

In adult ruminants, probiotics have mostly been selected to target the rumen compartment, which is the main site of feed digestion. The most common marketed products for ruminants are live yeast (*Saccharomyces cerevisiae*) preparations. In young pre-ruminants, bacterial probiotics such as lactic acid bacteria (*Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Propionibacterium* spp.) or *Bacillus* spores generally target the small intestines, where they promote optimal maturation of the rumen microbiota and reduce risk of pathogen colonisation. Improved weight gain and rumen development in young calves have been reported with several products (Galvao et al., 2005; Adam et al., 2008). In poultry, benefits of probiotic supplementation (live yeast or bacteria) are reported in broiler performance and health, with evidence of increased resistance of chickens to *Salmonella, E. coli* or *C. perfringens* infections (Banjeree and Pradhan, 2006).

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1.5.3. Pharmaceutical industry

The US Food and Drug Administration (FDA) uses other terms for live microbes for regulatory purposes; live microbes used in animal feeds are called "direct-fed microbials", and when intended for use as human drugs, they are classified as "live biotherapeutics" (Sanders, 2008). The term live biotherapeutic products refers to products containing whole, live microorganisms (i.e. bacteria, yeasts) with an intended therapeutic or preventive effect in humans, regardless of the route of administration (oral, intra-vaginal, topic, etc) (Sreeja et al., 2013). A "biotherapeutic agent" has been used to describe a microbe having specific therapeutic activity against a specific disease. An example of effective use of a biotherapeutic agent is the oral administration of *Sacchoromyces boulardii* to treat recurrent *Clostridium difficile* associated disease. Some of the expected characteristics of probiotic strains or probiotic formulations as biological drugs are listed in Table 1.3.

Probiotic preparations vary in the way in which they are presented; they may be in the form of powders, capsules, tablets, drops, chewing gum, lozenges with different excipients to maintain the preparation in the required condition. The microbial content of the preparations vary, with some containing only one organism while others have up to seven different species (Fuller, 1997). In addition to probiotic strains, supplement formulations may also be added with vitamins and prebiotics. With regards to dosage, it is generally considered that doses between 10^6 and 10^9 colony forming units (cfu) daily are required, and most clinical trials use doses within these ranges (Saavedra, 2001).

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Table 1.3: Expected characteristics of probiotic strains or products as biological drugs

• Selected strain should help in the healthy functioning of human body systems
• Strain used should be fully characterized using scientifically valid techniques to confirm the strain identity and its critical characteristics
• Each strain should be pure and when used in combination of pure strains, the proportion should be known
• The strain should be non-pathogenic and non-toxic
• The strain must be able to attach and adhere to intestinal epithelium
• The strain should have the ability to pass through digestive system in live condition and possibility of its intestinal implantation
• The strain should have undergone required *in vitro* and *in vivo* clinical studies to confirm its effect on the particular clinical condition
• Capable of delivering an accurate dosage form and content
• Potency of the formulation with respect to its ability to effect a given result
• Stability throughout its specified shelf life in a specified matrix

1.5.4. Food industry

Probiotics have rapidly gained interest in the area of self-care and complementary medicine under the general term "functional foods". Probiotic foods are a group of functional foods with growing shares and large commercial interest. Microbes have been used for years in food and alcoholic fermentations but only recently have undergone scientific scrutiny to examine their possible health benefits (Mitropoulou et al., 2013). According to Sanders and Huis in't Veld, (1999) probiotic-containing products are common in Japan and Europe. Sanders (1999) reported that in the United States, probiotics are just now receiving attention by the food industry as healthful ingredients for an increasingly health-conscious consumer. The passage in 1994 of the Dietary Supplement Health and Education Act invigorated the sale of probiotic products as dietary supplements (Sanders, 1999).

An increase in knowledge of functional foods has led to development of foods with health benefits beyond adequate nutrition. The last 20 years have shown an increased interest among

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consumers in functional foods, including those containing probiotics. The presence of probiotics in commercial food products has been claimed for certain health benefits. This has led industries focusing on different applications of probiotics in food products and creating a new generation of 'probiotic health foods'.

Milk and its products is good vehicle of probiotic strains due to its inherent properties and due to the fact that most milk and milk products are stored at refrigerated temperatures. Dairy products play an important role in delivering probiotic bacteria to humans, as these products provide a suitable environment that support growth and viability (Phillips et al., 2006). Among probiotic carrier food products, dairy drinks were the first commercialized products, and they are still consumed in larger quantities than other probiotic beverages. *Lactobacillus rhamnosus GG* is the most widely used bacterium in the manufacturing of the dairy beverages. Yoghurt is one of the original sources of probiotics and is known for its nutritional value and health benefits. It is produced using cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophiles*. Cheese was introduced to the probiotic industry in 2006 when Danisco decided to test the growth and survival of probiotic strains in cheese (Makelainen et al., 2010).

Several aspects, including safety, functional and technological characteristics, have to be taken into consideration in the selection process of probiotic microorganisms. The functional criteria of probiotics should be established based on both *in vitro* and *in vivo* assays. To deliver the health benefits, probiotics should be able to survive the acidic conditions of the upper GIT and proliferate in the intestine. Even though a probiotic strain fulfils the necessary safety and functional criteria, its selection should also satisfy technological criteria, as aspects related to probiotic food production and processing are also very important. Viability of bacteria is important, as it is strongly suggested that probiotic products should contain an adequate amount of live bacteria (at least 10^6 - 10^7 cfu/g). The food industry has adopted the recommended level of 10^6 cfu/g of probiotic cells at the time of consumption (Boylston et al., 2004). Thus, a daily intake of at least 10^8 - 10^9 viable cells, which could be achieved with a daily consumption of 100 g of probiotic food, has been suggested as the minimum intake to provide a probiotic effect (Mitropoulou et al., 2013).

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1.6. Studies of probiotics from raw goats' milk

Goats' milk as a source of probiotic isolates was investigated in Algeria by Cheriguene et al. (2007), where they enumerated and identified lactic acid bacteria. The results indicated the predominance of lactic acid bacteria from raw goats' milk in Algeria. Muddathir (1996) investigated the microbiological quality of fresh goats' milk and revealed the presence of different species of lactic acid bacteria and yeasts in addition to some other contaminants. In Sudan, fifty four raw milk samples were collected from cows, goats, ewes and camels in different areas and analysed using microbiology techniques. The results revealed that, the dominant lactic acid bacteria in milk of different animals were *Streptococcus lactis, Lactobacillus fermentum, Lactobacillus planturum, Streptococcus cremoris, Lactobacillus acidophilus, Leuconostoc lactis.* The dominant yeasts were *Debaryomyces hansenii, Kluyveromyces lactis* and *Saccharomyces rouxii.* The results obtained showed that, the lactobacilli and lactococci counts were in the range of 3.50-6.30 and 3.48-6.31 log/ml^{-1} respectively (Elgadi et al., 2008). The results are in agreement with those of other studies, undertaken to enumerate and isolate lactic acid bacteria from fermented milks. According to Beukes et al. (2001), the number of lactic bacteria largely exceeds that of the other microflora in traditional fermented milk in South Africa.

In 2010, Tambekar and Bhutada analysed 120 milk samples from domestic animals (40 each from buffalo, cow, and goat). The results revealed that 110 *Lactobacillus* species were isolated and identified as *L. acidophilus* (13 %), *L. brevis* (10 %), *L. bulgaricus* (9 %), *L. lactis* (19 %), *L. plantarum* (15 %), *L. rhamnosus* (14 %), *L. helveticus* (2 %), *L. casei* (17 %), and *L. fermentum* (1 %). Out of these 110 isolates, 3 were identified as excellent probiotics on the basis of their acid and bile tolerance, antibacterial activity, antibiotic resistance, antibacterial potential of bacteriocin, acid, alkali and high temperature tolerance of bacteriocin. The 3 best probiotics were *L. rhamnosus, L. plantarum* from goats' milk and *L. plantarum* from cow milk. In another study done in Algeria by Marroki et al. (2011), 19 strains of *Lactobacillus* were isolated from goats' milk. Isolates were identified by phenotypic, physiological and genotypic methods. Results obtained with phenotypic methods correlated with the genotypic characterization and 13 isolates were identified as *L. plantarum,* 2 isolates as *L. rhamnosus* and 1 isolate as *L. fermentum.* Three isolates identified as *L. plantarum* by phenotypic characterization were found to be *L. pentosus* by the genotypic methods. Based on these results,

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2 strains of *L. plantarum* and 1 strain of *L. rhamnosus* have been selected and are used as starter cultures in the manufacture of artisanal fermented dairy products in Algeria.

1.7. Conclusion

In South Africa, the isolation of potential probiotic bacteria from raw goats' milk has not been investigated. Beukes et al. (2001) isolated lactic acid bacteria from traditional fermented milk not from raw milk. Most studies done on raw goats' milk focused on identification of pathogenic microorganisms. To the best of our knowledge, this is the first study conducted in South Africa to generate data on the potential probiotic bacteria present in raw goats' milk of Saanen breed.

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CHAPTER 2

Isolation and identification of potential probiotic bacteria from South African Saanen goats' milk

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2.1. Abstract

Identification and further taxonomic classification of lactic acid bacteria is essential not only for understanding their individual contributions to fermentation processes, but also to reveal their roles in industrial and therapeutic applications and to study probiotic candidature. In this study, probiotic bacteria found in raw goats' milk were isolated and identified. Out of a total of 34 isolates, 17 isolates passed the initial selection criteria as putative probiotics. Analyses for the biochemical properties included catalase test, determination of growth at temperatures 10°C and 45°C and CO_2 production from glucose. The isolates were identified using API 50 CH kit and further characterized by 16S rDNA gene sequencing, followed by confirmation of results using MALDI-TOF MS. The seventeen isolates were identified by phenotypic characterization as *Lactobacillus plantarum* (16) and *Lactobacillus rhamnosus* (1). Molecular identification based on amplification of 1.5 kilobase region of the 16S ribosomal DNA (rDNA), identified seven of the isolates as *Lactobacillus plantarum* and ten as *Lactobacillus pentosus*. Using MALDI-TOF MS, 94 % of the isolates were correctly identified to the species level as *Lactobacillus plantarum* (15) and *Pediococcus acidilactici* (2). Phenotypic characterization and MALDI-TOF identified *Lactobacillus plantarum* as the dominant species found in raw goats' milk. Whereas the 16S rDNA gene sequencing identified *Lactobacillus pentosus* as the dominant species. *Lactobacillus plantarum* strains were identified by phenotypic characterization and MALDI-TOF MS as the dominant LAB found in South African Saanen raw goats' milk.

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2.2. Introduction

Interest in microorganisms from food sources is increasing due to the potential of finding new bacterial species and strains of importance. Over the past decades, a number of strains of lactic acid bacteria (LAB) have been incorporated in a wide range of food products for human and animal nutrition. As the probiotic capacities are strain-dependent, methods for reliable identification of LAB at the strain level are of great importance, especially for quality control of approved strains to avoid health risks, and misleading claims as well as for the description of new strains (Chandran, 2015).

Identification of lactobacilli has previously been based mainly on phenotypic properties, e.g., fermentation of carbohydrates, morphology, growth at different temperatures and lactic acid configuration (Holzapfel et al., 2001). However, the characterization of some *Lactobacillus* to species level by biochemical methods alone is not reliable because of the considerable variations in biochemical attributes between strains currently considered to belong to the same species (Schleifer et al., 1995). In fact, some species are not easily distinguishable in terms of phenotypic characteristics (Coeuret et al., 2003). Phenotypic methods have inherent limitations such as their poor reproducibility, the ambiguity of some techniques (largely resulting from the plasticity of bacterial growth), the extensive logistics for large-scale investigations and their poor discriminatory power. Mohania et al. (2008) mentioned another disadvantage of phenotypic analysis being that the whole information potential of a genome is never expressed, that is, gene expression is directly related to the environmental conditions (e. g the growth conditions in the laboratory).

In recent years, phenotypic properties are complemented or have been replaced by different molecular techniques such as DNA-DNA hybridization experiments, DNA sequence analysis, or PCR assays. These methods have been developed in order to obtain more consistent and accurate identification of lactic acid bacteria. Species level identification of LAB often relies mostly on determination of the phylogenetic position using 16S rDNA gene sequence analysis and further genotypic or phenotypic comparison with those held in data banks (Patel et al., 2012). Molecular methods are expensive, requires normally an average of 24 to 36 hours, and need technical expertise. The new technologies for accurate and rapid identification of bacteria are essential to different fields in microbiology. The recent development of matrix-assisted

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laser desorption ionization-time of flight (MALDI-TOF) has been shown to be a useful method for identification of different microorganisms.

Rapid and precise species identification of food-associated bacteria is also of crucial importance for food processing and product quality (Pavlovic et al., 2013). MALDI-TOF based identification technique, has been shown to be rapid, accurate and cost-effective. It has the potential to replace and/or complement conventional phenotypic methods. Compared with conventional phenotypic or PCR-based identification, MALDI-TOF MS shows rapid turnaround times, low sample volume requirements and modest reagent costs. Identification relies on mass spectral patterns, mostly composed of highly abundant proteins including many ribosomal proteins, which are assumed to be characteristic for bacterial species (Cherkaoui et al., 2010).

Isolation and identification of probiotic bacteria present in raw goats' milk has never been investigated in South Africa (SA). This implies that in SA, there is still lack of potential probiotic cultures that can be used in goats' farming as direct fed microbials. This is in line with the observation that the success of probiotic cultures depends on the original host from which they were isolated, being more effective in a host similar to their source, as well as being affected by geographical location from where the isolates came from. Thus, in SA, there is lack of knowledge about probiotic cultures that can be used in goats' production. Therefore, the present study focused on the isolation and identification of probiotic bacteria found in raw goats' milk using conventional microbiological methods, 16S rDNA gene sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry.

2.3. Materials and Methods

2.3.1. Milk samples collection

A total of 40 raw goats' milk samples were collected from Small-stock Division in Agricultural Research Council-Animal Production Institute, Irene, South Africa. Milk samples were obtained under hygienic conditions from healthy animals, by hand milking. Aliquots of 200 ml sample per animal were collected into sterile Schott bottles and then transported to the laboratory for analyses within 2 hours.

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2.3.2. Isolation and enumeration of total bacterial counts and coliforms

Total viable bacterial count and coliform counts of the milk samples were determined. Each milk sample was properly mixed to ensure homogenisation of the microbes present in the milk. For each of the sample, 1 ml of milk was pipetted aseptically into 9 ml (1:10 dilution) of sterile saline solution (0.85% w/v NaCl) in a test tube. The mixture was then vortexed (Heidolph REAX 2000, Germany) for 5 minutes. Tenfold serial dilutions up to 10^{-6} was prepared using sterile saline solution. Then 1 ml sample from each dilution was plated out using the pour plate method onto nutrient agar (Biolab) and violet red bile agar (Biolab) in triplicates for total counts and coliforms, respectively. The plates were incubated at 37°C for 48 hours. The total colony forming units per millilitre (cfu/ml) of aerobic bacteria and coliforms were recorded.

2.3.3. Isolation of lactic acid bacteria

One millilitre of each milk sample was aseptically added into 9 ml of sterile saline solution and mixed thoroughly to a homogenous suspension. Serial dilutions (10^{-1} - 10^{-7}) was performed and 1 ml aliquots from the dilutions was plated out onto (De Man, Rogosa and Sharpe) MRS agar supplemented with 0.05 g/L cysteine-HCL (MRS-cysHCL) and M17 agar plates by pour plate method in triplicates. MRS-cysHCL (Oxoid, UK) agar plates were incubated anaerobically in an Anaerobic Gas-Pack system (Oxoid) for 48 hours at 37°C for isolation of lactobacilli. M17 (Oxoid, UK) agar plates were incubated aerobically for 48 hours at 30°C for isolation of lactococci (Badis et al., 2004). Once single colonies were obtained, colonies were randomly selected from high dilution plates. Purity was checked by streaking on M17 agar and on MRS-cysHCL agar. The stock cultures of the purified colonies were stored in MRS-cysHCL broth and M17 broth supplemented with 10 % glycerol at -20°C.

2.3.4. Identification of microorganisms using conventional methods

2.3.4.1. Gram staining test

The gram reaction of the isolates was determined using a gram staining kit (Sigma-Aldrich Chemie GmbH, Switzerland) according to the manufacturer's instructions. A smear of each colony was prepared by mounting, a single colony of bacterium from the agar plates onto glass

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slides with one drop of water. After air drying, the smears were heat fixed and then stained by flooding with gram's crystal violet for 60 seconds. The slides were washed with water and then flooded with gram's iodine solution for 60 seconds before washing the smear with water. Then the slides were decolorized with gram's decolorizer solution and gently rinsed with water. The final step involved using counterstaining with gram's safranin solution for 60 seconds and then washing off excess safranin with water. Before microscopic examination, the slides were air-dried to remove excess water. The prepared slides were observed under a light microscope (Nikon Eclipse, TS 100) with a magnification of 1000X using oil immersion.

2.3.4.2. Catalase test

To check for production of enzyme catalase by the isolates, a drop of 3 % (v/v) hydrogen peroxide was placed on a clean microscopic slide. A loop full of bacterial culture was thoroughly mixed with the hydrogen peroxide on the slide. The mixture was observed for the production of gas bubbles (Nelson and George, 1995).

2.3.4.3. CO_2 production from glucose

In order to determine the homofermentative and heterofermentative characteristics of isolates, CO_2 production from glucose assay was performed using Muller (1990) method. Fifty microlitres of overnight cultures were transferred into 8 ml of MRS-cysHCL broth in test tubes containing inverted Durham tubes. The tubes were then incubated anaerobically at 30°C in anaerobic jars (Oxoid) containing AnaeroGen™ 2.5 L sachet (Thermo Scientific) for 5 days. After incubation, gas accumulation in Durham tubes was taken as the evidence for CO_2 production from glucose.

2.3.4.4. Growth determination at different temperatures

The growth temperatures of the isolates were determined using the method of Kavitha and Devasena (2013). One millilitre of fresh overnight cultures were inoculated into 100 ml of sterile MRS-cysHCL broths. The inoculated broths were incubated in anaerobic jars (Oxoid) containing AnaeroGen™ 2.5 L sachet (Thermo Scientific) for 72 hours at 10°C and 45°C. The growth of bacterial strains were visually confirmed by the changes in turbidity of MRS broth.

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2.3.4.5. API System

The ability to ferment carbohydrate substrates was studied using the API 50 CH kit (BioMerieux, France). All the tests were conducted in accordance with manufacturer's instructions. A swab of each LAB isolates grown on MRS-cysHCL agar plates (incubated anaerobically at 37°C for 24 hours) was suspended in API 50 CHL medium. Using a sterile pipette, the homogenized cell suspension was distributed into each of the 50 wells on the strips. All the wells were overlaid with sterile paraffin oil (BioMerieux, France) to affect anaerobiosis. The strips were moistened and covered with the lid, and incubated at 37°C for 48 hours. The change in colour from violet to yellow were monitored after 24 and 48 hours. The results, which form biochemical profiles, were identified using an apiwebTM software version 5.1. These profiles were then compared to those listed in the API 50 CH Analytical Profile Index.

2.3.5. Molecular identification

2.3.5.1. Genomic DNA Isolation

Total genomic DNA of isolates was extracted using, the MasterPure DNA Purification kit, Epicentre, Madison, WI, USA according to the manufacturer's instructions, with minor modifications. Briefly, cells from bacterial culture (1.5 ml of overnight culture) were harvested by centrifugation at 10,000 rpm for 10 minutes at room temperature. The pellet was then suspended in 300 µl of a mixture containing Tissue and Cell Lysis Solution and Proteinase K and mixed thoroughly by inverting the tubes. The samples were incubated at 70°C for 20 minutes and then treated with RNase A for 30 minutes at 37°C. The samples were then placed on ice for 5 minutes followed by addition of MP Protein Precipitation Reagent and then centrifuged at $\geq$ 10,000 x g in a microcentrifuge to precipitate proteins. The supernatant was collected in a clean tube. For precipitation of DNA, 100 % isopropanol was added to the supernatant and centrifuged at 4°C for 10 minutes at 10,000 rpm. The DNA pellet was washed twice with 70 % ethanol and resuspended in 35 µl of Tris-EDTA buffer.

2.3.5.2. Amplification of 16S rDNA Region by Polymerase Chain Reaction

PCR amplification of the 16S rDNA gene from each sample was performed using a thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad). Each reaction mixture (final volume 50

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µl) contained 5 µl Standard Taq Reaction buffer (10X), 1 µl of Deoxynucleotide solution mix (10 Mm), 1 µl of both primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3) and 1525R (5'-AGG GAG GTG WTC CAR CCG CA-3) (10 µM), 0.25 µl Taq DNA polymerase (1.25 U), 1 µl DNA template and 40.75 µl ddH_2O. The primers 27F and 1525R (Marroki et al., 2011) were used to amplify a 1.5 kilobase (kb) fragment of part of the 16S rDNA gene of the selected bacteria. The PCR conditions were as followed: initial denaturation for 5 min at 95°C, then 30 cycles of denaturation for 30 sec at 95°C, annealing for 1 min at 52°C, extension for 1 min at 68°C and final extension for 5 min at 68°C. The PCR products were mixed with GelRed 20X (Biotium Inc, Hayward, CA, USA) at 5:1 proportions and separated by electrophoresis in 1 % (w/v) agarose gel in 1 % TBE (tris boric-EDTA) buffer and visualized under UV light. The DNA fragments from the PCR reactions were purified using DNA Clean and Concentrator™ 25(Zymo Research) according to the manufacture's instruction.

2.3.5.3. PCR Sequencing

The sequencing reaction mixture was prepared by adding 4 µl of the purified PCR amplicon, 6 µl of Big-Dye, 3 µl of 5X Sequencing buffer, 3 µl of 27F primer and 6 µl of nuclease-free water. The sequencing PCR reaction was performed in a cycler set as follows: 25 cycles of 96°C (10 sec), 55°C (5 sec) and 60°C (4 min). After the cycles, the samples were cooled to 4°C. Sequencing products were purified by manual sodium acetate-ethanol precipitation. Sequences of the fragments, were determined using the automatic Big-Dye Sequencer ABIPRISM 3130xl. The sequence data for the 16S rDNA genes were compared with those of the GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and identified according to the closest relative.

2.3.6. Identification using MALDI-TOF

Bacterial isolates were streaked onto MRS agar plates to obtain single colonies, and then single colonies were used for MALDI-TOF analysis in a MALDI BIOTYPER MICROFLEX LT (Bruker Daltonik, Bremen, Germany). For the acquisition of the mass spectra, the manufacturer's instructions were followed. The BioTyper 3.0 software was used to compare the obtained spectra with reference strains in the database and the resulting similarity value was expressed as a log score. A score of ≥ 2.000 indicated identification on the species level, a score of ≥ 1.700 indicated identification on the genus level whereas any score under 1.700

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meant no significant similarity of the obtained spectrum with any database entry (Anderson et al., 2014).

2.4. Results and Discussion

2.4.1. Isolation and enumeration of total counts, coliforms and LAB

The total viable bacterial count is the number of bacteria in a sample that can grow and form countable colonies on Nutrient agar after being held at 37ºC for 24 hours. The average total viable bacterial count (TVBC) was 2.33 $x10^2$ cfu/ml. The Foodstuffs, Cosmetics and Disinfectants Act No. 54 of 1977 has established the minimum legal standards for raw milk to be < than 50 000 cfu/ml of TVBC. This result showed conformity with the Act. Contamination of milk and milk products is largely due to human factors and unhygienic conditions. Coliforms, particularly *Escherichia coli*, are frequently used in the microbiological analysis of food as an indicator of poor hygienic condition (Parekh and Subhash, 2008). No coliforms were detected in any of the raw goats' milk analysed in the current study. This indicated that good herd hygiene, uncontaminated water, properly hygienic procedures during milking, and properly washed and maintained equipment were used when milking. Lactic acid bacteria were predominant among the bacteria isolated, with the average of 1.9 $x10^2$ cfu/ml. Delavenne et al. (2012) reported similar results for the population of LAB in raw goats' milk. According to Medina et al. (2011), the main microorganisms in raw goats' milk and goat dairy products are LAB from Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, and Streptococcus species.

2.4.2. Phenotypic identification of isolates

The initial isolation and identification was based on morphological appearance and catalase test. After conducting some preliminary tests (Gram staining and catalase), a total of 17 isolates were picked for further identification. All the 17 isolates were found to be Gram positive, catalase negative cocci and rods (Table 2.1). The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to its simplicity. In performing catalase test, no bubbles were observed indicating that the isolated bacteria are catalase negative and could not mediate the decomposition of hydrogen peroxide (H_2O_2) to produce carbon-dioxide (CO_2). According to Salminen et al. (2004), LAB are Gram positive rod or cocci shaped bacteria and are catalase negative. All the isolates were characterized further using biochemical and

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physiological tests. The physiological tests include examining the influence of temperature on the growth of the isolates. The test on examining the influence of temperature was aimed to understand the type of bacteria, whether it belong to mesophilic or thermophilic groups. The results indicated that only one isolate was able to grow at 45^oC after 24 hours incubation (Table 2.1), therefore identified as a thermophic coccus. All the 16 other isolates were unable to grow at 45^oC and 10^oC after 96 hours incubation, therefore identified as mesophiles rods. The results obtained for the physiological tests correlated with reports by researchers elsewhere. Moulay et al. (2013) had found that LAB isolated from goats milk had the ability to grow at 30^oC, but not all isolates could grow well at 45^oC. Furthermore, it was observed that none of the isolates produced CO_2 from glucose (Table 2.1), this characteristic suggests their classification as facultative homofermentative lactobacilli. Homofermentative LAB ferment glucose to produce mainly lactic acid via the glycolytic pathway, without production of gas (Madigan et al., 2010).

The ability of LAB isolates to ferment oligosaccharides represents one of the desirable probiotic characteristics because the mono-saccharine that exist in the gastrointestinal tract will affect the life of microorganisms in the intestine (Kaplan and Hutkins, 2000). The capability of isolates to ferment carbohydrates was shown by the discoloration of the purple basal medium to a yellow colour. It was found that not all the carbohydrates could be fermented by selected isolates. Table 2.2 shows that all the 17 isolates could not ferment the following carbohydrates D-xylose, D-adonitol, dulcitol, inositol L-sorbose, glycerol, erythritol and D-arabinose.

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Table 2.1: **Morphological and physiological characterization of isolates from raw goats' milk**

Isolates	Cell shape	Gram staining	Catalase activity	CO_2 production	Growth at different temperatures	
					10°C	45°C
1	Rod	+	-	-	-	-
2	Rod	+	-	-	-	-
3	Rod	+	-	-	-	-
4	Rod	+	-	-	-	-
5	Rod	+	-	-	-	-
6	Rod	+	-	-	-	-
7	Rod	+	-	-	-	-
8	Rod	+	-	-	-	-
9	Rod	+	-	-	-	-
10	Rod	+	-	-	-	-
11	Rod	+	-	-	-	-
12	Rod	+	-	-	-	-
13	Rod	+	-	-	-	-
14	Rod	+	-	-	-	-
15	Rod	+	-	-	-	-
16	Rod	+	-	-	-	-
17	Cocci	+	-	-	-	+
Control	Rod	+	-	-	-	-

— = negative reaction and + = positive reaction

This condition was due to the lack of capability enzyme produced by isolates to decompose the sugar in the basal medium. As indicated in Table 2.2, all the 17 strains were able to ferment D-ribose, D-maltose, D-mannose, D-glucose, D-fructose, N-Acetyl-glucosamine, arbutin, and salicin. The other observation found, was that isolate 17 was able to ferment D-tagatose and D-darabinose which all the other 16 isolates could not ferment. After preliminary phenotypic characterization tests and interpretation of the API database, the isolates were identified as *Lactobacillus rhamnosus* and *Lactobacillus plantarum.* Fifteen of the isolates were satisfactorily identified as shown in Table 2.2, while for 2 isolates (isolate 12 and 17) a doubtful

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identification was obtained. The isolate 17 was identified incorrectly because although it had a round shaped structure that forms clusters, it was identified as *L. rhamnosus,* which is rod shaped. The isolate 12 was identified as *L. plantarum* but the identification percentage was not mentioned. Bill et al. (1992) and Klinger et al. (1992) indicated that some commercial identification systems often yield good results regarding genus identification but they are not fully adequate at the species level. Moreover, phenotypic methods rely on the availability of pure culture and are dependent on subsequent growth characteristics and biochemical profiling.

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Table 2. 2: Fermentation profile of LAB isolated from raw goats' milk by API 50 CHL kit incubated at 37°C

Substrate	Strain number																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
L-arabinose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-αD-Mannopyranoside	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Methyl-αD-Glucopyranoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-AcetylGlucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin ferric citrate	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Celiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+
Inulin	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-
D-Melezitose	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
D-Raffinose	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	+	-
Amidon	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
Xylitol	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-
Gentiobiose	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+
D-Turanose	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D-Fucose	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium Gluconate	-	-	+	-	-	+	-	-	+	+	-	-	-	+	-	+	+
Potassium 2-Ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium 5-Ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Identification %	99.9	99.9	99.9	99.9	99.7	99.9	99.9	99.9	99.9	99.9	99.7	99.4	-	99.9	99.9	99.9	-

Isolate 1-16 =*Lactobacillus plantarum*

Isolate 17= *Lactobacillus rhamnosus*

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2.4.3. Genotypic identification of the isolates by 16S rDNA sequencing

The 17 isolates were subjected to 16S rDNA sequencing for species level identification based on the 16S rDNA amplified by 27F and 1525R as reported by Marroki et al. (2011). A single discrete PCR amplicon band of approximately 1.5 kilobase (kb) was observed and purified to remove contaminants. In this study, the size of the amplicons of all the strains investigated corresponded with the size of the amplicon obtained for the *L. plantarum* (ATCC 8014), which was used as the reference strain (Figures 2.1A and 2.2B). The purified PCR products were used in sequencing for the identification at the species level. Only the forward primer 27F was for the 16S rDNA partial sequencing. Based on the 16S rDNA partial sequencing it was found that the dominant population bacteria found in raw goats' milk were *Lactobacillus* strains. Ten isolates were identified as *Lactobacillus pentosus* and seven as *Lactobacillus plantarum* (Table 2.3).

Bacterial differentiation at the species level is not always possible with the commonly applied methods for bacterial identification. Thus, the analysis of the 16S rDNA gene sequencing resulted to be complicated due to the high similarity of sequences of species of the same genus. On the bases of information from the 16S rRNA gene sequencing, the isolates were identified as *Lactobacillus pentosus* or *Lactobacillus plantarum*. However, 16S rRNA gene sequences are not suitable for discrimination of *L. pentosus* and *L. plantarum* species because of the high identity value (99 %) shared by the two species. Consequently, the definition of phylogenetic distances is also not feasible by such classic approach for the *L. plantarum* group species (Quere et al., 1997). However, the correct identification of the corresponding species is of great importance for food safety and quality. In order to get more information on the identity of the isolates, they were identified using MALDI-TOF.

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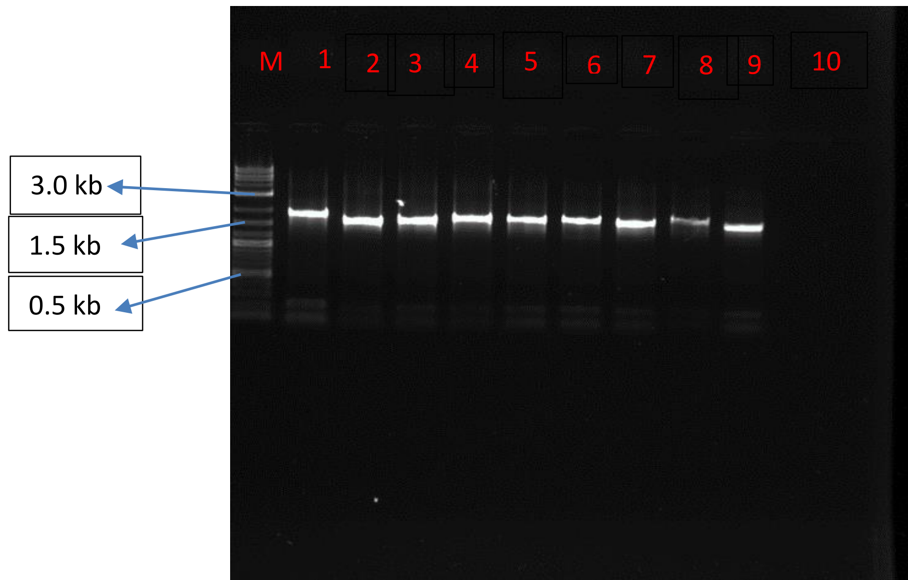

Figure 2.1A:Species-specific PCR amplification with primers 27F and 1525R. Lane M: (1 Kb ladder), Lane 1: (*Lactobacillus plantarum* ATCC 8014), Lane 2- 9: (Isolate 1-8), Lane 10: (Negative control)

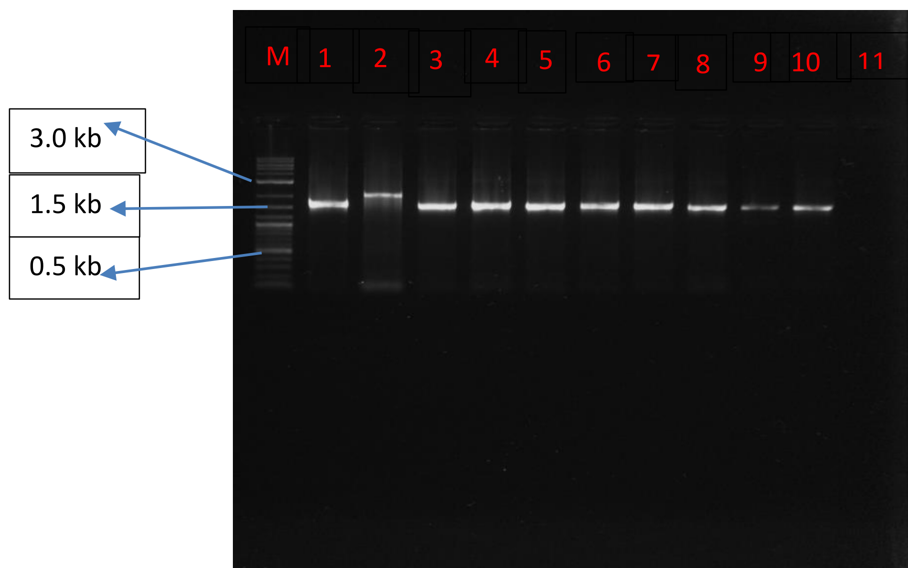

Figure 2.2B: Species-specific PCR amplification with primers 27F AND 1525R.
Lane M: (1 Kb ladder), Lane 1: (*Lactobacillus plantarum* ATCC 8014), Lane 2-10: (Isolate 9-17) Lane 11: (Negative control)

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Table 2.3: Percentage similarity of isolates to species in the NCBI nucleotide sequence database, based on partial 16S DNA

Isolates	Phylogenetic affiliation	Accession number	% similarity
1	*Lactobacillus plantarum*	KJ026587.1	95
2	*Lactobacillus pentosus*	AB362714.1	98
3	*Lactobacillus pentosus*	AB362714.1	98
4	*Lactobacillus plantarum*	KM207826.1	96
5	*Lactobacillus pentosus*	AB362714.1	98
6	*Lactobacillus pentosus*	AB362714.1	98
7	*Lactobacillus pentosus*	AB362714.1	97
8	*Lactobacillus plantarum*	KM207826.1	92
9	*Lactobacillus plantarum*	KM207826.1	94
10	*Lactobacillus plantarum*	KM207826.1	98
11	*Lactobacillus plantarum*	KC83663.1	97
12	*Lactobacillus plantarum*	KM207826.1	97
13	*Lactobacillus plantarum*	KM207826.1	95
14	*Lactobacillus plantarum*	KM207826.1	97
15	*Lactobacillus pentosus*	AB362714.1	98
16	*Lactobacillus plantarum*	KJ958428.1	97
17	*Lactobacillus pentosus*	AB362714.1	97

2.4.4. Identification of isolates using MALDI-TOF MS

The concept of bacterial differentiation/identification by detection of protein mass patterns is based on the principle that genomic sequences of organisms coding for the production of proteins are determinant for phylogenetic differences between organisms. Since proteins reflect the genomic differences, the mass spectra should be able to serve as a classification vector for bacteria (Hollard et al., 1996). The correct identification of the 17 isolates was dependent on the presence of the reference strains in the MALDI-bioTyper 3.0 database because the species of the reference strain will give the closest match for the identification of the tested strain. The use of MALDI-TOF MS is reported as suitable for the identification of anaerobic bacteria (Veloo et al., 2011). The results obtained by MALDI-TOF MS analysis (Table 2.4) enabled reliable identification of 5 selected strains, Biotyper log score >2.300, highly probable species identification. For 11 isolates, MALDI-TOF MS analysis yielded scores of ≥ 2.000 indicating secure genus or probable species identification. The remaining isolate was probable identified at genus level with score of > 1.700. From the total of 17 isolates, 15 (88 %) were accurately identified at species level as *Lactobacillus plantarum* with scores ≥ 2.000, and the 2 isolates

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(11.76 %) were identified at genus level as Pediococcus acidilactici with scores between 1.700 -2.000.

Table 2.4: Identification of isolates using MALDI-TOF MS

Analyte identity	Organism best match	BioTyper log score
1	*Lactobacillus plantarum*	2.307
2	*Lactobacillus plantarum*	2.377
3	*Lactobacillus plantarum*	2.312
4	*Lactobacillus plantarum*	2.241
5	*Lactobacillus plantarum*	2.178
6	*Lactobacillus plantarum*	2.255
7	*Lactobacillus plantarum*	2.260
8	*Lactobacillus plantarum*	2.227
9	*Lactobacillus plantarum*	2.224
10	*Pediococcus acidilactici*	1.986
11	*Lactobacillus plantarum*	2.231
12	*Lactobacillus plantarum*	2.210
13	*Lactobacillus plantarum*	2.056
14	*Lactobacillus plantarum*	2.321
15	*Lactobacillus plantarum*	2.328
16	*Lactobacillus plantarum*	2.262
17	*Pediococcus acidilactici*	2.060

Despite the fact that 16S rDNA gene sequencing is considered as the “gold standard” for the identification of anaerobic bacteria, that was not the case in this study. This result agrees with the report of Marroki et al. (2011) who reported a similar view stating that *L. plantarum* and *L. pentosus* have similar 16S rDNA sequences. Fei et al. (2014) also reported that 16S rDNA sequences are not suitable for discriminating *L. plantarum* and *L. pentosus* species, because of high sequence identity. This may be the reason why results obtained for 16S rDNA sequencing did not correlate with those obtained by phenotypic methods and MALDI-TOF MS. With MALDI-TOF MS fingerprinting, a higher discrimination potential has been described, allowing the differentiation and correct identification of much close bacterial species and even

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strains of the same species (Keys et al., 2004; Donohue et al., 2006; Vargha et al., 2006). Phenotypic method and MALDI-TOF MS analyses proved to be reliable to identify lactic acid bacteria to the species level. They both identified 94 % of the selected isolates correctly. MALDI-TOF MS fingerprinting proved to be applicable for bacterial identification at genus, species and even strain level. In this study, it demonstrated to be a rapid, cost-effective and accurate technique that achieved correct species identification of more than 94 % of the isolates. This is a significantly better result than the 16S rDNA gene sequencing. Furthermore, it has several advantages over other fast methods relying on genomics, such as DNA-microarrays, because fewer steps are necessary to achieve bacterial identification and thus, fewer errors are introduced along the analysing process.

2.5. Conclusion

The combination of applied methods for the identification of isolates has shown that *Lactobacillus plantarum* was the dominant species in raw goats' milk and *Pediococcus acidilactici* to a lesser extent. These indicates that the potential candidate probiotic bacteria to be used as direct fed microbials for goats production belong to these two closely related lactobacillus species. Since the beneficial effects of probiotics are mostly strain dependent, all the isolates will be screened for probiotic attributes.

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CHAPTER 3

In vitro screening of lactic acid bacteria isolated from raw goats' milk for probiotic attributes

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3.1. Abstract

Lactobacillus plantarum strains and *Pediococcus acidilactici* isolated from raw goats' milk were screened for selected probiotic characteristics. These isolates were evaluated for potential use as probiotics based on their adhesion to intestinal epithelial cells, resistance towards acidic and bile conditions, their antibiotic sensitivity profiles and production of antimicrobial peptides. All the 5 isolates exhibited similar adhesion properties to porcine ileum. Although most strains tolerated acidic conditions of pH 3, their viability was drastically reduced at pH 2. All the isolates could tolerate 0.3 % and 0.5 % bile salt concentration. They were resistant to nalidixic acid, vancomycin and gentamycin and susceptible to ampicillin, cephalothin, co-trimoxazol, erythromycin, oxytetracycline, penicillin G and tetracycline. Furthermore, they all showed antimicrobial activity towards intestinal pathogens, such as *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 33591), *Salmonella typhimurium* (ATCC 49416) and *Escherichia coli* (ATCC 35218). Based on these results, the 5 selected isolates have great potentials as candidate probiotic bacteria for prospective use in goats' farming.

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3.2. Introduction

Lactic acid bacteria (LAB) including *Lactobacillus* spp. are Generally Recognized as Safe (GRAS) bacteria that have been used in the processing of fermented foods. They occur naturally as indigenous microflora in raw milk and fermented milk products (Ali, 2011). Several criteria have been used for the selection of probiotic strains, the most commonly employed being the survival of the stressful gastrointestinal tract (GIT) conditions (i.e. low pH and high bile salt concentrations), the ability to temporarily colonize the GIT, which is related to their ability to adhere to mucus and /or intestinal epithelium, antibiotic resistance patterns and the antimicrobial activity through the production of antimicrobial molecules or the ability to inhibit or displace the adhesion of pathogens to the intestinal wall. Several *in vitro* and *in vivo* tests are employed for screening of these characteristics, although there is a lack of standardized or unified methodology for the assessment of probiotic functionality (Lee and Salminen, 2009).

To survive passage through the stomach and small intestine, probiotic strains must tolerate the acidic and protease-rich conditions of the stomach, and survive and grow in the presence of bile acids. Acid tolerance is also important for survival of the probiotics in foods. The dominant food vehicles for probiotics remain to be yoghurts and fermented milks, both of which provide a relatively low-pH environment in which probiotic bacteria must survive. Hence, acid tolerance is one of the first properties screened for when selecting probiotic strains (Tuomola et al., 2001). The ability to adhere to the intestinal mucosa is another important selection criterion for probiotics, because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization. Adhesion is also considered important for stimulation of the immune system (Tuomola et al., 2001).

In the complex GIT ecosystem, probiotics have developed mechanisms to survive in competition with other microorganisms. Essentially, the antagonism is exerted by competition for nutrients and for physical location, but also through production of antimicrobial substances. The ability of probiotics to produce antimicrobials is one mechanism to inhibit, exclude or compete with adherent enteropathogens for the ecological niche (Lee and Salminen, 2009). Another unique features of probiotics is their antibiotic resistance expression and transferability of resistance determinants to human pathogens and opportunistic bacteria (Ammor et al., 2007). Routine antibiotic susceptibility testing has been advocated as an essential selection criterion

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for potential probiotic cultures, to ensure that probiotic strains used are regarded as non-pathogenic and unlikely to participate in undesirable (e.g. antibiotic resistance) gene transfer cascades *in vivo*. The natural resistance of lactobacilli to a wide range of clinically important antibiotics may enable the development of antibiotic/probiotic combination therapies for such conditions as diarrhoea, female urogenital tract infection, and infective endocarditis (Charteris et al., 1998).

For several decades, antibiotics in prophylactic dosages have been used in animal feed to improve animal welfare and to obtain economic benefits in terms of improved animal performance. However, there is increasing concerns about the risk of developing cross-resistance and multiple antibiotic resistances in pathogenic bacteria in both human and livestock. The search for alternatives to antibiotics and new strains of potential probiotics in livestock has spurred interest into this study. The purpose of this study was to evaluate the probiotic potential of the lactic acid bacteria isolated from goats' raw milk under *in vitro* conditions. These isolates included the four *Lactobacillus plantarum* strains (KJ026587.1, KM207826.1, KC83663.1, and KJ958428.1) and *Pediococcus acidilactici*. The isolates were investigated for acid and bile tolerance, and then further screened for various functional properties, such as ability to adhere to the intestines, antibiotic resistance and production of antimicrobial activity.

3.3. Materials and Methods

3.3.1. Acid tolerance

The acid tolerance of the 5 isolates was studied using a modified method described by Liu et al. (2011). One millilitre aliquot of the overnight cultures (adjusted to approximately 1 x10^8 cfu/ml) were inoculated into 100 ml of MRS broth (Oxoid) supplemented with 0.05 g/L cysteine-HCL (MRS-cysHCL) adjusted to pH 1, 2, and 3 using hydrochloric acid (HCL). The cultures were then incubated anaerobically at 37°C in anaerobic jars (Oxoid) containing AnaeroGen™ 2.5 L sachet (Thermo Scientific). Then viable bacterial counts as a measure of bacterial growth were performed at 0, 1, 2, and 3 hours using the pour plate technique in MRS-cysHCL agar. *Lactobacillus plantarum* (ATCC 8014) was used as a control.

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3.3.2. Bile salt tolerance

The bile tolerance test was conducted using a modified method of Walker and Gilliland (1993) with minor modifications. Briefly, 1 ml aliquot (which corresponds to approximately 1 x10^8 cfu/ml) of overnight cultures were inoculated into 100ml MRS-cysHCL broth supplemented with 0.3 % and 0.5 % ox-gall (Biolab). The cultures were then incubated anaerobically at 37ºC for 24 hours in an anaerobic jar (Oxoid) with an AnaeoGen 2.5 L sachet (Thermo Scientific). Bacterial growth was monitored by viable plate counts after 0, 2, 4 and 24 hours incubation at 37ºC as was done for acid tolerance test. *Lactobacillus plantarum* (ATCC 8014) was used as a control.

3.3.3. Antibiotic susceptibility testing

The antibiotic susceptibility test of isolates was assessed using antibiotic discs diffusion method according to Charteris et al. (1998). The broth cultures of LAB were prepared using MRS-cysHCL and adjusted to 0.5 McFarland standards (equivalent to 1 x 10^8 cfu/ml). A 100 µl suspension of freshly grown bacterial cultures was spread on MRS-cysHCL agar plates. The antibiotic discs were placed on the surface of agar and the plates were incubated anaerobically in anaerobic jars (Oxoid) containing AnaeroGen™ 2.5 L sachet at 37ºC for 24 hours. Susceptibility pattern was assessed for vancomycin (30µg), ampicillin (10µg), cephalothin (30µg), co- trimoxazol (25µg), nalidixic acid (30µg), gentamycin (10µg), penicillin G (10µg), tetracycline (30µg), erythromycin (15µg), and oxytetracycline (30µg). The diameters of inhibition zones were measured and the results (average of three readings) were recorded.

3.3.4. Production of antimicrobial activities

The antimicrobial activity of the isolates was determined using agar well diffusion technique according to Mohankuman and Murugalatha (2011). Isolates were screened for production of antimicrobial activity against *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 6380), *Salmonella typhimurium* (ATCC 49416), and *Staphylococcus aureus* (ATCC 33591) as selected test pathogens. Selected isolates were grown in MRS-cysHCL broth for 48 hours at 37ºC. Cell free supernatants were obtained by centrifugation of cultures at 5000 rpm for 10 minutes at room temperature. The cell-free supernatant was divided into two parts, where one part was adjusted to pH 6.5 by adding 1 M

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NaOH and the other part was not adjusted. Around 6 mm diameter wells were made on the solidified Mueller Hinton Agar (Oxoid) seeded with test pathogens. Aliquots of the supernatant (100 µl) were dispensed in the wells, and the plates were incubated overnight at 37°C. The diameters of clear zones of growth inhibition around each well were measured.

3.3.5. Adhesion assay

Porcine ileum, collected from pigs immediately after slaughter, were aseptically dissected into 3 cm long sections and kept on ice for a maximum of 9 hours. The 5 bacterial isolates were inoculated into 250 ml MRS-cysHCL broth and incubated at 37°C to OD_{600} 1.2, equivalent to approximately 1x 10^8 cfu/ml of bacteria. A section of ileum was added to each of the latter cultures and incubated for 6 hours at 8°C on a rotary shaker. Samples of the culture were withdrawn every 2 hours, serially diluted and plated onto MRS-cysHCL agar. Colonies were counted after 24 hours of incubation at 37°C. Furthermore, the ileum sections were aseptically removed from the flasks and mucus layer carefully scraped off with a sterile glass slide. Preparations of the mucus samples on microscopic slides were treated with the BacLight viability probe (Molecular Probes Inc, Eugene, Oregon, USA) for visualization of adhered bacteria. The slides were incubated for 10 minutes in the dark at room temperature. Images of adhering bacterial cells were captured using a high-performance CCD camera, mounted on a Nikon Eclipse E400 epi-fluorescence microscope, equipped with a x60/1.4 Dic H oil objective and filters. Sections of ileum suspended in MRS-cysHCL broth not inoculated with any bacteria served as controls (Brink et al., 2006).

3.4. Results and Discussion

3.4.1. Acid tolerance

In order to exert their beneficial effects in the host, probiotics must remain alive during both ingestion and their transit prior to reaching the large intestines. Probiotics have to pass through the stressful conditions of the stomach with pH between 1.5 and 3.0, and in the upper intestine which contains bile (Lankaputhra and Shah, 1995; Corzo and Gilliland, 1999). Although stomach pH can be as low as 1.0, in most *in vitro* assays, pH 3.0 has been preferred (Garriga et al., 1998; Suskovic et al., 2001). The mean resident time of food in the stomach is 3 hours, and hence assays are normally run for that long. The effect of acidic conditions of pH 2, and 3

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on the viability of the LAB isolates is shown in Figure 3.1 and 3.2. In this study, all *Lactobacillus plantarum* strains and *Pediococcus acidilactici* isolated from goats' milk showed no tolerance in pH 1, with no growth observed after only 1 hour of incubation. There was a decline of viable cells in pH 2 within an hour of incubation for all the isolates, with a decrease much higher for *P. acidilactici.* There was no growth for all the isolates after 2 hours of incubation at pH 2. This phenomenon has been observed in a number of probiotic bacteria where substantial decrease in the viability of strains was often observed at pH 2.0 or below (Gupta et al., 1996). However, all the *L. plantarum* strains and *P. acidilactici* isolates showed resistance to exposure at pH 3. For all the *L. plantarum* strains, there was a decline of two logs in viable counts after 1 hour. The counts of *P. acidilactici* showed a decline of two logs after 2 hours. The residual viable counts for all the isolates were greater than 10^6 cfu/ml after 3 hours at pH 3. The observed results in this study, correlated with reports from Charteris et al. (1997). According to Charteris and co-workers, enteric lactobacilli are able to tolerate pH 3.0 for a few hours and pH 2.0 for several minutes, while viable counts *Lactobacillus* are destroyed at pH 1.0.

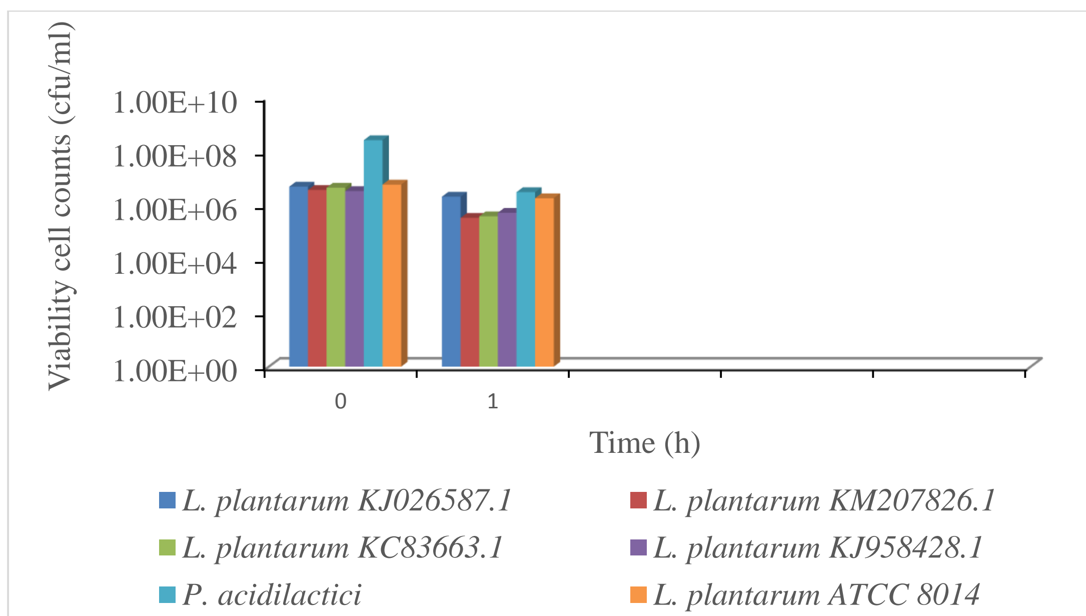

Figure 3.1: Survival of LAB isolates during 3 hours of incubation at pH 2

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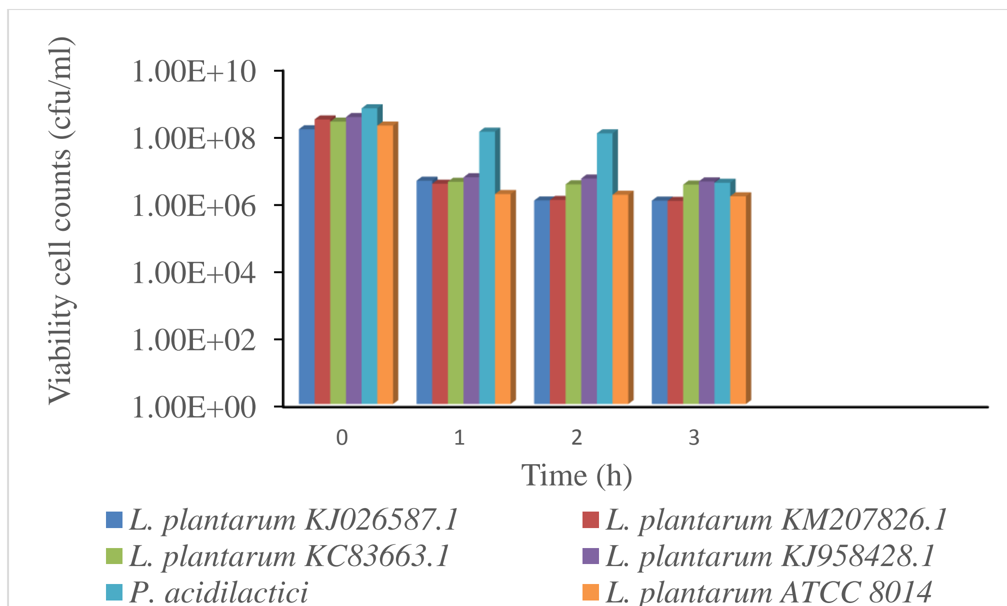

Figure 3.2: Survival of LAB isolates during 3 hours of incubation at pH 3

3.4.2. Bile tolerance

Bacteria to be used as probiotics should be able to resist inhibitory factors in the gastrointestinal tract such as bile salts. Bile salts are surface-active chemicals produced in the liver from the catabolism of cholesterol (Soomro and Masud, 2012). Bile tolerance has been described as an important factor in addition to pH tolerance for survival and growth of probiotics in the gastrointestinal tract. Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3 %, and the staying time is suggested to be 4 hours (Prasad et al., 1998; Dunne et al., 2001). The ability of all the isolates to resist bile salts was revealed after 24 hours of incubation at 37°C (Figure 3.3 and Figure 3.4). There was an increase in viable counts of all the isolates after culturing in 0.3 % and 0.5 % concentration of bile salts after 4 hours incubation. The data revealed a decrease in viable cell counts after 24 hours in 0.3 % bile salt concentration (Figure 3.3), but almost the same viable counts was obtained in 0.5 % bile concentration (Figure 3.4). The resistance of the isolates to high ox-gall concentrations is most likely due to the expression of bile-resistance related proteins in the bacterial cells (Hamon et al., 2011). Owing to the high tolerance of bile salt of all the isolates, we expect the strains to be effective in bile salt deconjugation and consequently effective in lowering serum cholesterol. Tolerance to bile salts is a prerequisite for colonization of bacteria in the small intestine of the host (Havenaar et al., 1992). Bile salt hydrolytic (BSH) activity may contribute to resistance of the isolates to the toxicity of conjugated bile salts in the

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duodenum and therefore is an important colonization factor. Therefore the tolerance of the isolates to bile salts may play a role in maintaining the equilibrium of the gut microflora. Earlier studies reported that the resistance ability is due to the presence of bile salt hydrolase (BSH), an enzyme that reduces toxic effects by conjugating bile (Rani and Pradeep, 2014).

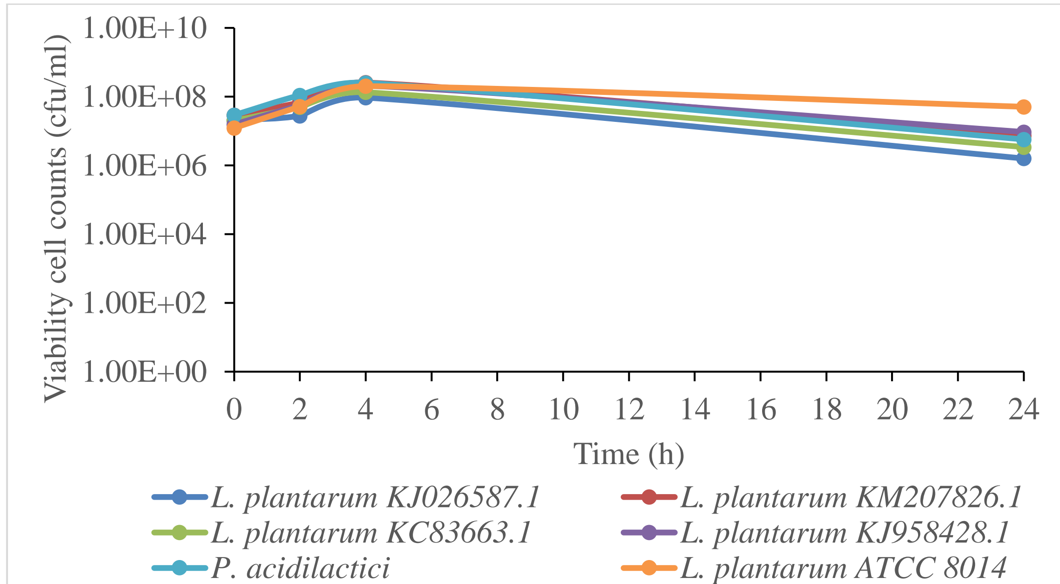

Figure 3.3: Growth pattern of LAB isolates on MRS medium supplemented with 0.3 % bile salt

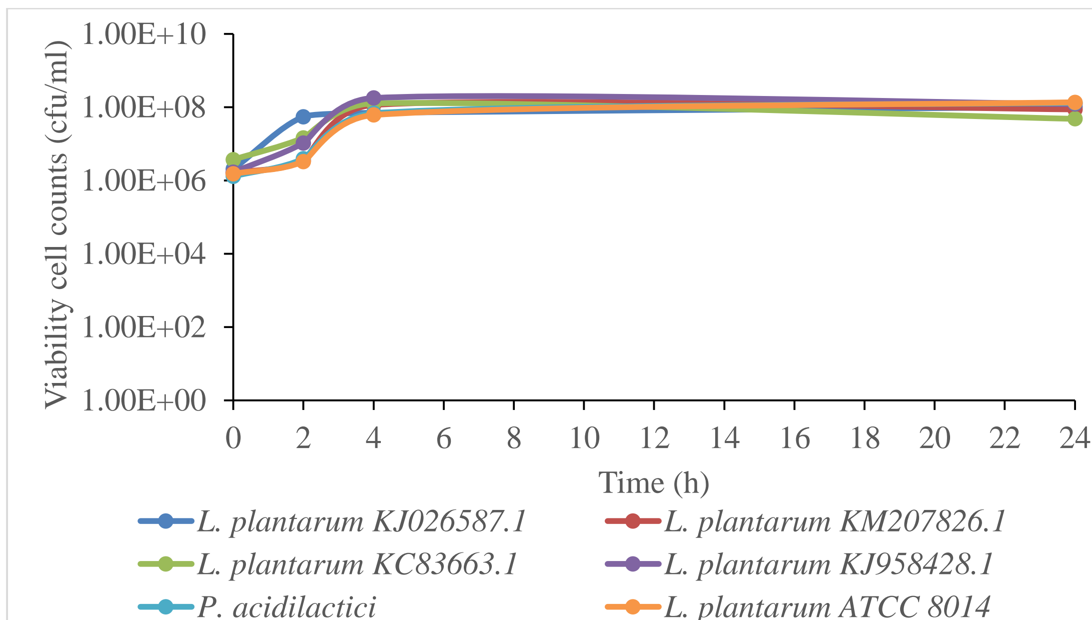

Figure 3.4: Growth pattern of LAB isolates on MRS medium supplemented with 0.5 % bile salt

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3.4.3. Antibiotic susceptibility

The overwhelming use of antibiotics over the past years in both animals and humans has played a significant role in the outspread emergence of antibiotic resistant bacteria (Ashraf and Shah, 2011). Subsequently, with the spread of antibiotic resistance in microbial communities, concerns have been raised about the existence of antibiotic resistance in beneficial bacterial species, which includes probiotic strains (Sharma et al., 2014). Probiotics are commonly used as feed additives to promote growth in livestock. A key requirement for probiotic strains is that they should not carry transferable antibiotic resistance genes (Aymerich et al., 2006). The importance of assessing the antibiotic resistance profile pattern of isolates is to restrict the use of probiotic cultures harbouring transferable antibiotic-resistance genes. In this study, all the 5 isolates from raw goats' milk were assayed for their susceptibility to ten antibiotics, using the disk diffusion method. Based on the results, all isolates showed resistance to gentamycin, nalidixic acid and vancomycin, whereas sensitive to the remaining antibiotics viz. ampicillin, cephalothin, co-trimoxazol, erythromycin, oxytetracycline, penicillin G and tetracycline (Table 3.1). Interestingly, their sensitivities to most of the antibiotics tested were very similar, except for co-trimoxazol, penicillin and ampicillin.

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Table 3.1: Antibiotic susceptibility profile of LAB isolates (Diameter of inhibition zone (mm))

LAB isolates	Antibiotics									
	TS	E	OT	KF	NA	VA	PG	T	AP	GM
	Concentration (µg)									
	30	15	30	30	30	30	10	30	10	10
	Diameter of inhibition zone (mm)									
***L. plantarum* KJ026587.1**	5	19	19	15	R	R	17	18.5	15	R
***L. plantarum* KM207826.1**	10	19	19	15	R	R	17	18.5	15	R
***L. plantarum* KC83663.1**	5	19	19	15	R	R	17	18.5	15	R
***L. plantarum* KJ958428.1**	10	19	16.5	15	R	R	17	18.5	22	R
P. acidilactici	10	19	19	15	R	R	17	18.5	22	R
***L. plantarum* ATCC 8014**	5	19	19	15	R	R	17	18.5	15	R

TS= Co-trimoxazol ; E= Erythromycin ; OT= Oxytetracycline ; KF= Cephalothin ; NA= Nalidixic acid ; VA= Vancomycin ; PG= Penicillin G ; T= Tetracycline ; AP= Ampicillin ; GM= Gentamycin, R= Resistant.

Lactobacilli display a wide range of antibiotic resistance naturally, but in most cases, antibiotic resistance is not of the transmissible type, and therefore does not usually create a safety concern (Saarela et al., 2002). In this study, all the isolates were resistant to nalidix acid, gentamycin and vancomycin. The results concur with the study of D' Aimmo et al. (2007), where high resistance to nalidixic acid was found among all the strains of *L. acidophilus* and *L. casei*. In the study of Liu et al. (2009), it was reported that 30 % of the *Lactobacillus* isolates were resistant to gentamycin. Several species of *Lactobacillus* including *L. rhamnosus* and *L. casei,* are intrinsically resistant to vancomycin. There is an underlying possibility that vancomycin resistance could be transferred to other bacteria but there are no such reports to date. In the study of Klein et al. (2000), all the isolated *Lactobacillus* strains, namely *L. reuteri* strains and *L. rhamnosus* strain were found resistant to vancomycin but susceptible to a broad range of antibiotics. None of the strains possessed the *vanA*, *vanB* or *vanC* gene, therefore this finding established the safety of the *Lactobacillus* strains for use as probiotics concerning their vancomycin resistance. The five isolates were found to be susceptible to the inhibitors of cell wall synthesis ampicillin. These results correlated with data reported by Danielsen and Wind (2003), indicating that most *Lactobacillus* strains are susceptible to β-lactams, especially

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penicillin. All the isolates were susceptible to tetracycline, as tetracycline resistance in *Lactobacillus* is considered as an acquired characteristic, which can be conjugally transferred to other genera of lactic acid bacteria (Gevers et al., 2003). The differences in the degree of inhibition with different antibiotics has been attributed to various modes of action on the cell components such as the cell wall, protein and DNA synthesis, and RNA polymerase (Neu, 1992).

3.4.4. Antimicrobial activity assay

Recent concerns on the rampant and indiscriminate use of antibiotics for disease treatment and growth promotion of livestock, and development of antibiotic resistant pathogens, have led to increased interest in the application of probiotics and their antimicrobial metabolites as alternative antimicrobial strategies for treatment and prevention of infections (Hemant and Harshada, 2015). Hence, antimicrobial activity against pathogens ravaging goats' production is a desirable property of a probiotic strain to be used in goats' farming. Antibacterial activity is vital for the successful colonization of lactobacilli in the intestinal mucosa as they provide a barrier effect and defence against pathogens (Vaughan et al., 1999). *Lactobacillus* may incur antimicrobial effect by producing some substances such as organic acids, (lactic, acetic propionic), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances such as bacteriocins, which may be continuously excreted by the bacteria to generate the inhibitor activity against the pathogens (Santos et al., 2003). The antibacterial activity of the cell free supernatants obtained from the candidate probiotics was tested by agar well diffusion method against *Escherichia coli* (ATCC C35218), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 6380), *Salmonella typhimurium* (ATCC 49416), and *Staphylococcus aureus* (ATCC 33591) as selected pathogens of importance in goats' farming. The diameters of inhibition zones showed that all the isolates have antibacterial effects against the tested pathogens (Table 3.2). All the 5 isolates have inhibition zones with diameter of 30 mm against *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 49416). There was absence of antimicrobial activity in neutralized (pH 7) cell free supernatants of all the isolates. The inhibitory activities of all the isolates in this study might be due to the production of organic acids since the activity was eliminated after neutralization with NaOH of the cultures supernatant. Schilliager et al. (1997), also reported the inhibition of Gram-negative pathogens attributed to production of organic acids by *Lactobacillus* strains isolated from dairy products.

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Table 3.2: Antimicrobial activity of LAB isolates

LAB isolates	Bacterial pathogens				
	E. coli	*S. aureus*	*P. aeruginosa*	*S. typhimurium*	*P. vulgaris*
	Diameter of inhibition zone (mm)				
***L. plantarum* KJ026587.1**	6	10	30	30	6
***L. plantarum* KM207826.1**	6	10	30	30	6
***L. plantarum* KC83663.1**	6	10	30	30	6
***L. plantarum* KJ958428.1**	6	10	30	30	6
P. acidilactici	6	10	30	30	6
***L. plantarum* ATCC 8014**	6	10	30	30	6

Mastitis, which is the inflammation of the mammary gland, is considered one of the most important diseases of domestic animals, caused by several etiologic agents. Transmission of the microorganisms primarily occurs by entering through the milk canal, and usually involves agents from animals and environmental origin and from milking process (Anderson et al., 2005). The most common bacteria that causes mastitis in goats are *Staphylococcus aureus,* followed by minor occurrence of those caused by *Escherichia coli, Clostridium perfringes, Streptococcus, Pseudomonas and Nocardia* genera (Bergonier et al., 2003). Leitner and Krifucks (2007) reported sporadic outbreak of clinical mastitis caused by *Pseudomonas aeruginosa* in Israeli dairy sheep and goats. For treatment of mastitis, the use of antibiotic therapy is recommended in some cases. However, consumption of antibiotics for treatment of mastitis might results in development of antibiotic resistant bacteria, and building up antibiotics in milk and meat that can be hazardous to humans. An effective treatment by other substances than antibiotics is needed. In this study, the production of antimicrobial activity by the probiotic isolates against the *S. aureus and P. aeruginosa* was investigated. The results demonstrated inhibitory activities against *S. aureus* and *P. aeruginosa* for all the isolates. Therefore *L. plantarum* and *P. acidilactici* isolated from raw goats' milk can be recommended for use in the treatment of mastitis in dairy goats.

Bacterial enteritis is the most important cause of diarrhoea in lambs and goats' kids. Enterotoxigenic *Escherichia coli* and *Cryptosporidium parvum* are considered among the most prevalent causative agents of enteritis in goats (Gerald et al., 1992). To prevent the onset of this disease, antibiotics have been added to the feedstock of livestock. However, the use of

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antibiotics in animal feed has been regulated and organic methods for livestock have been recommended because of problems such as advent of resistant bacteria and antibiotics residues within livestock products (McEwen and Fedorka-Cray, 2002). *Salmonella enteritidis* produces enterotoxins which are invasive to cause inflammatory change within the intestine leading to diarrhoea. Consumption of antibiotics for the treatment of such disease in animals results in appearance of multidrug-resistant (MDR) strains of *Salmonella.* The antimicrobial activity produced by the 5 isolates in this study, demonstrated inhibition of growth for both *S typhimurium* and *E. coli.* These results concur with Pascual et al. (1999), who reported *Lactobacillus* species as the most widely used probiotic bacteria for the control of *Salmonella* derived diseases as an alternative solution.

3.4.5. Adhesion assay

One of the most important properties of probiotic bacteria including lactobacilli is their ability to adhere to the target sites for the colonization in the gut for expressing optimal functionality. The adhesion of lactobacilli cells to intestinal mucus was used to evaluate the ability of strains to colonize the intestines. The bacteria must adhere to mucosal epithelial cells lining the gut to be designated as probiotic (Boonaert et al., 2000; van der Mei and Busscher, 2001), which also depends on the number of bacteria added. The level of adhesion of bacteria positively correlates with the number of bacteria added upon certain point when the saturation of potential binding sites on cell lines probably occurs (Matijasic et al., 2003). Adhesion of bacteria is a complex process involving contact between both the bacterial cell membrane and interacting surfaces (Duary et al., 2011). Staining with the BacLight viability probe revealed strong adhesion of the isolates to the ileum mucus. Based on the data obtained (Table 3.3), most of the isolates adhered to the mucus on the ileum during the 6 hours. The negative numbers of cells that adhered found at 2 and 4 hours could be caused by undercounting of bacteria because of chains or clumps formed when growing. However, concluded from the number of cells viable, all the isolates adhered similarly with a small difference to the mucus. Tuomola and Salminen (1998) also reported similar results where the difference in adhesion of probiotic isolates was small using LIVE/DEAD BacLight viability probe to study the adhesion of 12 different *Lactobacillus* strains.

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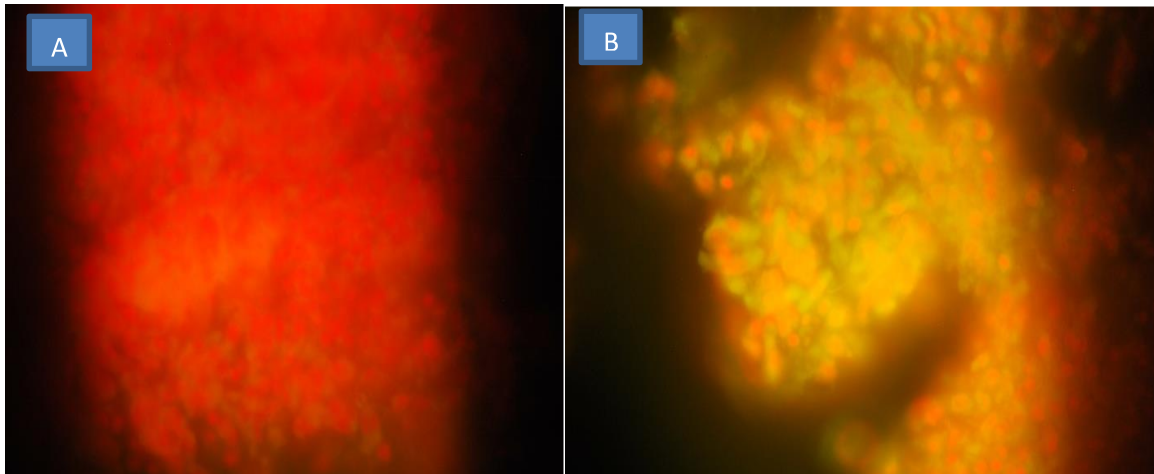

Figure 3.5: Representative images of sections of ileum suspended in MRS broth (A) without probiotics and (B) inoculated with probiotic isolates (viable cell fluorescence green)

Table 3.3: Number of probiotic cells adhering to mucus during incubation period

LAB isolates	Incubation time (hours)		
	2	4	6
	Number of cells adhering (cfu/ml)		
***L. plantarum* KJ026587.1**	- 2.3 X10^6	1.68 x10^7	6.78 x10^7
***L. plantarum* KM207826.1**	1.00 x10^7	-3.10 x10^6	5.54 x10^7
***L. plantarum* KC83663.1**	4.60 x10^6	4.30 x10^6	7.07 x10^7
***L. plantarum* KJ958428.1**	-1.25 x10^7	0	4.99 x10^7
P. acidilactici	3.00 x10^5	1.13 x10^7	6.37 x10^7
***L. plantarum* ATCC 8014**	2.80 x10^6	1.20 x10^6	5.64 x10^7

Expressed as the difference between the number of cells inoculated and the number of cells in suspension after the given incubation period.

3.5. Conclusion

The role of antibiotics to prevent infections caused by pathogenic bacteria, and also for their positive incidence on animal performance have been recognized. The use of antibiotics as growth stimulants is questionable and there are few antibiotics permitted (Draksler et al., 2004). The appearance of resistant bacterial populations, the presence of residual antibiotics in foods of animal origin and the increasing interest for organic products have led to the search for

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alternatives. The administration of probiotic foods to animals represents an excellent alternative to antibiotics. Satisfactory results have been obtained in calves, pigs, chickens and lambs, achieving a good general health and better animal productive performance (Lema et al., 2001). However, no research has been done on the isolation and the use of probiotics in South Africa on indigenous Saanen goat breed, therefore this research represents the first of its kind in South African goats' industry. The efficacy of probiotics depends on the adaptation of microbial species to the environmental conditions and their ability to survive and compete with pathogens in the gastrointestinal tract. The isolated *L. plantarum* strains and *P. acidilactici* have met most of the criteria used for probiotics. They have shown the ability to tolerate, survive the stressful gastrointestinal conditions, the ability to adhere to the intestinal epithelium and ability to produce antimicrobial activities against pathogens causing common diseases in the goats' industry. The results obtained from this study demonstrated the potential probiotic ability of the isolated *L. plantarum* strains and *P. acidilactici* from Saanen goats' raw milk.

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CHAPTER 4

Conclusions and Recommendations

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Conclusions

- Lactic acid bacteria present in milk from South African Saanen goats are dominated by *Lactobacillus plantarum* strains, but also contain *Pediococcus acidilactici* species.

- The selected bacterial isolates were able to tolerate acidic conditions of pH 3, and bile salt concentrations of 0.3 % and 0.5 %. Therefore, these isolates would be able to survive in the stressful conditions of the gastrointestinal tract, i.e. low pH and high bile salt concentrations in order to exert their beneficial effects.

- All the isolates displayed susceptibility to most antibiotics used in goats' feeds including, ampicillin, penicillin G, cephalothin, co-trimoxazol, erythromycin, tetracycline and oxytetracycline. They also displayed resistance to nalidixic acid, gentamycin and vancomycin antibiotics. However, this resistance might not pose problems as their genes have been shown not to be transferable and thus will unlikely participate in undesirable antibiotic resistance gene transfer.

- All the isolates showed antimicrobial activity towards some of the pathogens affecting the goats' industry including, *Proteus vulgaris*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Escherichia coli.* Therefore, the antimicrobial activity produced by all the isolates can be recommended for use as alternative to antibiotics in the treatment of mastitis and bacterial enteritis in dairy goats.

- Furthermore, these isolates exhibited very similar adhesion properties to porcine ileum. They would be able to colonize the goats' gastrointestinal tract temporarily without being washed out, and therefore, modulating immune responses and competitively exclude pathogens in dairy goats.

- The lactic acid bacteria isolated from raw goats' milk have great potential as candidate probiotics for prospective use in goats' farming.

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Recommendations for future work

- Future research should focus on the *in vivo* studies to evaluate the probiotic effects of the isolated bacteria in the animals. Optimisation of cell growth and shelf life stability of the probiotic bacteria for large scale production will be necessary in order to develop and produce these isolates as direct fed microbials to be used in goats feeding. The determination of the dosage form and size that will give positive probiotic effects to the livestock should also be done.

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