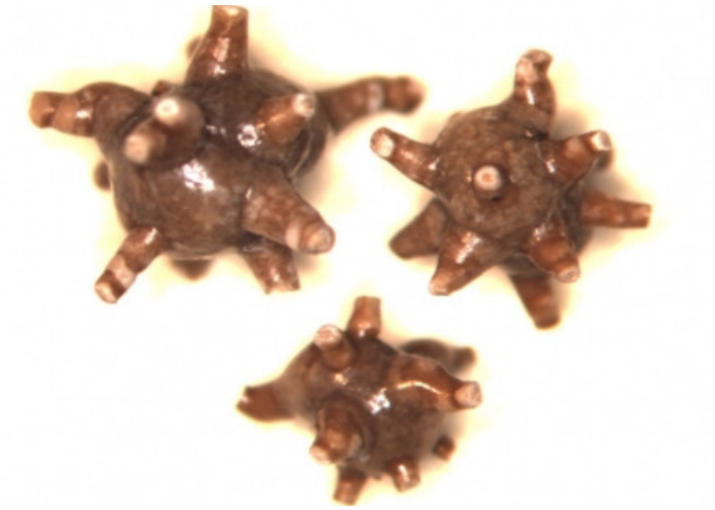
EVALUATING OXALATE-DEGRADING *LACTOBACILLUS* SPP. FOR THEIR ABILITY TO BE USED AS PROBIOTICS IN THE TREATMENT OF KIDNEY STONE DISEASE



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ABSTRACT

Kidney stone disease is a world-wide problem that affects about 10-15% of the general population. Although the direct cause of kidney stone formation is not known, reports have suggested it is probably a multifactorial disease. *Lactobacillus* strains which potentially had increased ability to degrade oxalate were previously isolated from a healthy low kidney stone risk group. The aim of this study was to identify these natural *Lactobacillus* strains and evaluate their potential for use as probiotics in reducing the risk of kidney stone disease. Identification was achieved by PCR amplification and sequencing of the 16S rRNA gene and the 16S-23S rRNA internal transcribed spacer (ITS) region. The strains were identified as follows; *Lactobacillus gasseri* 7(3), *L. gasseri* 17(4), *Lactobacillus reuteri* 17(7) and *L. reuteri* 16(9). Their probiotic characteristics were also evaluated, by determining their antibiotic susceptibility profiles, their antimicrobial activity, adhesion ability, and their acid and bile tolerance levels. The strains were also examined for their oxalate degrading activity, and overall they were shown to be potential probiotic strains with good oxalate degrading ability. The strains were also examined at the genetic level with regard to the regulation of the *oxc* gene encoding the oxalyl-CoA decarboxylase enzyme that assists in degrading oxalate. Among the 4 *Lactobacillus* strains, *L. gasseri* 7(3) strain possessed both good probiotic properties and had the highest oxalate degrading activity. Thus, it was selected for study of the further transcriptional regulation of *oxc* gene in the presence or absence of 10 mM ammonium oxalate under pH 6.8 and 5.5 conditions. Dot blot analysis confirmed that *oxc* gene was constitutively transcribed under both pH conditions in presence or absence of 10 mM oxalate. This suggests that *L. gasseri* 7(3) possesses a “generalist” oxalate-degrading character due to the fact that it can utilize alternative substrates as source of energy other than oxalate. This finding indicated that *L. gasseri* 7(3) could possibly be used as a potential probiotic bacterium in management of kidney stone disease.

March 2010

DECLARATION

I declare that *evaluating oxalate-degrading Lactobacillus spp. for their ability to be used as probiotics in the treatment of kidney stone disease* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Siti M Kabanda March 2010

Signed:

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ABBREVIATIONS

BHI	brain heart infusion
BLAST	basic local alignment search tool
bp	base pair (s)
C	cytosine
CFU	colony forming units
CH_3COOH	acetic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
G	guanine
g	grams
GRAS	generally recognized as safe
h	hour
HCL	hydrochloric acid
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase pair (s)
LAB	lactic acid bacteria

LB	Luria Bertani Medium
Log	logarithimic
mg	milligram
min	minute (s)
ml	millilitre
mM	millimolar
MRS	de Man, Rogosa, Sharpe broth
NaOH	sodium hydroxide
NCBI	national center for biotechnology information
ng	nanogram
nm	nanometer
OD	optical Density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
RNA	ribonucleic acid
rpm	revolutions per minute

rRNA	ribosomal RNA
RT-PCR	reverse trascriptase PCR
RT-qPCR	quantitative RT-PCR
sec	seconds
spp.	species
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
U	units
UV	ultra violet
w/v	weight/volume
X-gal	bromo-chloro-indolyl-galactopyranoside
µl	microliters
λ	lambda
%	percentage

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CHAPTER 1

LITERATURE REVIEW

1.1. Kidney stones

Kidney stone disease (or nephrolithiasis) is a common, worldwide problem that affects approximately 6-10% of the general population (Mittal and Kumar, 2004). Treatment is costly and the disease is defined as a painful urological disorder (Taylor and Curhan, 2007). It is estimated that more men have kidney stones than women (Bihl and Meyers, 2001). In South Africa, the problem extends to race, with 15% of white men and 5% of white women being prone to kidney stone formation, as opposed to less than 1% in the black population (Whalley *et al*., 1998). The reasons for the disparity of kidney stone formation between the two population groups are not so far understood, as the direct cause of stones formation is unknown and probably multifactorial (Heller, 1999). One of the contributing factors is likely to be the supersaturation of urine with the respect to its constituents such as calcium, oxalate and uric acid among others (Woolfson and Mansell, 1994). This supersaturation may lead to the formation of crystals, which attach to the inner surface of the kidney and build up into kidney stones. However, it should be noted that, supersaturation is not the only contributing factor to the formation of kidney stones, as there are other factors involved in stone formation (see section 1.1.1).

Several types of stones can be formed and these are categorized based on their chemical composition. Approximately 80% of stones are made up of calcium oxalate and, less often, calcium phosphate. Of the remaining, 10% are composed of struvite (which forms in the presence of urine infection and is made up of a mixture of magnesium, ammonia and phosphate), 9% contain uric acid (which occurs when the acidity level in urine is high), and only 1% contain cystine. The latter is a rare phenomenon and occurs mainly in an inherited condition called cystinuria (Coe *et al*., 2005).

1.2. The risk factors for kidney stones

There are numerous factors that may increase the risk of kidney stone formation, such as gender, age, ethnicity, family history, geographical factors, diet or medicine, as reviewed in detail below.

1.2.1 Gender and Age

As previously explained, gender has been shown to have an effect on the formation of kidney stones, with the condition affecting men more than women (Bergsland *et al*., 2002). This could possibly be due to higher supersaturation in the urine of men as compared to women (Parks *et al*., 1997). Other studies have suggested that oestrogen may also play a role in the reduced risk of kidney stones in women (Heller *et al*., 2002). Oestrogen is a female hormone that has been suggested to shield women from the formation of kidney stone. Age is also another factor that may influence the kidney stone formation. Studies have shown that older individuals have a higher incidence of kidney stone disease than young adults; however, the cause of this age effect is not clearly understood (Yagisawa *et al*., 1999).

1.2.2. Ethnicity and Family history

According to Soucie *et al*. (1994), the prevalence of kidney stones in United States of America was higher among white people as compared to black people. This has also been observed in South Africa where approximately, 15% of the male white population are at risk of formation of kidney stones as compared to less than 1% of the black population (Whalley *et al*., 1998). Family history is considered to be another contributing factor. Curhan *et al*. (1997) reported that, the prevalence of kidney stones is higher among people whose families have had a history of kidney stones as compared to those whose families had no kidney stones. The causes of this phenomenon have, however, not yet been understood.

1.2.3. Geographical factors

The geographical difference in the pattern of kidney stone disease incidence has been examined for decades. It is thought that the kidney stone disease is more commonly found in dry regions as compared to wet regions (Chandrajith *et al*., 2006). For instance, in the south-eastern part of Turkey, urolithiasis has been shown to be endemic, probably due to the hot climate, which may lead to dehydration or fluid loss (Ece *et al*., 2000). It is believed that the dehydration process could lead to the increase in urinary concentration and thereby result in kidney stone formation. Interestingly, in other parts of the world such as South Africa, the hot climate has not been shown to play a role in kidney stone disease, as the incidence of disease is low (Whalley *et al*., 1998). This may indicate that the assumption of a correlation between climate and kidney stone incidence is not valid.

1.2.4. Diet

Numerous studies have reported that there is a link between diet and kidney stones. Curhan *et al*. (1993) found that high intake of calcium in the diet lowered the risk of kidney stone disease. On the other hand, a low-calcium diet has been shown to possibly lead to greater absorption of oxalate from the bowel into the urine, and this leads to calcium oxalate formation (von Unruh *et al.,* 2004). Previous studies have determined that the risk of developing hyperoxaluria (excessive oxalate present in the urine) is caused by high intake of oxalate, which may result in excessive absorption of oxalate due to low availability of calcium in the intestine (Siener *et al*., 2003) as illustrated in Fig. 1.1. It is understood that the combination of oxalate and calcium in the gut reduces the risk of kidney stone formation, as it lessens the availability of oxalate for absorption in the intestine. In addition, patients who suffer from kidney stone disease are advised to maintain a high fluid intake to lessen the incidence of concentrated urine. Taylor *et al*. (2004) reported that high intake of magnesium was also associated with the decreased risk of kidney stone disease.

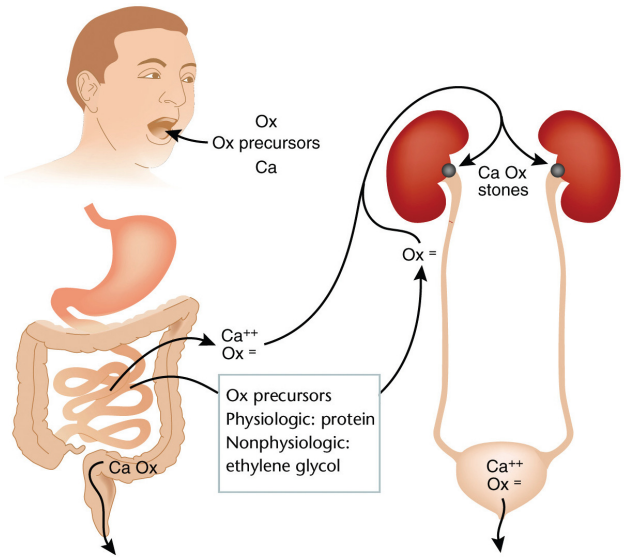

Figure 1.1. The route shows the intake of the ingested oxalate or oxalate precursors (Kleinman, 2007).

1.2.5. Medication

Several authors have indicated that some medications may increase the risk of kidney stone formation. Kuo *et al*. (2002) reviewed cases of topiramate-induced nephrolithiasis as described in the urologic literature. Topiramate is a drug which is commonly prescribed for the treatment of migraine headaches, and seizure disorders. It was found that treatment with topiramate might cause metabolic acidosis in patients by inhibiting the activity of carbonic anhydrase. Moreover, topimarate may cause distal tubular acidification which may eventually lead to kidney stone formation. Indinavir has also been shown to contribute in the increased risk of developing kidney stone. Indinavir is an HIV-1 protease inhibitor, a medicine that is used to treat patients with HIV infection (Hogg *et al*., 1997; Saltel *et al*., 2000).

1.3. Treating and prevention of kidney stone formation

For many decades, kidney stones have been treated by means of therapeutic drugs, surgery and other preventative measures. Moreover, the primary treatment given to most patients with kidney stones, is to consume large amounts of fluids or be given water by intravenous infusion, if nauseous (Bihl and Meyers, 2001). Patients are also encouraged by their doctors to eat food that is rich in calcium and avoid food rich in oxalate such as nuts, chocolate or spinach (Woolfson and Mansell, 1994). Therapeutic drugs such as thiazides are the most commonly used class of drugs, but potassium citrate, tiopronin and allopurinol may also be helpful to treat kidney stones (Ingelfinger, 2002).

In some cases, it may be difficult to manage the stone especially if it is larger than 5 mm or blocks the flow of urine. Urological procedures such as extracorporeal shockwave lithotripsy (ESWL), percutaneous nephrolithotomy and ureteroscopy have, therefore, been introduced to get rid of the stones (Lingeman *et al*., 2003; Knoll, 2007). However, all these treatments may have their side effects and may cause substantial renal or ureteral damage. Therefore, an alternative option for the treatment of kidney stone disease without the use of drugs or surgery is needed. Treatment with oxalate degrading bacteria could be a new therapeutic choice for the treatment of kidney stone disease.

1.4. Oxalate and kidney stone disease

Oxalate is a toxic compound found in variety of food such as sweet potato, chocolate and tea (Stewart *et al*., 2004; Holmes and Kennedy, 2000). Humans lack the enzymes needed to metabolize oxalate. It is therefore, excreted unchanged in the urine by the kidneys or eliminated in the faeces. (Morton *et al*., 2002; Kolandaswamy *et al*., 2009). However, the increased absorption of oxalate-rich food in the gut could be dangerous, as this may lead to the formation of calcium oxalate stones (Argenzio *et al*., 1988). The oxalate-degrading ability of several bacteria such as *Oxalobacter*

formigenes, has therefore, been studied with a view to their possibility use in the prevention of kidney stone disease.

1.5. *Oxalobacter formigenes*

The oxalate degrading activity of bacteria is known to be essential in regulating oxalate homeostasis (Sidhu e*t al*., 1998; Stewart *et al*., 2004). *O. formigenes* in particular has attracted world-wide attention for its oxalate-degrading ability (Allison *et al*., 1985). This bacterium uses oxalate as its source of energy; furthermore, numerous studies have suggested that there is a correlation between the absence of *O. formigenes* in the gut and higher urinary oxalate concentration and increased risk of hyperoxaluria (Anantharam *et al*., 1989; Troxel *et al*., 2003; Sidhu *et al*., 1998). *O. formigenes* has been shown to use two enzymes for the degradation of oxalate. These are oxalyl-CoA decarboxylase, which decarboxylates an activated oxalate molecule, and formyl-CoA transferase, which activates an oxalate molecule by cycling the CoA moiety from formyl-CoA (Sidhu *et al*., 1997). This reaction is illustrated in Fig. 1.2.

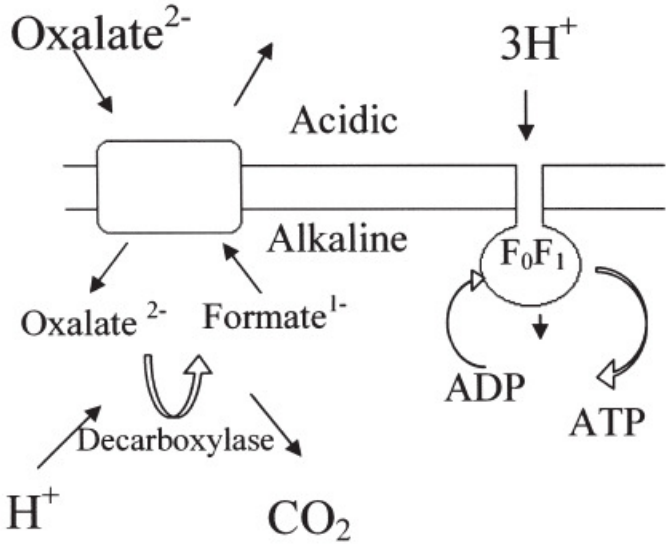

Figure 1.2. Oxalate catabolism of *O. formigenes* (Mittal and Kumar, 2004).

Oxalate catabolism by *O. formigenes* is triggered by the transport of oxalate into the cell. It is then converted to oxalyl-CoA by the transfer of Coenzyme A from formyl-CoA (catalysed by the formyl-CoA transferase enzyme). Thereafter, oxalyl-CoA is decarboxylated to formyl-CoA and CO_2 by oxalyl-CoA decarboxylase, with diffusion of CO_2 out of the cell. The CoA is transferred from formyl-CoA to an incoming molecule of oxalate and formate is released from the cell through an antiporter. An antiporter (OxlT) is a membrane protein that mediates the exchange of oxalate and formate (Ruan *et al*., 1992). However, for every molecule that is oxalyl-CoA decarboxylated, there is proton consumption, and this leads to the maintenance of a proton gradient that generates ATP synthesis (Mittal and Kumar, 2004). The genes encoding the oxalyl-CoA decarboxylase and the formyl-CoA transferase were identified as the *oxc* and *frc* genes, respectively (Lung *et al*., 1994; Sidhu *et al*., 1997). The antiporter protein, *oxlT* gene has also been identified in *O. formigenes* (Abe *et al*., 1996). *Oxc* is the most studied gene, and thus it was of special interest to study and evaluate its ability to reduce increased risk of kidney stone disease.

1.6. Other oxalate degrading bacteria

Besides *O. formigenes*, there are other enteric bacteria such as, *Enterococcus faecalis* (Hokama *et al*., 2000), *Providentia rettgeri* (Hokama *et al*., 2005), *Eubacterium lentum* (Ito *et al*., 1996) *Bifidobacterium infantis* (Campieri *et al*., 2001), *Lactobacillus* and *Bifidobacterium spp* (Campieri *et al*., 2001; Lewanika *et al*., 2007; Turroni *et al*., 2007) that have shown their potential ability to degrade oxalate. Among the aforementioned bacteria, *O. formigenes* is considered as a "specialist" oxalotroph, as it uses oxalate as its sole source of energy or carbon (Sahin, 2003; Allison *et al*., 1985). The other bacteria are defined as "generalist" oxalotrophs and they are able to use a variety of substrates as well as oxalate as their source of energy.

The focus of the present study is, however, on *Lactobacillus* species, and the possible role they may play in protecting the South African black population group from the increased risk of kidney stone disease.

1.7. *Lactobacillus* Genus

Lactic acid bacteria (LAB) are microorganisms that have traditionally been used in fermented foods and beverages, and they are documented as generally regarded a safe (GRAS) for use in these foodstuffs (Klaenhammer *et al*., 2005). The *Lactobacillus* genus is a heterogeneous microbial group containing approximately 135 species and 27 subspecies, whose classification is constantly being redistributed (Bernardeau *et al*., 2008). They are Gram-positive, nonspore-forming, rod shape bacteria with no flagella, which ferment sugars to produce lactic acid. Furthermore, these lactobacilli are regarded as strictly fermentative, aerotolerant and acidophilic and have a GC content that ranges between 33 mol% and 55 mol% (Stiles and Holzapfel, 1997). There are a number of *Lactobacillus* strains that exert health-benefits on the host (Heller, 2001), which possess desirable and functional characteristics for use as probiotics. These include *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus johnsonii* among others (see section 1.7). However, the identification of *Lactobacillus* species using traditional phenotypic methods is generally difficult and unreliable because most of these strains have similar nutritional and physiological characteristics (Quere *et al*., 1997; Kao *et al*., 2007). Studies have, therefore, focused on the use of molecular methods to assist in discerning closely related species, as they offer high sensitivity and discriminatory power for identification of probiotic microorganisms (Holzapfel *et al*., 2001).

Several studies have been done on probiotic lactobacilli strains to determine their potential to degrade oxalate in preventing kidney stone. Campieri *et al*. (2001) found reduced levels of oxaluria

in patients with calcium-oxalate urinary stone disease and mild hyperoxaluria who were treated with an oral mixture of freeze-dried oxalate degrading lactic acid bacteria. Turroni *et al*. (2007) evaluated the oxalate degrading activity in several *Lactobacillus* species used as probiotics and showed that this was high. This demonstrated that *Lactobacillus* species could possibly be used in a probiotic approach for treatment of kidney stone disease.

1.8. Mechanism of oxalate metabolism in *Lactobacillus* species

Campieri *et al*. (2001) investigated the presence of homologues of the *O. formigenes oxlT*, *oxc* and *frc* genes in *Lactobacillus* species by PCR amplification of genomic DNA using gene specific primers. No amplification products were observed, indicating that they had failed to detect the genes under the PCR conditions used. However, in the recent studies, Turroni *et al*. (2007) used different primers and PCR conditions and reported the presence of *oxc* and *frc* genes in *Lactobacillus* strains. The *oxc* and *frc* genes encoding oxalyl-CoA decarboxylase and formly-CoA transferase respectively were also identified in *L. acidophilus* NCFM by Azcarate-Peril *et al*. (2006). Transcriptional analysis revealed that mildly acidic conditions were a prerequisite for transcription of these genes. This was further confirmed by Lewanika *et al*. (2007), who reported that these genes were regulated under mildly acidic conditions in *Lactobacillus gasseri* Gasser AM63^{T}.

1.9. *Lactobacillus* species as probiotics

Probiotics are defined as live microorganisms, that when ingested, provide a health benefit on the host (Reid *et al*., 2003) and hence they promote intestinal microbial balance. LAB species are frequently used as probiotics, especially *Lactobacillus* species due to its beneficial properties and the important role it plays in maintaining the intestinal environment and in stimulating the immune system of the host (Reid, 1999). For these bacteria to be considered as probiotics, there are several

aspects to be studied. These include their response to antibiotics (sensitivity or resistance), their ability to inhibit pathogenic bacteria, adhesion to the host tissue, tolerance to gastric transit, and bile salts tolerance (Charteris *et al*., 1998a; Rönkä *et al*., 2003). These aspects are reviewed in detail below.

1.9.1. Antibiotic susceptibility

The ecological balance of the intestinal microbiota is essential for the health of the host and helps to prevent colonisation by pathogenic microorganisms (D'Aimmo *et al*., 2007). Antibiotics have traditionally been used in the treatment of microbial diseases (Mathur and Singh, 2005). However, the intake of antibiotics may interfere with the microbial balance of human microbiota (Rafii *et al*., 2008). For instance, it may lead to the increase of antibiotic-resistant microorganisms due to either misuse or excessive use of antibiotics (Mathur and Singh, 2005). This would be detrimental to human health since resistance would prevent the functionality of antibiotics in treating infections (Sullivan *et al*., 2001). Bacterial resistance to antibiotics can be either be intrinsic or acquired. Intrinsic resistance is defined as a naturally occurring feature which is present in all strains of a particular species or genus. Acquired resistance may be caused by genetic mutation or acquisition of foreign material (DNA) from other bacteria through plasmid or transfer of genomic DNA (Saarela *et al*., 2000; Courvalin, 2006). Probiotics that are resistant to antibiotics may possibly be harmful to the human host since they may have the capacity to transfer resistance genes to pathogenic bacteria (Curragh and Collins, 1992). However, the advantage of probiotics that are resistant to antibiotics is that they could be administered during antibiotic treatment and still survive and be beneficial to people whose normal intestinal microbiota is low in number due to the effects of the antibiotic (Cebeci and Gürakan, 2003; Salminen *et al.*, 1998). It is therefore, essential to characterise the antibiotic resistance capabilities of potential probiotics in order for them to be used appropriately.

1.9.2. Antimicrobial activity

Lactobacillus species play a protective role in the gut by hindering the growth of pathogenic bacteria through the production of a variety of compounds with inhibitory activity (Piard and Desmazeaud, 1992). The inhibitory activity is due to the production of organic acids (such as lactic acid), hydrogen peroxide, bacteriocins (Aroutcheva *et al*., 2001), reuterin (Axelsson *et al*., 1989) and diacetyl (Jay, 1982). Interestingly, Annuk *et al*., (2003) stated that these antimicrobial compounds produced by LAB, have been shown to exert a specific antagonistic action against Gram-positive and Gram-negative pathogens. For instance, Gram-positive bacteria have been shown to be particularly sensitive to bacteriocins (Abee *et al*., 1995). On the other hand, Jay (1982) showed that Gram-negative bacteria were more affected by diacetyl produced by *Lactobacillus* species than Gram-positive bacteria. There are also other mechanisms that have been proposed to be responsible for eradicating pathogens. These include competition for adhesion in inhibiting pathogens present in epithelia and mucosal surfaces, and stimulation of mucosal immunity (Servin, 2004). Therefore, the antagonistic properties of probiotics could be beneficial to the host in treatment of bacterial infection caused by possible pathogens.

1.9.3. Low pH

Acid resistance is considered as one of the desirable properties when selecting potential probiotic bacteria (Guo *et al*., 2009). The ability of probiotic bacteria to withstand an acidic environment is vital, especially when passing through the stomach and into the gut in order to exert beneficial effects on the host. Several mechanisms are available for bacteria to tolerate and shield themselves in a low pH environment. Gram-positive bacteria, in particular, survive acidic environments by means of several possible mechanisms (Fig. 1.3). Proton pumps can be used to regulate the cytoplasmic pH and play a crucial role in acid tolerance. Proteins involved in the repair or degradation of damaged

cell may be produced. The cell envelope may be altered such that the cells are protected by changing their architecture, composition, stability, and activity. Finally, cells may produce alkaline compounds to neutralize the acid environment. These mechanisms are discussed in greater depth below (Cotter and Hill, 2003).

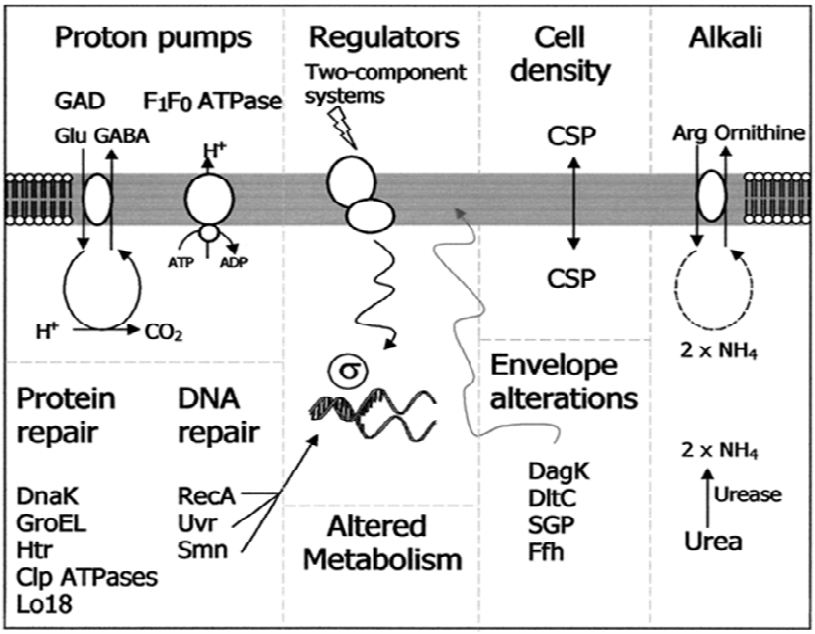

Figure 1.3. Mechanisms of resistance to low pH in Gram-positive bacteria (Cotter and Hill, 2003).

Microbial resistance to low pH involves various mechanisms, but F_1F_0 ATPase has been shown to be an important component in response and tolerance of Gram-positive bacteria to low pH. The enzyme is involved in pumping protons from the cytoplasm to the exterior of the cell, thereby maintaining the internal pH within the acidic environment (Cotter and Hill, 2003). In an alternative mechanism, glutamate decarboxylase (GAD) is the enzyme responsible for consumption of protons this results in the rise of intracellular pH, thus controlling the bacterial pH environment (Cotter and Hill, 2003). This works together with γ-aminobutyrate (GABA) which is the product produced when glutamate is decarboxylated in the cytoplasm (Cotter and Hill, 2003) (Fig. 1.3).

Several enzymes are involved in the repair of pH-induced damage. RecA is essential for the repair of DNA and restarting stalled replication forks (Cotter and Hill, 2003; Cox *et al*., 2000). Uvr is also involved in DNA repair, which assists the bacterial survival at low pH (Hartke *et al*., 1996). DnaK is a member of class I heat shock proteins which participates in the protein folding and protection of denatured proteins (Bukau and Walker, 1989). The gene encoding DnaK is induced by acid shock, increasing the level of DnaK protein to maintain the proteins in the acidic environment. GroEL is another example of a protein that participates in protein folding, which is induced in response to acid stress condition (Fayet *et al*., 1989). The Htr protease degrades abnormal proteins produced during stress conditions, while the Clp ATPases function in the degradation of misfolded proteins (Cotter and Hill, 2003). Lo18 is another type of stress protein with a chaperone-like function that is induced by several stresses, including low pH (Guzzo *et al*., 1997). DltC is a protein that is encoded by the *dlt* operon and is involved in the synthesis and secretion of activated D-alanine (Cotter and Hill, 2003). Inactivation of *dlt*C gene results in the generation of an acid-sensitive *Streptococcus mutans* strain, which shows in increased proton permeability and a failure to induce a significant acid tolerance response (ATR), both of which ensure survival at a low pH (Boyd *et al*., 2000).

Diacylglycerol kinase (DagK) is an enzyme involved in phospholipid metabolism, and in *Streptococcus mutans* mutants, its deficiency has an effect on the membrane architecture and composition (Yamashita *et al*., 1993). Ffh is a type of chaperonin which participates in the translocation of protein and membrane biogenesis (Luirink and Dobberstein, 1994). *Streptococcus* GTP-binding protein (SGP) is a protein located in the membrane of *S. mutans*, which is induced during stress response by *S. mutans* (Baev *et al*., 1999). However, the function of this protein is not known.

Urease and arginine deiminase pathways are other mechanisms by means of which bacteria can maintain the intracellular pH. This involves the production of ammonia (NH_3) which combines with proteins and leads to production of ammonium (NH_4^+) in the cytoplasm elevating the internal pH (Cotter and Hill, 2003). Two component signal transduction systems and sigma factors have been shown to be involved in the regulation of these mechanisms by which bacteria respond to low pH (Cotter and Hill, 2003). Cell density has been found to influence cell-to-cell communication. Modification of cell envelope is also another means of defence from extremely acidic pH (Cotter and Hill, 2003). However, above all, F_1F_0 ATPase, GAD, GABA, DnaK and GroEL, arginine deiminase pathway have been identified in *Lactobacillus* species, and have been shown to contribute in the acid-stress response mechanism (Cotter and Hill, 2003; Lim *et al*., 2000).

1.9.4. Bile

The ability to tolerate bile is an important criterion that bacteria should fulfil in order to be used as probiotics (Charteris *et al*., 1998a). The presence of bile in the gut presents several challenges to bacteria due to its antimicrobial action. These include damage to the cell membrane and modification of macromolecules through DNA damage or incorrect protein folding (Begley *et al*., 2005). Gram-positive bacteria are known to be more sensitive than Gram-negative bacteria to the detrimental effect of bile (Begley *et al*., 2005; Floch, 2002).

Bile salts are effective detergents that promote emulsification and digestion of fats (Ridlon *et al*., 2006). They are synthesised from cholesterol in the liver and are secreted as the glycine or taurine conjugated form into the duodenum, where they play a part in absorption of fats (Noriega *et al*., 2006). Thereafter, bile salts are reabsorbed in the ileum and colon after deconjugation by intestinal microbiota (such as *Lactobacillus* species) and returned to the liver (Bron *et al*., 2004).

The deconjugation of the bile salts is carried out by bile salt hydrolases (BSH). The mechanism of how BSH activity contributes to the usefulness of intestinal bacteria in the gastrointestinal tract has not yet been entirely understood (Noriega *et al*., 2006). However, a review by Begley *et al.* (2005) reported that, there are several hypotheses concerning this issue. One hypothesis proposes that deconjugation may exert a beneficial advantage in bacteria of some species which may utilize liberated amino acids taurine as an electron acceptor. The second hypothesis proposed that BSH assists the inclusion of cholesterol or bile into the bacterial membrane. This may increase the strength of the membranes or alter the membrane characteristics that may affect the sensitivity of host defence molecules. The final hypothesis states that deconjugation may decrease the toxicity of conjugated bile salts, increasing the chances for the survival of bacteria in the gastrointestinal tract. BSH activity has been found in certain *Lactobacillus* species, however, the resistance to bile salts encountered in the intestine varies greatly between the species and strains (Erkkilä and Petäjä, 2000; Chateu *et al*., 1994) and the mechanism is not yet fully understood (Šušković *et al*., 2000).

1.9.5. Adhesion

The ability of microorganisms to attach to intestinal epithelial cells is regarded as one of the essential criteria when selecting potential probiotic bacteria (Buck *et al*., 2005). Adhesion plays a significant role in the ability of bacteria to colonize the human gut and thereby exert health-promoting effects on the host. Therefore, when potential probiotic bacteria are ingested they need to establish themselves permanently in the human gut (Zhao *et al*., 2007). In order to do this they should have the ability to adhere to the intestinal epithelium to prevent being washed away by the normal flow of human gut fluids (Salminen *et al*., 1998). There is very little information on the mechanisms through which intestinal microflora become established and endure in the intestine (Adlerberth *et al*., 1996). However, two mechanisms were proposed by means of which probiotic microbiota adhere to the host

intestinal epithelium (Charteris *et al*., 1998a). These are non-specific adhesion mediated by surface charge interactions, and specific hydrophobic interaction (receptor-specific binding) mediated by exocellular polysaccharide, lipotechoic acid and proteins. Some studies have, however, shown that adhesion in *Lactobacillus* species could be mediated by proteins (Conway and Kjelleberg, 1989; Cocconnier *et al*., 1992) or possibly, carbohydrates (Henriksson *et al*., 1991).

Overall, an organism can only be considered to be a probiotic once these properties have been identified. The consumption of this product is aimed at promoting beneficial attributes of the host, by the use of microbiota that resides in the gastrointestinal tract. This requires detailed information of the microbiota that will be developed as a probiotic.

1.10. Research objectives

As previously discussed, many *Lactobacillus* species have been shown to possess beneficial properties, which make them ideal candidates for use as probiotics. This study aims at identifying new *Lactobacillus* strains isolated from healthy human faecal samples that have the ability to degrade oxalate and may possibly be useful in the development of probiotics for the treatment of kidney stone disease.

Related work conducted at the University of Cape Town looked at the diversity of total faecal *Lactobacillus* species between a high kidney stone group risk and low risk group with the aim of identifying possible species involved in oxalate degradation in the gut (Magwira, 2008). Findings revealed that *Lactobacillus* species from the low risk group exhibited higher oxalate degrading activity as compared to those from high risk population, suggesting that *Lactobacillus* species may perhaps play a role in protecting individuals from developing kidney stones.

In a pilot study, four *Lactobacillus* strains isolated from healthy, low risk subjects seemed to show an increased ability to degrade oxalate. In this study, the aim was to identify these *Lactobacillus* strains to the species level with the use of molecular tools, to confirm their ability to degrade oxalate, and to investigate their probiotic characteristics such as antibiotic resistance, their ability to inhibit the growth of pathogens, their adhesion ability, and their acid and bile tolerance. The strains were also examined at the genetic level with respect to the regulation of genes encoding enzymes involved in oxalate degradation.

CHAPTER 2

IDENTIFICATION OF *LACTOBACILLUS* ISOLATES

2.0. Summary

Four *Lactobacillus* strains previously isolated from healthy human fecal samples were identified using molecular based methods. The strains were initially identified by 16S rRNA gene sequencing and the identity confirmed by amplification of the 16S-23S rRNA gene spacer region. These regions are known to be highly conserved and discriminative. A phylogenetic tree was also constructed to understand the relationships between the *Lactobacillus* strains as compared with reference strains from the current *Lactobacillus* taxonomy group. The resulting identity of the strains were as follows; *L. gasseri* 7(3), *L. gasseri* 17(4), *L. reuteri* 17(7) and *L. reuteri* 16(9).

2.1. Introduction

The four bacterial isolates used in this study were, in a previous study (Magwira, 2008) putatively identified as members of the genus *Lactobacillus*. A detailed identification was, therefore, necessary to enable their development as possible probiotic strains. *Lactobacillus* has traditionally been identified based on cell and colony morphology, as well as biochemical tests (Zhong *et al*., 1998). However, these phenotypic methods have been shown to be disadvantageous due to the fact that, they are labour-intensive and may be inaccurate because of the related nutritional and growth needs of many different *Lactobacillus* species (O'Sullivan, 2000; Kao *et al*., 2007).

Studies have, therefore, shifted towards using molecular-based methods, which have been used by several authors in the identification of *Lactobacillus* species and to understand the phylogenetic relationships among species (Zhong *et al*., 1998; Olsen and Woese, 1993). These methods include; 16S rRNA gene sequencing (Morotomi *et al*., 2002), 16S-23S rRNA intergenic region sequencing (Tannock *et al*., 1999), randomly amplified polymorphic DNA (RAPD)-PCR (Tilsala-Timisjärvi and Alatossava, 1998), pulsed field gel electrophoresis (PFGE) (Roy *et al*., 1999) and DNA-DNA hybridization (Schillinger, 1999). Such methods have been shown to be consistent, reproducible and efficient enough to discriminate between closely related species (Singh *et al*., 2009). RAPD analysis is a PCR-based technique that helps to distinguish between closely related bacteria with the use of short arbitrary primers that will randomly bind to the genomic DNA of microorganisms to produce a DNA fingerprint (Tilsala-Timisjärvi and Alatossava, 1998; Welsh and McClelland, 1990). PGFE is a technique based on the use of genomic DNA digested with restriction enzymes, separated into large DNA fragments to distinguish among closely related *Lactobacillus* species (Tynkkynen *et al*., 1998). The DNA-DNA hybridization method determines the similarity between two DNA samples and can show the taxonomic relationship between closely related species (Kwon *et al*., 2004)

In this study, the use of 16S rRNA and 16S-23S rRNA gene spacer region sequencing was applied to identify the 4 *Lactobacillus* isolates, because they are regarded as sufficiently sensitive to discriminate between closely related species.

Ribosomes are composed of protein and RNA moieties and are regarded as essential components of prokayortic and eurkaryotic cells (Barry *et al*., 1991). In prokaryotes, the rRNA genetic loci consist of conserved genes (16S, 23S and 5S rRNA genes) that are located within an operon on the bacterial chromosome and separated by spacer regions (Jensen *et al*., 1993; Tannock, 1999) (Fig. 2.1).

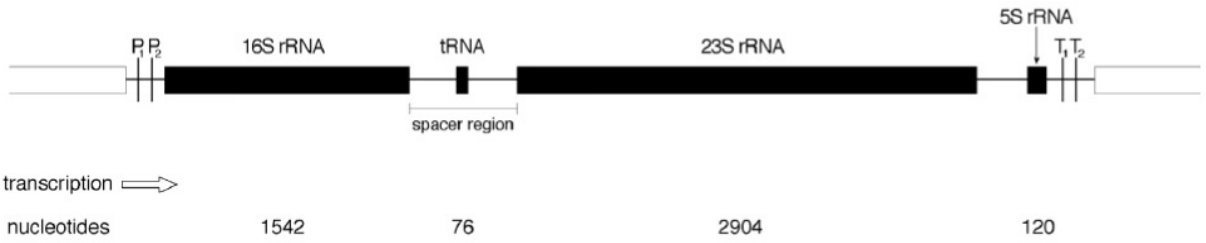

Figure 2.1. The rRNA operon of bacteria showing the 16S-23S rRNA gene spacer region. P_1P_2 = promoters; T_1T_2 = terminators (Tannock, 1999).

Sequence analysis of the 16S rRNA gene is known to be an accurate and useful technique that has been used widely to study the phylogenetic, inter and intragenic relationships among multiple genera (Leblond-Bourget *et al*., 1996; O'Sullivan, 2000). Because it is highly conserved, the 16S rRNA gene is known to possess a similar length (approximately 1.5 kb) throughout the entire bacterial kingdom (Gürtler and Stanisich, 1996). However, as the evolutionary distance between bacteria decreases, differences in the 16S rRNA gene sequences has been shown to be insufficient in discriminating between closely related species (Leblond-Bourget *et al*., 1996). Other studies have used the 23S rRNA genes sequence for bacterial identification, but because of its large size (3 kb), it

is a less useful gene to target (Berthier and Ehrlich, 1998). Moreover, neither the 23S nor 5S rRNA gene sequences have been extensively used for phylogenetic studies.

The 16S-23S rRNA gene spacer region, however, has been regarded as a more accurate tool in bacterial identification because of the polymorphisms between closely related species (Gürtler and Stanisich, 1996). These polymorphisms within the spacer region can be due to the number and type of tRNA sequences that occur there. For instance, the majority of Gram-negative bacteria contain $tRNA^{Ala}$ and $tRNA^{ile}$ genes, whereas others may contain only $tRNA^{Glu}$ (Osorio *et al*., 2005). However, in the case of Gram-positive bacteria, the spacer region may contain either $tRNA^{Ala}$ or $tRNA^{ile}$ or both of the genes (Nour, 1998). The 16S-23S rRNA gene spacer region is commonly amplified by carrying out a PCR, using primers from highly conserved flanking sequences (Jensen *et al*., 1993), thereby discriminating between closely related species.

The aim of this study was to identify the four putative *Lactobacillus* isolates to the species level, and to establish a phylogenetic tree of the isolates to show their relationship to other *Lactobacillus* species by analysing the sequences of their 16S rRNA genes and 16S-23S rRNA gene spacer regions.

2.2. Materials and methods

2.2.1. Strains, media and growth conditions

The following putative *Lactobacillus* strains isolated from human faecal samples were used in this study: B7(3), B17(4), B17(7) and B16(9). They were stored at -70°C in de Man Rogosa Sharpe (MRS) broth (Biolab) with 50% glycerol. Cultures were grown anaerobically in MRS medium at 37°C and subcultured twice in MRS before use. *Escherichia coli* was grown aerobically in Luria-

Bertani medium (LB). The anaerobic cultivation was carried out in an anaerobic chamber (Forma Scientific, model 1024) in an atomosphere consisting of 5% hydrogen, 10% carbon dioxide and 85% nitrogen.

2.2.2. Isolation of genomic DNA from the bacterial isolates

The *Lactobacillus* isolates were streaked on MRS agar and incubated anaerobically for 48 h. Single colonies were then inoculated into 5 ml MRS broth and incubated anaerobically at 37^{o}C for 18 h. This culture (2 ml) was centrifuged at 14.000 rpm for 2 mins, to collect the cells. The pellet was resuspended in 200 µl of lysis buffer (20 mM Tris-Cl [pH 8.0], 2 mM EDTA, 1.2% Triton, and 20 mg/ml lysozyme) and incubated for 30 mins. Proteinase K (2 µl) was added and the mixture was incubated for another 30 mins. The genomic DNA extraction was carried out with the Genomic DNA Purification Kit (Fermentas Life Science) according to the instruction manual, with 10 µg/ml of RNAse treatment prior to the precipitation step.The genomic DNA was eluted with 30 µl of sterile distilled water and stored at -20^{o}C. The DNA quality and concentration was determined by agarose gel electrophoresis and spectrophotometric measurement using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies).

2.2.3. 16S rRNA gene PCR

The 16S rRNA gene was amplified in a 50 µl PCR reaction, containing 200 ng of genomic DNA, 2.5 µl of the universal primers F27 (F) and R5 (R) at 10 µM (Table 2.1), 25 µl of Kapa Ready Mix (Kapa Biosystems) and 18 µl of sterile distilled water. As a negative control, PCR was performed using water instead of DNA, and for positive control, DNA from *Lactobacillus plantarum* KLDS 1.0728 was used. The amplification was performed using a thermocycler (GeneAmp® PCR system 9700, Applied Biosystems) under the following conditions: pre-denaturation at 96^{o}C for 2 mins,

followed by 25 cycles of denaturation at 95°C for 30 sec, annealing temperature of 55°C for 30 sec, elongation temperature at 72°C for 1 min and final elongation at 72°C for 3 mins. The PCR products were separated by electrophoresis on an 0.8% agarose gel containing ethidium bromide. The PCR products were purified using the Biospin PCR purification kit (BioFlux) and then sequenced.

Table 2.1. Description of primers used in this study

Name	Primer sequences (5′-3′)	PCR Fragment size (bp)	References
F27 (F) R5 (R) } 16S rRNA	AGAGTTTGATCCTGGCTCAG ACGGITACCTTGTTACGACTT	1500	Lane, 1991 Alm *et al.*, 1996
16A (F) 23B (R) } 16S-23S rRNA	GAATCGCTAGTAATCG GGGTTCCCCCATTCGGA	400	Tannock *et al.*, 1999

2.2.4. 16S-23S rRNA spacer region PCR

For the amplification of 16S-23S rRNA spacer region, the PCR mixture (50 µl) consisted of 200 ng of genomic DNA, 2.5 µl of 16A (F) and 23B (R) primers at 10 µM (Table 2.1), 25 µl of Kapa Ready Mix (Kapa Biosystems) and 18 µl of sterile distilled water. Amplification was performed using a thermocycler (GeneAmp® PCR system 9700, Applied Biosystems) with initial denaturation at 94°C for 5 mins, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 53.5°C for 30 sec and elongation at 72°C for 30 sec, with a final elongation at 72°C for 5 mins. The PCR products were visualised on a 2% agarose gel stained with ethidium bromide. Three bands were observed on the gel, and the smallest band (400 bp) was excised from the agarose gel with a sterile blade and extracted with the BioSpin gel extraction kit (BioFlux). This DNA was used as a template in a second round of PCR. The amplified PCR product was purified again and cloned into *Escherichia coli* DH5α competent cells (Sambrook *et al*., 1989), using the pTZ57R/T vector (Fermentas Life Science), following the protocol provided by InsTAclone PCR Cloning Kit (Fermentas Life

Science). The transformants were screened on LB agar plates containing 250 µg/ml ampicillin, 500 µg/ml X-Gal and 0.25 mM IPTG. The white colonies were isolated and screened to confirm the presence of inserts using colony PCR (Sambrook *et al*., 1989). Three positive clones were picked and each clone was grown in 5 ml LB medium with 5 µl of ampicillin, the plasmid DNA extracted using the peqGOLD Plasmid Miniprep Kit I (peQLab Biotechnologie GmbH) and the inserts were sequenced.

2.2.5. DNA sequencing and phylogenetic analysis

The nucleotide sequencing was carried out using the fluorescent dideoxynucleotide chain termination method (Sanger *et al*., 1977) and the MegaBACE 500 DNA sequencer (Amersham Pharmacia Biotech, Buckinghamshire, UK). The nucleotide sequence obtained was analysed and edited using the DNAMAN program package (Lynnon Corporation, Quebec, Canada) and further analysed on-line using the BLAST server at the NCBI sequence database (http://www.ncbi.nlm.nih.gov). A similarity of > 98% to the 16S rRNA gene or 16S-23S rRNA gene spacer region sequences of the strains was used as the standard for the identification. Nucleotide sequence alignments were performed using the ClustalW program (Thompson *et al*., 1994). The phylogenetic trees were constructed using the program MEGA 4.0 (Tamura *et al*., 2007). The bootstrap test of Phylogeny-Neighbor Joining Tree option with 1000 iterations was used to produce phylogenetic trees.

2.3. Results and Discussions

2.3.1. 16S rRNA

The 16S rRNA gene of the putative *Lactobacillus* isolates was amplified using F27 (F) and R5 (R) primers (Table 2.1). All the isolates produced a single PCR product of approximately 1500 base pairs (Fig. 2.2). The positive control (Lane 5) produced the expected product size, confirming the sizes of

the 4 *Lactobacillus* strains. No amplification product was observed in the negative control (Lane C), indicating the absence of contaminating DNA.

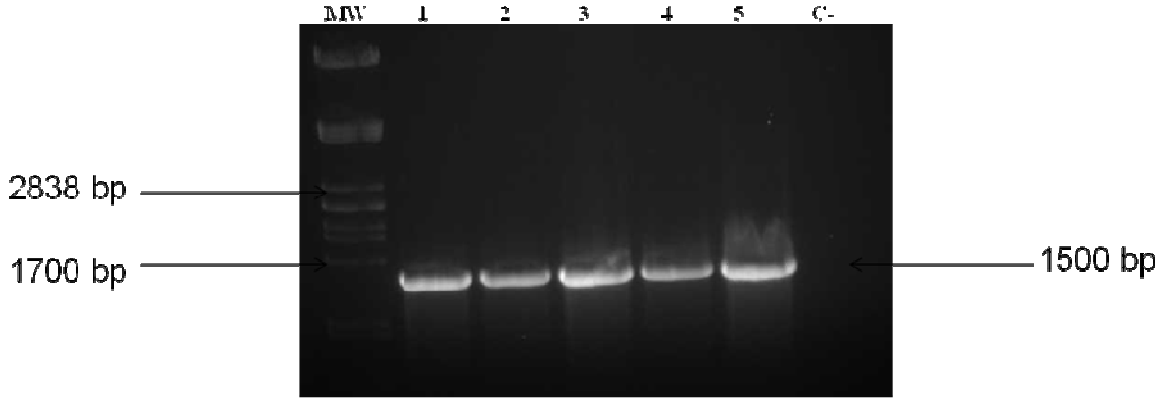

Figure 2.2. Agarose gel electrophoresis of PCR products of the 16S rRNA gene from *Lactobacillus* isolates. Lane MW, Molecular size marker (λ DNA digested with *Pst*I); 1, B7(3); 2, B17(4); 3, B17(7); 4, B (16)9; 5, Positive control (*L. plantarum* KLDS 1.0728); C-: Negative control.

DNA sequencing of 16S rRNA gene PCR products revealed the identity of the isolates (Table 2.2). Isolates B7(3) and B17(4) had 100% identity to *L. gasseri* NCC2857 (FJ557005.1), a novel *Lactobacillus* spp. isolated in Spain. The two isolates may be identical to each other, although they were isolated from different individuals, denoted by B7 and B17. Isolate B17(7) showed 99% identity to *L. reuteri* LU3 (AY735406.1), isolated from food from Korea and B16(9) showed 98% identity to *L. vaginalis* DoxG3 (GQ422709.1) isolated from dental plaque.

Table 2.2. Percentage identity of the *Lactobacillus* isolates using 16S rRNA gene sequence analysis.

Isolate	**Close relative**	**Accession number**	**% Identity**
B7(3)	*L. gasseri* NCC2857	FJ557005.1	100
B17(4)	*L. gasseri* NCC2857	FJ557005.1	100
B17(7)	*L. reuteri* LU3	AY735406.1	99
B16(9)	*L. vaginalis* DoxG3	GQ422709.1	98

A 16S rRNA based phylogenetic tree was constructed with the Phylogeny program (MEGA 4.0 version). This analysis is essential as it provides information regarding the relationship of the strains with reference strains (Olsen *et al*., 1994). The phylogenetic relationship was observed between the 4 *Lactobacillus* isolates in comparison with other *Lactobacillus* species (Fig. 2.3).

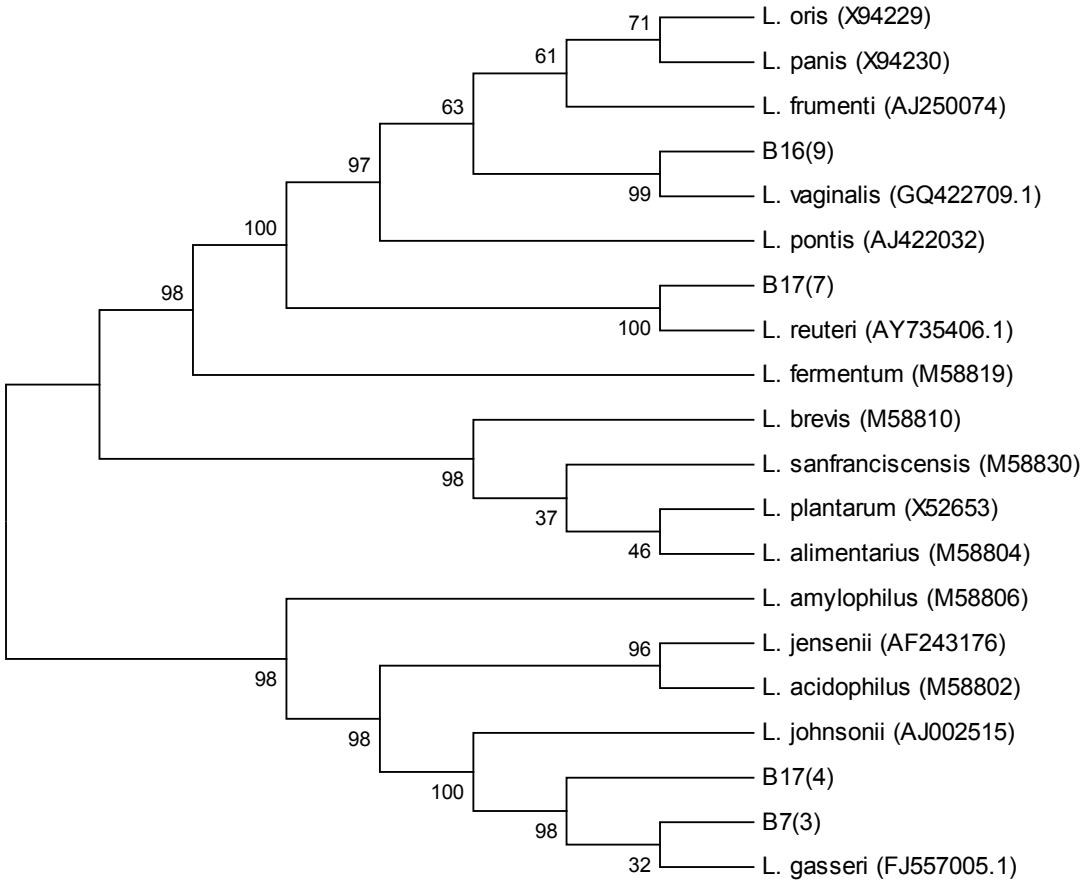

Figure 2.3. Phylogenetic tree based on the 16S rRNA gene sequences demonstrating the relationship of the *Lactobacillus* isolates in comparison with other *Lactobacillus* species. The GenBank accession numbers of the used 16S rRNA gene sequences of the *Lactobacillus* species are in paranthesis. Numbers at each branch-point represent the bootstrap values. This figure was generated using Mega 4.0 (Tamura *et al*., 2007).

Isolates B7(3) and B17(4), clustered together with the *L. gasseri* NCC2857 strain (FJ557005.1), demonstrating a close relationship between the three *Lactobacillus* strains. However, these two isolates were shown to be clearly distinct from B17(7) and B16(9) isolates, as they clustered in

different branches. The B17(7) isolate was shown to be closely related to *L. reuteri* LU3 (AY735406.1), whereas the B16(9) isolate matched closely to *L. vaginalis* DoxG3 (GQ422709.1). It is important to note that the genus *Lactobacillus* consists of several groups composed of species that have been defined based on their 16S rRNA gene sequences. These include the *L. acidophilus* group (*L. johnsonii*, *L. gasseri*, *L. acidophilus* and *L. jensenii*), the *L. casei* group (*L. rhamnosus*, *L. sakei* subspecies and *L. curvatus*), the *L. plantarum* group (*L. plantarum* and *L. paraplantarum*), the *L. buchneri* group (*L. brevis*, *L. bruchneri* and *L. spicheri*) the *L. salivarius* group (*L. salivarius* and *L. animalis*) and the *L. reuteri* group (*L. vaginalis*, *L. fermentum*, *L. frumenti* and *L. reuteri*) (Canchaya *et al*., 2006). Since the *L. reuteri* group clearly demonstrates that *L. vaginalis* and *L. reuteri* are closely related, this may explain the phylogenetic relationship seen between the B17(7) and B16(9) isolates. However, studies have reported that, 16S rRNA gene sequencing may not always considered to be the best approach for classifying, due to the high similarity in this gene among closely related species in the various *Lactobacillus* groups (Kao *et al*., 2007). Amplification of 16S-23S rRNA spacer region can however, provide better identification of closely related species.

2.3.2. 16S-23S rRNA spacer region

As explained previously, the amplification of 16S-23S rRNA spacer region is known to be more discriminatory than the amplification of 16S rRNA gene. Therefore, the putative *Lactobacillus* isolates were further examined by amplifying the 16S-23S rRNA spacer region, using the 16A (F) and 23B (R) primers (Table 2.1) to determine their identity. The PCR products yielded 3 main bands (Fig. 2.4). Interestingly, the banding pattern for lane 1 and 2 differed marginally from that of lane 3 and 4, in that the band sizes varied. This demonstrated that there are differences among the isolates. It should be noted that a typical pattern of *Lactobacillus* strain consist of 3 bands, the small, medium

and large spacer region, as reported by Moreira *et al*. (2005). Moreover, the variation in these lengths could be due to either the number or type of tRNA sequences present in this region.

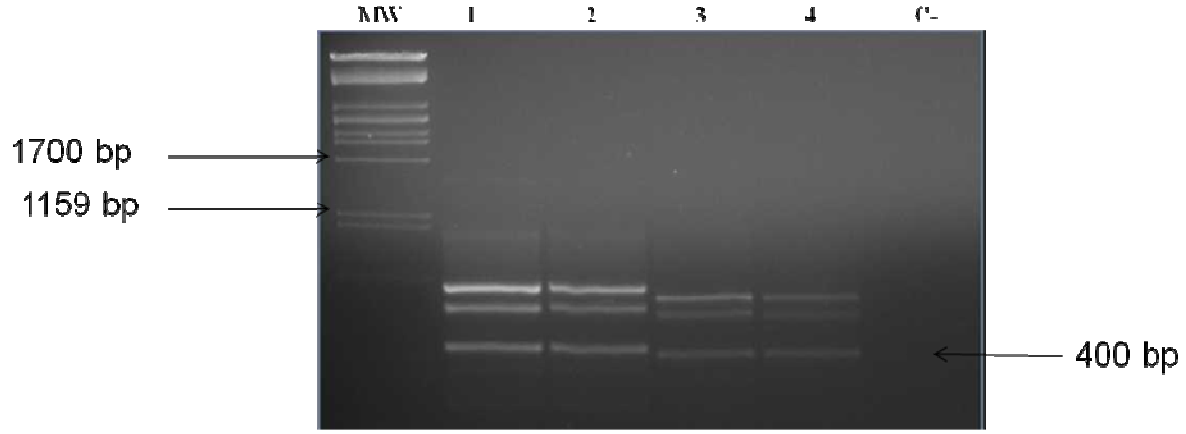

Figure 2.4. Agarose gel electrophoresis of PCR of the 16S-23S rRNA spacer region from 4 *Lactobacillus* isolates. Lane MW: Molecular size marker (λ DNA digested with *Pst*I); 1, B7(3); 2, B17(4); 3,B17(7); 4, B (16); C-, Negative control.

The small (400 bp) bands of the *Lactobacillus* isolates were excised and cloned into the pTZ57R/T vector as previously described. The inserts were confirmed and sequenced. The nucleotide sequences obtained were analysed, aligned and a phylogenetic tree was constructed. The sequences revealed by BLAST analysis confirmed that isolates B7(3) and B17(4) had a 99% identity with *L. gasseri* ATCC 33323 (CP000413.1) (Table 2.3). B17(7) displayed 100% identity with *L. reuteri* DSM 20016 (CP000705.1), whereas B16(9) isolate displayed 100% identity with *L. reuteri* JCM 1112 (AP007281.1).

Table 2.3. Percentage identity of the *Lactobacillus* isolates using 16S-23S rRNA gene spacer region sequence analysis.

Isolate	**Close relative**	**Accession number**	**% Identity**
B7(3)	*L. gasseri* ATCC 33323	CP000413.1	99
B17(4)	*L. gasseri* ATCC 33323	CP000413.1	99
B17(7)	*L. reuteri* DSM 20016	CP000705.1	100
B16(9)	*L. reuteri* JCM 1112	AP007281.1	100

The phylogenetic tree was constructed to determine the phylogenetic relationship among the *Lactobacillus* isolates in comparison with the reference *Lactobacillus* species (Fig. 2.5).

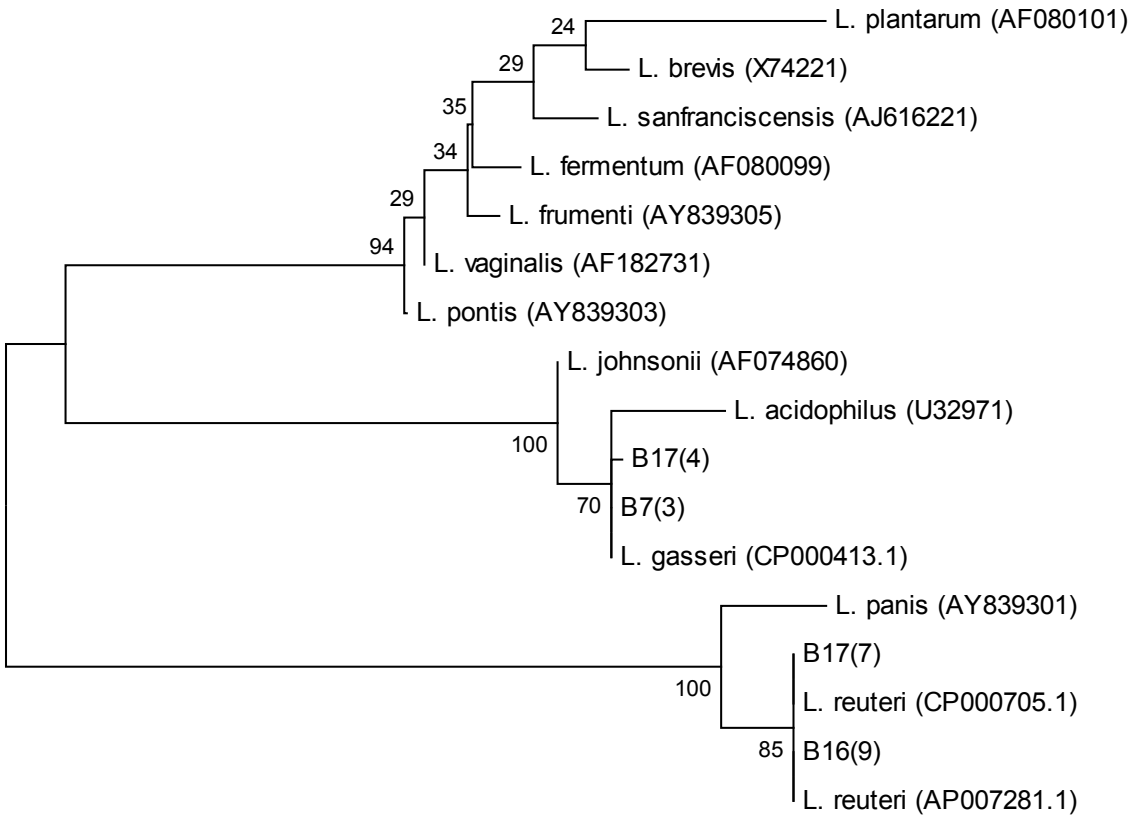

Figure 2.5. Phylogenetic tree based on the 16S-23S spacer region sequences demonstrating the relationship of the putative *Lactobacillus* isolates in comparison with other *Lactobacillus* species. The GenBank accession numbers of the used 16S-23S rRNA spacer region sequences of the *Lactobacillus* species are in paranthesis. Numbers at each branch-point represent the bootstrap values.

The B7(3) and B17(4) isolates consistently clustered together and were closely related to the *L. gasseri* ATCC 33323 strain (CP000413.1). The two isolates also clustered with *L. acidophilus* and *L. johnsonii* strains, suggesting that the strains may also be closely related. The B17(7) and B16(9) isolates clustered with *L. reuteri* strain DSM 20016 (CP000705.1) and *L. reuteri* JCM 1112 (AP007281.1) as expected demonstrating a very close relationship. Using the 16S rRNA gene sequence, the B16(9) isolate was identified as *L. vaginalis*, however, using the 16S-23S rRNA spacer region sequence, it was identified as *L. reuteri*. This could possibly be due to the fact that the 16S-23S rRNA gene spacer region is more accurate in discriminating closely related species (Gürtler and Stanisich, 1996). Taking the 16S rRNA gene sequence and 16S-23S rRNA spacer region sequence data together, the 4 putative *Lactobacillus* strains were identified as *L. gasseri* 7(3), *L. gasseri* 17(4), *L. reuteri* 17(7) and *L. reuteri* 16(9).

As explained previously, the genus *Lactobacillus* represents a variety of species, which have been isolated from several habitats, such as the gastrointestinal tract of animals and humans, oral and genital tracts (Roos *et al*., 2000). However, among this genus, certain *Lactobacillus* species are recognised as truly autochthonous (natural) gut inhabitants, such as *L. salivarius*, *L. reuteri*, *and L. gasseri*, while others species such as *L. plantarum* and *L. brevis* are considered as transient (allochthonous) gut inhabitants of the host (Dal Bello *et al*., 2003). This study revealed the identity of the *Lactobacillus* isolates, *L. gasseri* 7(3), *L. gasseri* 17(4), *L. reuteri* 17(7) and *L. reuteri* 16(9), indicating that these strains could be referred as autochthonous strains, due to the fact that they are natural isolates. Molecular analysis has demonstrated that individual humans usually differ in their microflora (Kimura *et al*., 1997). Nonetheless, in this study the strains isolated from 4 different individuals were closely related. This could be due to either consumption of similar diet or geographic location.

Previous studies have been reported that the human intestinal microflora compromises of several microbial species that are commonly detected in the human faeces. However, among the microbial species, *L. gasseri* and *L. reuteri* strains have been shown to be one of the predominant *Lactobacillus* species in the human gastrointestinal tract of neonates (Wall *et al*., 2007) and adults (Reuter, 2000), the oral cavity (Munson *et al*., 2004), the vaginal tract (Pavlova *et al*., 2002), the small intestine of a pig (Wadström *et al*., 1987), human faeces, (Tannock *et al*., 1999), and the gastrointestinal tract and faeces of mouse (Hata *et al*., 2007). These strains have previously been demonstrated to have health-promoting effects and are used as probiotics, which when ingested, exert positive effect on the host. Health benefits attributed to the consumption of *L. gasseri* include, boosting the immune system of healthy humans (*L. gasseri* CECT 5714) (Olivares *et al*., 2006), assisting in suppressing *Helicobacter pylori* infection and reducing gastric mucosal inflammation (*L. gasseri* OLL 2716) (Sakamoto *et al*., 2001). *L. reuteri*, has been shown to improve human gastrointestinal health by reducing the incidence and severity of diarrhoea (*L. reuteri* ATCC 55730) (Valeur *et al*., 2004; Shornikova *et al*., 1997) and may also prevent infection caused by *Cryptosporidium parvum* in immunodeficient individuals (Alak *et al*., 1997). Other studies have recently reported that, to understand the impact of gut microflora on human health and well-being, it is important to assess the content, diversity and functioning of the microbial gut community (Qin *et al*., 2010).

2.4. Conclusion

This study revealed the identity of the 4 *Lactobacillus* isolates based on 16S rRNA gene sequencing as well as the sequence of the 16S-23S rRNA spacer region. The methodology based on targeting the conserved ribosomal gene has been shown to be accurate in identifying and discriminately closely related species. However, Tilsala-Timisjärvi and Alatossava (1998) indicated that these regions are not sensitive enough to differentiate bacteria below the species level. Therefore, in order to validate

and extend the results obtained from the 16S rRNA gene and 16S-23S rRNA spacer region, it would have been interesting to apply other molecular tools such as RAPD and PGFE techniques. However, due to time-constraints, the experiments could not be performed in this study. These methods have been used for better discrimination of *Lactobacillus* strains, on the basis of their genomic profiles (Lortal *et al*., 1997; Roy *et al*, 1999). DNA-DNA hybridization is another technique that helps to distinguish closely related species at their genomic level. It determines the genetic similarity by measuring the degree of overall nucleotide difference between two pools of DNA (Ruvolo and Smith, 1986). Future work should include applying these techniques for further identification of the 4 *Lacobacillus* isolates used in this study.

Due to their potential health-promoting effects and the fact that they were isolated from their natural ecological environment, it was of interest to determine whether the 4 *Lactobacillus* strains used in this study had appropriate probiotic characteristics. It was also especially important to determine their ability to degrade oxalate in the context of their possible use in the prevention of kidney stone disease. These studies are described in Chapter 3.

CHAPTER 3

CHARACTERIZATION OF POTENTIAL PROBIOTIC STRAINS

3.0. Summary

The four *Lactobacillus* strains were examined for their potential as probiotics by measuring their antibiotic resistance, antimicrobial activity, adhesion capacity, and acid and bile tolerance. They were also tested to determine their oxalate degrading activity. The *Lactobacillus* strains were shown to comply with most of the criteria required to be classified as probiotics. The strains were most sensitive to penicillin and highly resistant to kanamycin. They demonstrated good antimicrobial activity against pathogenic bacteria, with high antimicrobial activity against *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*. They also displayed good adhesion properties, as determined by yeast agglutination and autoaggregation assays. *L. reuteri* 17(7) possessed the best adhesion properties as compared to the other strains. The strains were also tested for their oxalate degrading ability and *L. gasseri* 7(3) displayed the highest oxalate degrading activity, of more than 30%. Among the *Lactobacillus* strains, *L. gasseri* 7(3) and *L. reuteri* 17(7) were selected to test their acid (pH 2 and pH 4) and bile (0.5% and 1%) tolerance. These strains were sensitive at pH 2, but tolerated pH 4 conditions well. Furthermore, these strains were shown to tolerate high bile salt concentrations well, with *L. reuteri* 17(7) able to grow as well as the control culture at 1% bile concentrations. Overall, the strains appeared to be good probiotic candidates.

3.1. Introduction

There are numerous microorganisms that have either been used or identified for use as potential probiotics (Rolfe, 2000). Amongst these microorganisms, *Lactobacillus* and *Bifidobacterium* species are the common bacteria traditionally used as probiotics, since they exert beneficial effects on the host (Rolfe, 2000). For probiotic bacteria to do this, they need to meet several criteria. These include appropriate resistance to antibiotic treatment, the ability to inhibit the growth of pathogenic bacteria by producing antimicrobial substances, efficient adhesion to the host tissue, tolerance of low pH during gastric transit, and tolerance of bile salts found in the gut (Charteris *et al*., 1998a; Rönkä *et al*., 2003). In the current study, the ability to degrade oxalate to reduce risk of kidney stone disease was also of interest (Azcarate-Peril *et al*., 2006). The possible scientific approaches that have been used to evaluate these features are reviewed below.

3.1.1. Antibiotic susceptibility

It is important to ascertain the antibiotic resistance or susceptibility of the strains of interest, because this may have an advantageous or disadvantageous effect. For example, probiotic bacteria that are resistant to antibiotics could possibly be given during antibiotic treatment of a disease (Vanderhoof *et al*., 1999). However, it should be noted that resistance could also be problematic, since the resistance determinants may be transferred to a pathogen with detrimental outcome for the host (Curragh and Collins, 1992). Conversely, when a probiotic bacterium is sensitive to antibiotics, it could be unfavourable, as it may not have a significant protecting effect in patients that are under antibiotic treatment (Gould and Short, 2008). Safety assessments should, therefore, be performed to fully understand the antibiotic resistance patterns of potential probiotic bacteria.

There are several methods that have been used to determine the antibiotic susceptibility of *Lactobacillus* species. These include: Agar disk diffusion (Zhou *et al*., 2005; Temmerman *et al*., 2003), the use of E-test strips, (Danielsen and Wind, 2003; Katla *et al*., 2001), broth dilution (Mayrhofer *et al*., 2008) and agar dilution (King and Philips, 2001). However, comparing the results obtained by these methods can be problematic, because certain methods have their own limitations. For instance, certain techniques have been shown to be unreliable for particular antibiotics tested, or the culture medium used might affect the outcome of the antibiotic susceptibility testing (Ammor *et al*., 2007). It is, therefore, important to select an appropriate method for antibiotic susceptibility testing. In this study, the well-described disk diffusion method was used (Zhou *et al*., 2005).

3.1.2. Antimicrobial activity

Lactic acid bacteria (LAB) play a protective role through hindering the growth of pathogenic bacteria by producing diverse inhibitory compounds (Piard and Desmazeaud, 1992). Several reports have described different techniques that may be used to detect the antimicrobial activity of probiotic bacteria, including the agar spot method, the well-diffusion technique, and the microtiter well method (Jacobsen *et al*., 1999; Dimitonova *et al*., 2007; Maragkoudakis *et al*., 2006). However, the agar spot method has been shown to be an accurate and reliable method and it was, therefore, used in this study (Jacobsen *et al*., 1999).

3.1.3. Adhesion studies

Numerous reports have investigated the adhesion of lactobacilli to gastrointestinal tissue or epithelial cells. Studies have successfully used intestinal mucus and enterocyte-like Caco-2 cells to evaluate the adhesion ability of probiotic bacteria (Ouwehand *et al*., 1999; Buck *et al*., 2005). However,

alternative *in vitro* methods linked to adhesion have also been described, such as determining the ability of the probiotic cells to agglutinate yeast as well as their efficiency of autoaggregation.

Adlerberth *et al*. (1996) found that certain *Lactobacillus plantarum* strains have been shown to adhere specifically to mannose-containing polysaccharides in human colonic cell lines. These authors concluded that this characteristic may provide an advantage in adhesion which may potentially be involved in the ability of the bacteria to colonize the intestine. The yeast agglutination method, therefore, is based on studying the capacity of bacteria to adhere to mannose residues found on the cell wall of *Saccharomyces cerevisae* (Adlerberth *et al*., 1996). The addition of mannose-adhering bacteria to yeast cells results in the agglutination of the yeast, which can be observed by light microscopy (Pretzer *et al*., 2005). Studies have shown that carbohydrate binding lectins and protein moieties on bacterial surface mediate *Lactobacillus* adhesion (Henriksson and Conway, 1992; Conway and Kjelleberg, 1989; Cocconnier *et al*., 1992). Further studies have shown that carbohydrates such as mannose inhibit (Bar-Shavit *et al*., 1977) the adherence of bacteria to mammalian cells (Oyofo *et al*., 1989).

The ability of probiotic strains to autoaggregate is another important property linked to adhesion to the intestinal epithelium. Their coaggregation ability with pathogens on the other hand, allows them to form a barrier which assists in preventing colonization of epithelium by the pathogenic bacteria (Kos *et al*., 2003; Del Re *et al*., 2000). Reniero *et al*. (1992) did a study to identify possible extracellular factor(s) in the culture supernatant responsible for mediating cell aggregation. The results indicated that the aggregation mechanism usually involved the interaction of bacterial cell surface components such as protein, lipoteichoic acids, and carbohydrates as mediators of adherence (Reniero *et al*., 1992; Gusils *et al*., 2002).

3.1.4. Oxalate-degrading ability

In this study, the ability to degrade oxalate is another essential requirement for the probiotic bacteria under investigation, since it would allow them to prevent the development of pathological disorders such as hyperoxaluria (excess urinary oxalate), and kidney stone formation (Azcarate-Peril *et al*., 2006). There are numerous methods available that can be used to determine oxalate degradation by bacteria. These include the measurement of oxalate levels by gas-liquid chromatography, ion chromatography, high performance liquid chromatography, mass spectrometry and enzymatic assays (Çalişkan, 2000). In this study, the enzymatic assay was used due to its simplicity, rapidity and reproducibility (Liu *et al*., 2009). This assay is commonly used for measurement of urinary oxalate to evaluate the possibility of recurrent stone formation in patients with nephrolithiasis (kidney stone disease).

3.1.5. Low pH and bile tolerance

The survival capacity of probiotic bacteria in the gut depends on their ability to tolerate low pH during gastric transit (Pennacchia *et al*., 2003). Several authors have used hydrochloric acid (HCl), acetic acid (CH_3COOH) or sodium hydroxide (NaOH) to adjust the pH medium of the growth in order to investigate acid resistance of bacteria (Gupta *et al*., 1996; du Toit *et al*., 1998 and Delgado *et al*., 2007). Studies are generally carried out in microwell plates or universal bottles with MRS broth adjusted to a range of pH values. Tolerance to bile is one of the factors that determine the survival of the bacteria in the gut. Several bile preparations have been used to evaluate the bile resistance of probiotic bacteria. These include Oxgall (derived from bovine bile) cholic acid and taurocholic acid (Liong and Shah, 2005).

3.2. Materials and Methods

3.2.1. Antibiotic susceptibility testing

The antibiotic susceptibility of the *Lactobacillus* strains was determined on MRS agar (Biolab) using the antibiotic disk diffusion method. It was done according to Zhou *et al*. (2005) except that the bacterial strains were initially grown for 18 h at 37^{o}C in 5 ml MRS broth under anaerobic conditions. The optical density (OD_{600}) of the cell cultures was adjusted to 0.2 and 100 µl of the culture was spread on the MRS agar. Disks (Oxoid) impregnated with their specific antibiotics: rifampicin (5 µg), chloramphenicol (30 µg), streptomycin (10 µg), ampicillin (10 µg), vancomycin, (30 µg) cephalothin (30 µg), kanamycin (30 µg), erythromycin (30 µg), penicillin G (10 µg) and tetracycline (30 µg), were placed aseptically on the MRS agar surface. After 48 h anaerobic incubation at 37^{o}C, the diameter of the zones of inhibition was measured (mm). The experiment was done in biological triplicates with two technical repeats.

3.2.2. Antimicrobial activity assay

The antimicrobial activity of the *Lactobacillus* strains against 7 pathogens was evaluated using the agar spot method as described by Jacobsen *et al*. (1999), except that 5 µl of the *Lactobacillus* test culture (grown for 18 h in MRS broth) was spotted on an MRS agar plate and incubated for 48 h under anaerobic conditions. The pathogens tested were *Escherichia coli* ATCC 25922, *Staphylococcus aureus*, *Salmonella typhimurium*, grown in Luria medium (McCaffrey *et al*., 1992) under aerobic conditions, and *Enterococcus faecium*, *Enterococcus faecalis*, *Clostridium perfrigens* and *Clostridium botulinum*, grown anaerobically in Brain heart infusion medium (BHI). All these were pre-grown for 18 h and incubated at 37^{o}C. The MRS agar preinoculated with the *Lactobacillus* test culture was overlaid with soft agar (0.8%) inoculated with the pathogenic bacteria. Thereafter,

the plates were examined for zones of inhibiton. The experiment was performed as biological triplicates and two technical replicates.

3.2.3. Adhesion assay

The ability of *Lactobacillus* strains to agglutinate yeast cells was evaluated as described by Adlerberth *et al*. (1996) and Pretzer *et al*. (2005) with the following modifications: *Saccharomyces cerevisiae* was grown for 20 h in YPD (10 g yeast extract, 20 g peptone and 20 g glucose) medium (Biolab) under aerobic conditions at 30^{o}C. Yeast cells were harvested by centrifugation, washed with 1X Phosphate buffered saline (PBS) (pH 7.4) and stained with safranin solution and resuspended in 1 ml of PBS or D-mannose (BDH, Biochemicals Limited) solution (1M). The bacterial strains were grown for 18 h at 37^{o}C in 5 ml MRS broth. Thereafter, 2 ml of each culture was centrifuged at 10,000 × g for 10 mins. The supernatant was filter sterilized using a 0.4 micron filter, and placed in a sterile 2 ml Eppendorf tube. The bacterial cell pellet was resuspended in 1 ml of 1X PBS or culture supernatant. The bacterial suspension (20 µl) was placed on a glass slide, mixed with 20 µl safranin stained yeast cells resuspended in either PBS or mannose (1 M), and observed for yeast agglutination using a light microscope at a magnification of 40X (Leitz). Agglutination was also observed by mixing 20 µl of bacterial culture resuspended in culture supernatant with 20 µl safranin stained yeast cells. Yeast cells (20 µl) mixed with water (20 µl) was used as a control.

To test the ability of the bacterial cells to autoaggregate, the cultures were prepared as for the yeast aggregation assay (above) except that the cell pellet was resuspended in a 2 ml of 0.85% sodium chloride (NaCl) solution and vortexed for a minute. The cell suspension was incubated in the presence or absence of 1 ml supernatant at room temperature. The autoaggregation ability was

observed visually for sand-like particles settling to the bottom of the tubes (Kmet and Lucchini, 1999). The experiment was done as biological triplicates and two technical repeats.

3.2.4. Oxalate-degrading ability of the *Lactobacillus* strains

The strains were inoculated into ½ strength MRS broths (half w/v) supplemented with 10 mM ammonium oxalate which had been filter-sterilized and added after autoclaving. After 2 days of incubation at 37^{o}C, 100 µl of the culture was transferred into fresh 5 ml ½ strength MRS broth with 10 mM of ammonium oxalate and incubated for a further 2 days. The cell density was then adjusted to OD_{600} 0.5 in sterile PBS buffer (pH 7.2) and 100 µl of this suspension was inoculated into 10 ml ½ strength MRS broth (supplemented with 10 mM ammonium oxalate) and incubated anaerobically for 5 days at 37^{o}C. On days, 2 and 5, 1 ml aliquots of the culture were centrifuged and the supernatant retained (stored at -20^{o}C). This experiment was done in duplicate and ½ strength MRS broth with no added ammonium oxalate was used as the control. For the oxalate assay, the concentration of oxalate present in the supernatant fractions from days 2 and 5 was measured using the enzymatic assay kit (Trinity Biotech plc, Ireland).

3.2.5. pH and Bile tolerance

The pH and bile tolerance of the candidate bacteria was measured using a protocol based on the work of Prasad *et al*. (1998) except that the cultures were adjusted to an OD_{600} approximately 0.5 and used to inoculate fresh 20 ml MRS broths adjusted to pH 2 and 4 with HCl. To determine cell growth in the presence of bile, the 16 h culture was also adjusted to an OD_{560} of approximately 0.5 and used to inoculate fresh 20 ml MRS broth with Oxgall at concentrations of 0.5% and 1% (w/v). All the cultures were incubated anaerobically at 37^{o}C, and after every hour, the optical density was measured using S1000 Diode Array Spectrophotometer (Labotec). Viability of the cells was

determined by diluting the cultures and plating on MRS agar plates at 0 and 6 h. The MRS agar plates were incubated at 37^{o}C for 2 days under anaerobic conditions. The experiment was done as three biological experiments and two technical repeats.

3.3. Results and Discussions

3.3.1. Antibiotic susceptibility testing

The antibiotic disk diffusion method was used to determine the susceptibility of the *Lactobacillus* strains against 11 antibiotics. The *Lactobacillus* strains were susceptible, to varying extents, to the following antibiotics; rifampicin, chloramphenicol, ampicillin, cephalothin, erythromycin, penicillin and tetracycline (Fig 3.1). All the test strains were most sensitive to penicillin, having the greatest zone of inhibition. *Lactobacillus reuteri* 17(7) and *Lactobacillus reuteri* 16(9) were both resistant to vancomycin, whereas *Lactobacillus gasseri* 7(3) and *Lactobacillus gasseri* 17(4) were both slightly sensitive to vancomycin. A similar trend was seen in the responses of the strains to streptomycin. All the test strains were resistant to kanamycin, confirming the findings of Danielsen and Wind (2003) who showed that a number of *Lactobacillus* strains were naturally resistant to kanamycin. Elkins and Mullis (2004) also reported that lactobacilli naturally show intrinsic kanamycin resistance and this could be due to membrane impermeability, which may possibly be complemented by potential efflux mechanisms. *L. reuteri* 16(9) was more sensitive to tetracycline in comparison with the other strains.

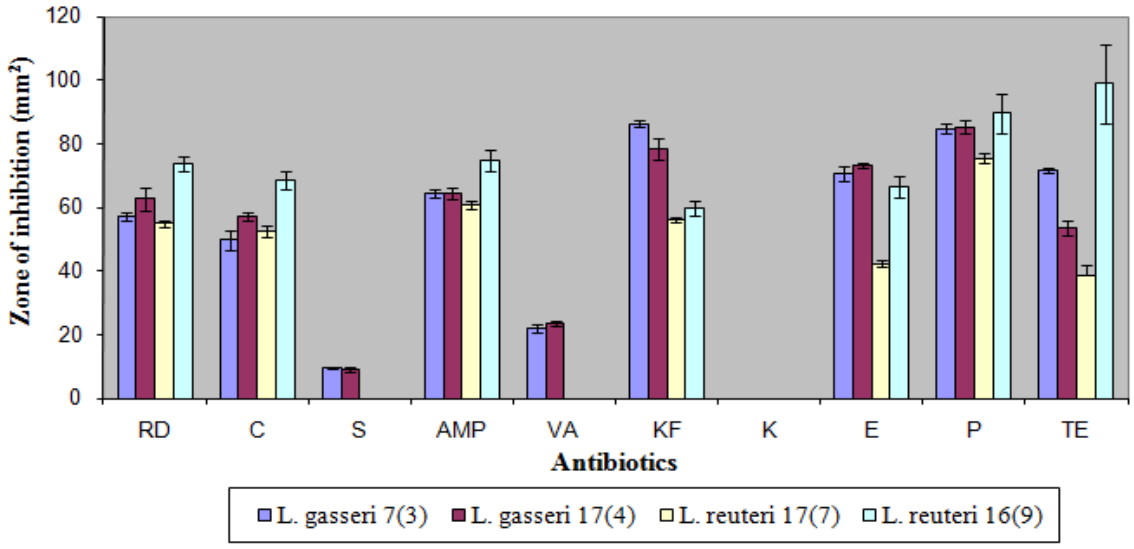

Figure 3.1. Antibiotic susceptibility of *Lactobacillus* strains.
RD, Rifampicin; C, Chloramphenicol; S, Streptomycin; AMP, Ampicillin; VA, Vancomycin; KF, Cephalothin; K, Kanamycin; E, Erythromycin; P, Pencillin G and TE, Tetracycline. The error bars indicate the standard error calculated from three replicates of the data.

These results were similar to a study reported by Zhou *et al*. (2005), who established the antibiotic susceptibility profiles of 7 *Lactobacillus* species to 18 antibiotics. These species were sensitive to ampicillin, cephalothin, pencillin, erythromycin, chloramphenicol, rifampicin and tetracycline.

Homofermentative *Lactobacillus* species have been shown to be sensitive to vancomycin, while heterofermentative *Lactobacillus* species are naturally resistant to vancomycin (Ammor *et al*., 2007). The findings reported here are in agreement with the above mentioned statement, where the two *L. gasseri* strains were shown to be sensitive to vancomycin, while the two *L. reuteri* were resistant to vancomycin. *L. gasseri* strains are known to be homofermentative (more energy efficient than heterofermentative), whereas *L. reuteri* are heterofermentative (Morelli *et al*., 1998). Interestingly, the vancomycin-susceptibility results of *L. gasseri* 7(3) and *L. gasseri* 17(4) agree with the findings of Kõll *et al*. (2008), who used the E-test method to demonstrate that their *L. gasseri* strain was

sensitive to vancomycin. Generally, the vancomycin resistance is based in the production of cell wall peptidoglycan precursors, which terminates with D-Alanyl-D-lactate as a substitute of D-Ala-D-Ala. Thus, the modified D-ala-D-lactate does not bind vancomycin (Tykkynen *et al*., 1998).

In this study, the *Lactobacillus* strains that exhibited resistance to kanamycin, streptomycin, vancomycin and less susceptibility to chloramphenicol, rifampicin and erythromycin could possibly be given to patient during treatment with these antibiotics. However, due to the concerns on safety of probiotics, further studies should investigate the possibility of resistance transfer from a probiotic strain to human bacterial pathogens.

3.3.2. Antimicrobial activity

The *Lactobacillus* strains were analyzed by the agar overlay method to test their ability to inhibit the growth of pathogens. It was found that most of the strains inhibited the pathogens tested to varying extents (Fig. 3.2). The Gram-negative pathogens were generally inhibited to a greater extent by the *Lactobacillus* strains than the Gram-positive pathogens. *E. faecium* and *E. faecalis* were not inhibited at all by *L. gasseri* 7(3), whereas *L. gasseri* 17(4) did inhibit these species. This indicates that their antagonistic activity was strain specific. Interestingly, the *Lactobacillus* strains showed a strong inhibition of *E. coli*, *S. aureus* and *S. typhimurium*. Overall, *L. reuteri* 17(7) and *L. reuteri* 16(9) appeared to inhibit the growth of these pathogens to a greater extent than the *L. gasseri* strains. This was possibly due to the production of reuterin (Talarico and Dobrogo, 1989), which is a low-molecular weight, non-protein, pH-neutral and water-soluble substance produced by some strains of *L. reuteri*. Reuterin has been shown to have antibacterial, antimycotic and antiprotozal activities (Sung *et al*., 2002; Axelsson *et al*., 1989).

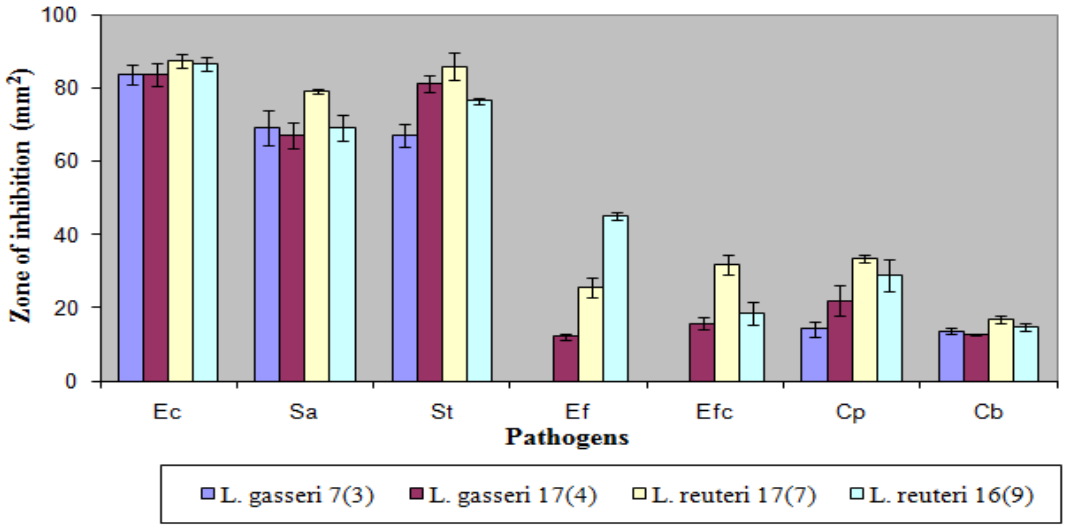

Figure 3.2. Antimicrobial activity of *Lactobacillus* strains against pathogens. The error bars indicate the standard error calculated from three replicates of the data.
Ec, *Escherichia coli*; Sa, *Staphyloccus aureus*; St, *Salmonella typhimurium*; Ef, *Enterococcus faecium*; Efc, *Enterococcus faecalis*; Cp, *Clostridium perfrigens* and Cb, *Clostridium botulinum.*

In a previous study, Jacobsen *et al*. (1999) reported that *L. reuteri* DSM 12246 showed a strong inhibition of all the pathogenic bacteria tested. In the present study, the *Lactobacillus* strains inhibited the growth of most pathogens tested, suggesting that these strains would possibly be helpful in protection from diseases or infections caused by pathogens. Future work on the nature of the antimicrobial agents produced by the test strains would be of interest.

3.3.3. Yeast agglutination

The colonization of bacteria on epithelial cells is regarded as an important criterion for selecting potential probiotic bacteria. In this experiment, a yeast agglutination assay was used to determine the potential adherence capacity of the candidate *Lactobacillus* strains. Agglutination of the cells with or without supernatant was observed using a light microscope (Fig. 3.3) and the results tabulated (Table 3.1).

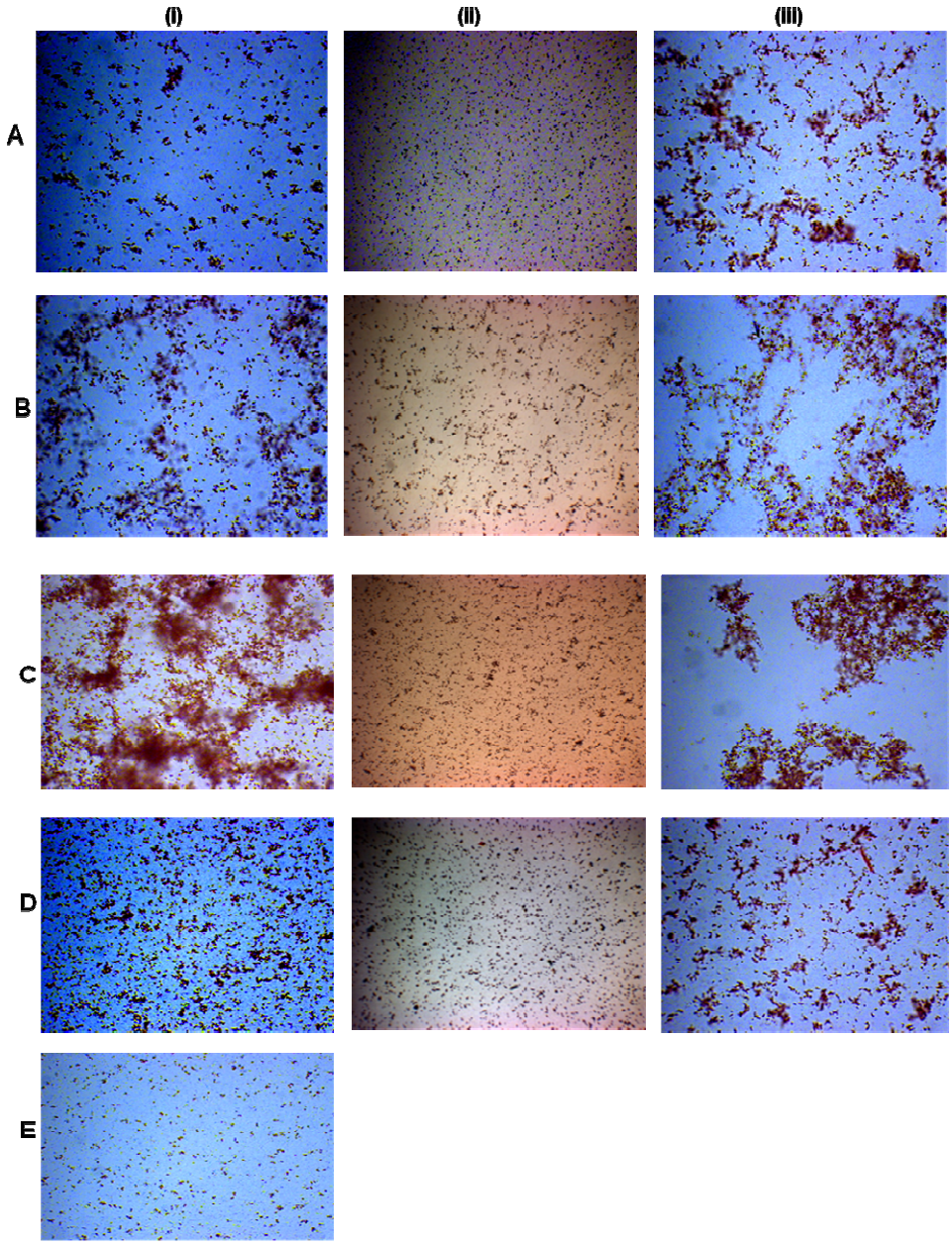

Figure 3.3. Yeast agglutination of the *Lactobacillus* species. (A) *L. gasseri* 7(3); (B) *L. gasseri* 17(4); (C) *L. reuteri* 17(7); (D) *L. reuteri* 16(9); (E) negative control (i) with safranin stained yeast cells (ii) with mannose (iii) with culture supernatant. The experiment was done in duplicate.

The negative control was prepared by resuspending the yeast cells with water. Positive agglutination was observed in all the strains, with *L. gasseri* 17(4) and *L. reuteri* 17(7) showing the strongest

agglutination irrespective of whether culture supernatant was added to the cells or not. *L. gasseri* 7(3) and *L. reuteri* 16(9), showed weak agglutination in the absence of culture supernatant, however, it was enhanced by the addition of this solution. This could possibly be due to the presence of certain proteins moieties in the supernatant (Conway and Kjelleberg, 1989). The ability of these strains to display strong agglutination indicates that, they may contain surface receptors that could recognize host tissue receptors, thereby promoting binding to the epithelium.

Table 3.1. Yeast agglutination efficiency

Strains	**Cells**	**Cells with mannose**	**Cells and supernatant**
L. gasseri 7(3)	+	-	++
L. gasseri 17(4)	++	-	++
L. reuteri 17(7)	++	-	++
L. reuteri 16(9)	+	-	++

(-) no agglutination, (+) weak agglutination, (++) strong agglutination

The assay was repeated by resuspending the cells in 1M solution of D-Mannose and no agglutination was observed. This suggested that the D-mannose inhibited the adhesion of the bacterial cells to the yeast cells, thus verifying the role of the sugar moieties in the assay. A mannose specific adhesion mechanism has previously been studied by Adlerberth *et al*. (1996), who observed an interaction between *L. plantarum* strains with human colonic cell line HT-29. It was found that the adhesins could have been involved in mediating the adhesion. Pretzer *et al*. (2005) has, however, recently identified the mannose-specific adhesin (Msa; gene name, *msa*) of some *L. plantarum* strains with the ability to agglutinate *Saccharomyces cerevisiae*. It was, therefore, confirmed that the manose-specific adhesins were likely to be involved in the interaction of *L. plantarum* with its host in the intestinal tract, although the adhesion was found to be phenotypically variable among *L. plantarum* strains.

The adhesins present on the bacterial cell surface have been shown to agglutinate *Saccharomyces cerevisiae* cells, which are largely composed of mannose-containing polysaccharide (mannan) on their cell surface (Cawley and Ballou, 1972), this could be responsible for the excellent agglutination of *L. gasseri* 17(4) and *L. reuteri* 17(7) bacterial strains.

3.3.4. Autoaggregation

The autoaggregation ability of the bacterial strains was investigated by visual observation based on the rate at which they sedimented at the bottom of the tube (Fig. 3.4).

The cells with strong auto-aggregation ability formed sand-like particles by settling to the bottom of the tube, resulting in a clear solution. The strain without auto-aggregation ability showed continued turbidity. Of the strains tested, *L. reuteri* 17(7) showed rapid autoaggregation within 5 mins (Table 3.2). Strains *L. gasseri* 7(3), *L. gasseri* 17(4) and *L. reuteri* 16(9), (Table 3.2) however, were unable to autoaggregate within 2 h, but did so after 16 h.

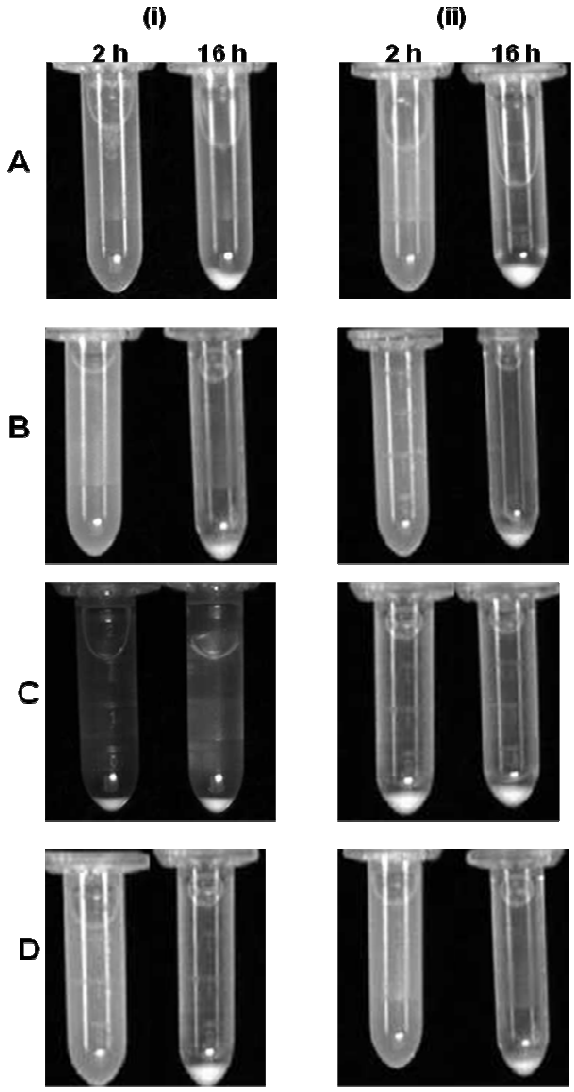

Figure 3.4. Aggregation properties of 4 *Lactobacillus* strains

(A) *L. gasseri* 7(3); (B) *L. gasseri* 17(4); (C) *L. reuteri* 17(7); (D) *L. reuteri* 16(9). (i) with no supernatant (ii) with supernatant. The experiment was done in duplicate.

Several reports have shown that filter-sterilized culture supernatant can contain proteins that promote the aggregation ability of *Lactobacillus* species (Reniero *et al*., 1992; Roos *et al*., 1999). When the experiment was repeated by resuspending the bacterial cells with their supernatant, there was no change or increase in their aggregation ability when measured after 2 h as compared to the results in

the absence of supernatant (Table 3.2). However, after incubating cells with supernatant, there was increased aggregation as measured at 16 h, which was annotated as (++).

Table 3.2: Autoaggregation efficiency

Visual score				
Strains	**Cells (After 2 h)**	**Cells (After 16 h)**	**Cells and supernatant (After 2 h)**	**Cells and supernatant (After 16 h)**
L. gasseri 7(3)	-	+	-	++
L. gasseri 17(4)	-	+	-	++
L. reuteri 17(7)	++	++	++	++
L. reuteri 16(9)	-	+	-	++

(-) no aggregation, (+) aggregation, (++) rapid aggregation.

The rate at which the bacterial cells settled to bottom of the tube was more rapid in comparison with cells in the absence of supernatant (Table 3.2). This suggests that there may be proteins present in the supernatant that could be responsible for the aggregation ability of the tested strains. Overall, *L. reuteri* 17(7) was found to be potentially the most adhesive strains, due to its high immediate auto-aggregation ability. Although the other 3 strains auto-aggregated slowly, they still might possess sufficiently good auto-aggregation ability in later stages of colonization.

3.3.5. Oxalate-degrading ability of *Lactobacillus* strains

The identification of strains with oxalate degrading ability might be beneficial to individuals who suffer from kidney stone disease or related intestinal disorders, since they could potentially be used to reduce the levels of oxalate in the gut.

The four *Lactobacillus* strains were tested to determine their ability to degrade ammonium oxalate (Fig. 3.5). The induced strains were grown in ½ strength MRS broth containing 10 mM ammonium

oxalate for 2 consecutive days to stimulate their cells to produce the necessary enzymes to utilize oxalate. Subsequently, the residual oxalate in fresh broth cultures was measured after 2 and 5 days of incubation using the oxalate assay kit (Trinity Biotech plc, Ireland). Half strength MRS broths were used in order for the strains to initially utilize alternative carbon sources to promote growth, and then allowing them to use ammonium oxalate when the other carbon sources are depleted.

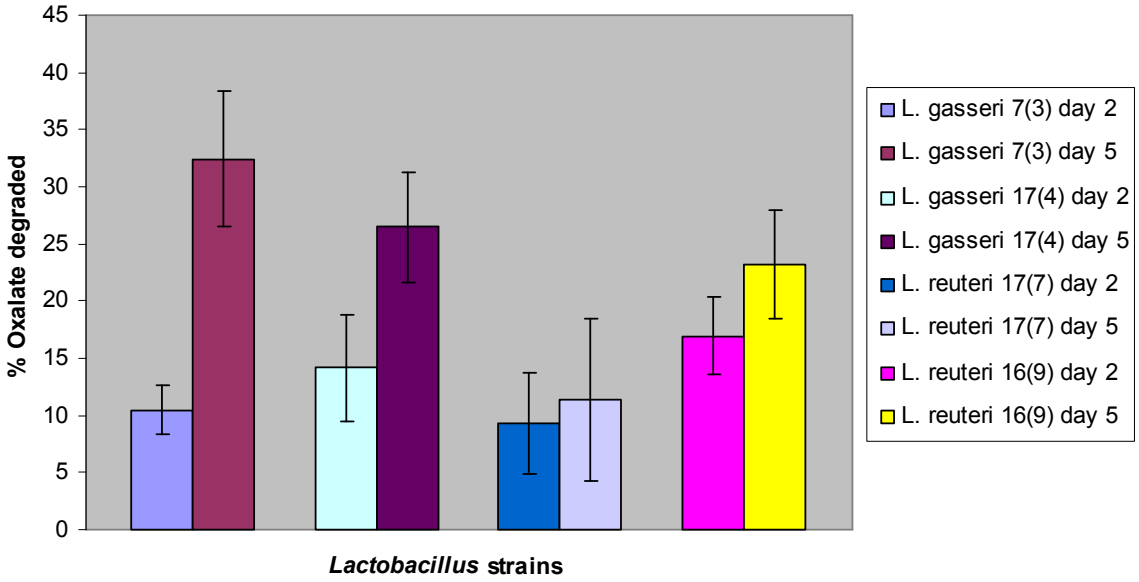

Figure 3.5. Oxalate degrading activity of the *Lactobacillus* strains grown in ½ strength MRS broths supplemented with 10 mM of ammonium oxalate. Activity expressed as a percentage of oxalate in uninoculated medium. The error bars indicate the standard error calculated from three biological replicates of the data.

All the strains grew in ½ strength MRS broth supplemented with 10 mM ammonium oxalate, and reduced the amount of oxalate in the medium over a period of 5 days as compared to uninoculated culture medium. The growth of these strains in the presence of 10 mM ammonium oxalate demonstrated that oxalate at this concentration was not toxic to *Lactobacillus* species. *L. gasseri* 7(3), *L. gasseri* 17(4) and *L. reuteri* 16(9) showed good oxalate degradation, reducing the oxalate concentration by 25-35%. *L. reuteri* 17(7) had lower oxalate degrading activity of approximately

10% degradation. Campieri *et al*. (2001) evaluated the *in vitro* oxalate degradation in *Lactobacillus* species, and *L. acidophilus* strain showed good oxalate degradation, whereas *L. brevis* and *L. plantarum* demonstrated a modest ability to degrade oxalate. In another study, Turroni *et al*. (2007) also evaluated the oxalate degrading activity of several *Lactobacillus* species; some strains demonstrated little or no degradation. This suggests that the ability of lactobacilli to degrade oxalate is both species and strain specific.

Several methods have been proposed for the measurement of oxalate, each with its advantages and disadvantages. The oxalate assay used in this study is based on oxidation of oxalate catalysed by oxalate oxidase to form hydrogen peroxide and carbon dioxide. The hydrogen peroxide reacts with 3 methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino) benzoic acid in the presence of peroxidase to yield an indamine dye, which has an absorbance maximum at 590 nm. A major problem encountered with this assay (enzymatic assay) is the interference by substances such as ascorbate. However, in this study interfering substances such as ascorbate, were eliminated by treating the diluted sample with activated charcoal (Keevil and Thornton, 2006; Li and Madappally, 1989).

Turroni *et al*. (2007) and Federici *et al*. (2004) noted that the enzymatic method showed low accuracy as observed from the recovery tests wherein 90-110% or 93-107% of added oxalate was recovered. Nonetheless, several studies have applied this method to evaluate oxalate degradation activity in small or large scale (Lewanika *et al*., 2007; Turroni *et al*., 2007). In this study, the validity of the screening has not been found to be compromised by the error measurement of oxalate degrading capacity; instead 3 biological experiments with technical replicates were performed to produce reproducible results. This assay was, therefore, considered to be suitable to detect the

oxalate degrading activity of the 4 *Lactobacillus* strains, although the values obtained should not be compared to studies by other authors, using different conditions.

3.3.6. Effect of pH on bacterial growth and viability

Two *Lactobacillus* strains were selected for further investigation of their ability to withstand the harsh conditions of certain regions of the gut, by exposing them to low pH. *L. gasseri* 7(3) was selected for further study because it was the most efficient degrader of oxalate (Fig. 3.5), while *L. reuteri* 17(7) was also examined because despite its slightly lower oxalate degrading capacity, it possessed other good probiotic features such as strong autoaggregation ability and good antimicrobial activity.

The 2 strains were tested to study their acid tolerance under conditions that mimic the gastrointestinal tract environment by exposing them to different pH conditions (Fig. 3.6). The strains were grown in MRS broth medium at pH 2 and pH 4 which was adjusted with HCl. The initial OD_{600} for the two strains at 0 h was 0.1. The two strains hardly grew at pH 2, and the OD only increased to 0.175 and 0.153 for *L. gasseri* 7(3) strain and *L. reuteri* 17(4), respectively, after 7 h of incubation. The cell growth of both strains was slightly higher at pH 4 than at pH 2, with *L. gasseri* 7(3) and *L. reuteri* 17(7) showing an OD increasing to 0.332 and 1.95, respectively. This was substantially lower than the control cells (pH 6.8) which reached final OD values of 2.623 and 4.837 for *L. gasseri* 7(3) and *L. reuteri* 17(7), respectively.

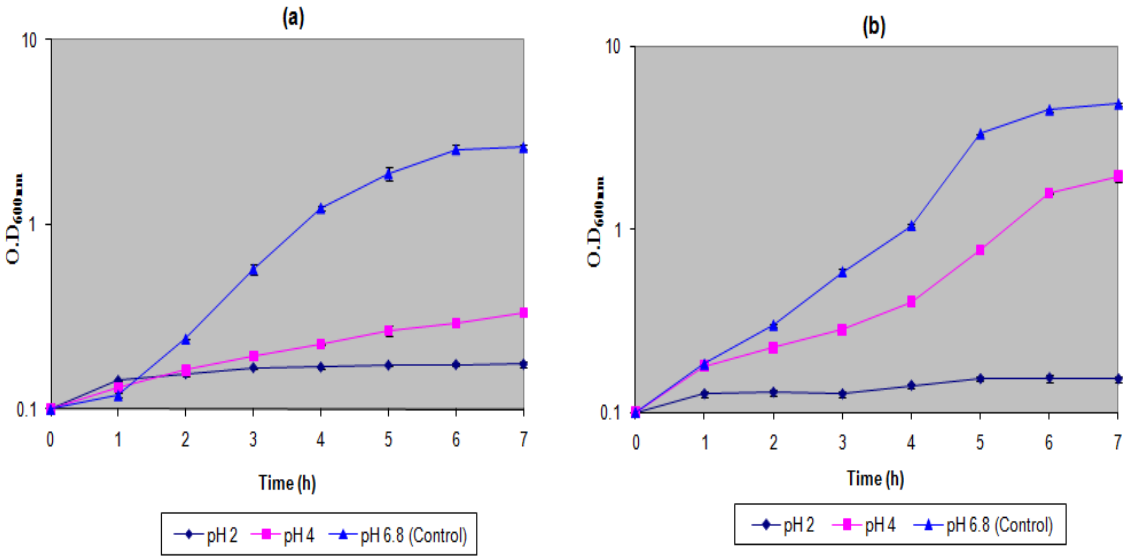

Figure 3.6. Growth of *Lactobacillus* strains in the presence of acid.
(a) *L. gasseri* 7(3) and (b) *L. reuteri* 17(7). The error bars indicate the standard error calculated from three replicates of the data.

Even if the bacteria are unable to grow at low pH, their ability to remain viable under acidic conditions during transit through the stomach of the host, is one of the major criteria for selecting a potential probiotic (Tuomola *et al*., 2001). For that reason, the effect of acidic conditions on the viability of the two strains was evaluated under the various pH conditions (Fig. 3.7). The numbers of viable bacteria were determined at 0 and 6 h. At the beginning of the experiment, the viable count of the *L. gasseri* 7(3) and *L. reuteri* 17(7) was 7.6×10^7 and 4.91×10^7 c.f.u. ml^{-1}, respectively. After 6 h of incubation at pH 2, this decreased to 5.53×10^5 and 1.87×10^6 c.f.u. ml^{-1}, respectively.

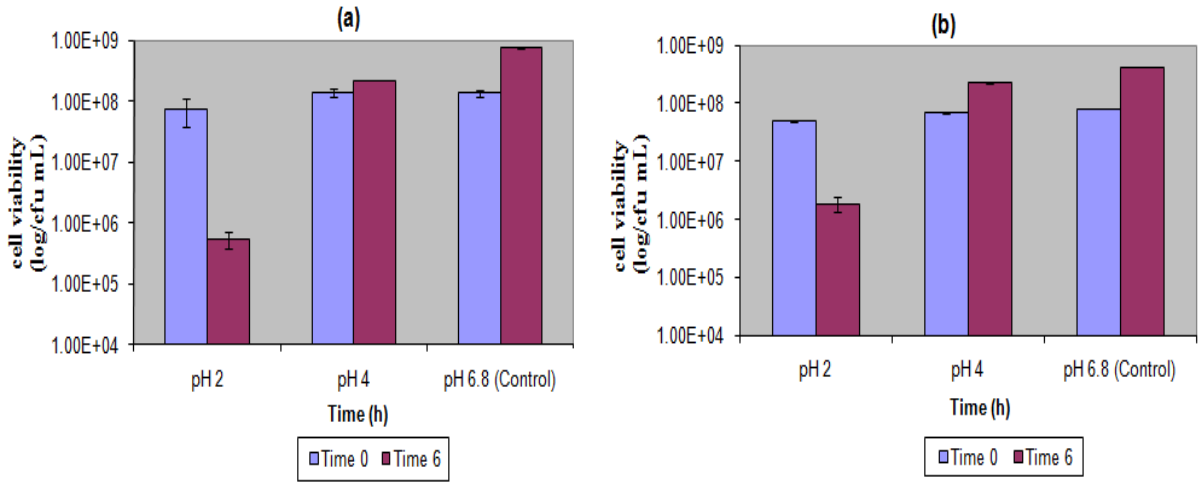

Figure 3.7. The effect of pH on the viability of the *Lactobacillus* strains.
(a) *L. gasseri* 7(3) and (b) *L. reuteri* 17(7) at pH 2 and 4. The error bars indicate the standard error calculated from three replicates of the data.

At pH 4, however, the two strains maintained viability from time 0 to 6 h of incubation in a similar way to the control (pH 6.8). Taken together, Fig. 3.6 and Fig. 3.7, illustrate that exposure to pH 2 not only inhibits growth of the both strains, but it also actively kills the cells. At pH 4, there was a slight increase in cell viability of both strains with an increase in the OD of the cultures. Although, the stomach pH can be as low as 1, the fact that these strains maintain some viability at pH 2 is thought to be a suitable criterion for selecting potential probiotic bacteria (Prasad *et al*., 1998). Moreover, once the food is ingested, the pH level in the stomach is raised, possibly due to the buffering capacity of the food. Hence the probiotic strains are unlikely to be exposed directly to a low pH environment like the stomach (Prasad *et al*., 1998). The ability of these strains to survive such conditions, could be based on maintaining the stable gradient between extracellular and cytoplasmic pH of the cell (Corcoran *et al*., 2005) (See Chapter 1). F_0F_1-ATPase is one of common mechanisms used by lactobacilli to protect themselves against acidic conditions (Cotter and Hill, 2003). Such mechanism

may have possibly assisted the *L. reuteri* 17(7) strain to resist low pH better than the *L. gasseri* 7(3) strain.

3.3.7. Effect of bile salts on bacterial growth and viability

For a bacterium to be used as a probiotic, it should be able to resist the effects of bile salts and bile secretions present in the small intestine. The concentration of bile in the intestine is appproximately between 0.2 to 2% (Gunn, 2000). Bile is known to be toxic to the bacterial cells, and thus it is important for the bacteria to develop a mechanism to survive in such hostile environment. *L. gasseri* 7(3) and *L. reuteri* 17(7) strains were, therefore, screened to test their ability to tolerate bile salts.

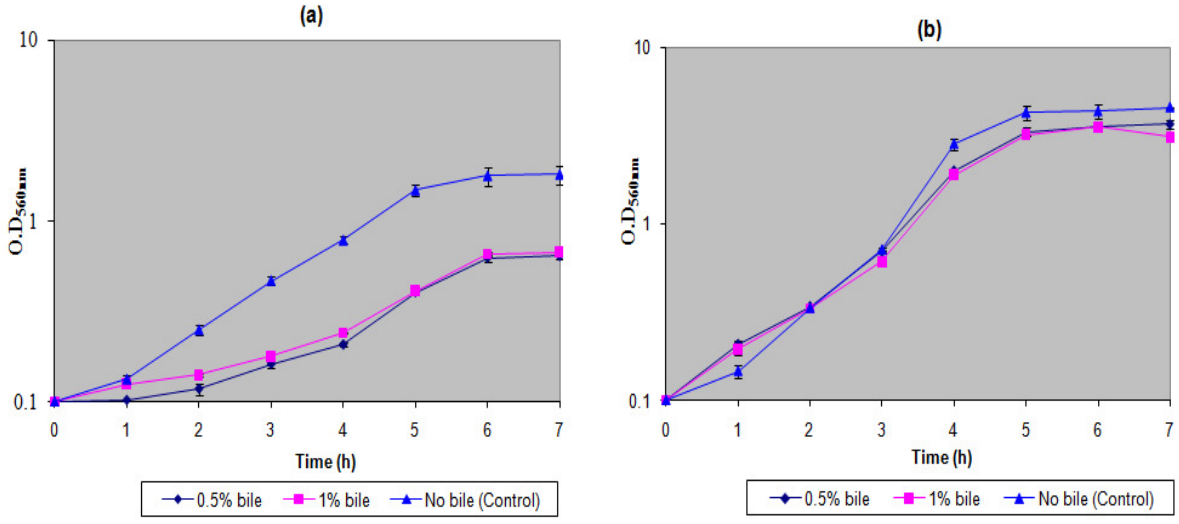

Figure 3.8. Growth of *Lactobacillus* strains in the presence of Oxgall bile salts.
(a) *L. gasseri* 7(3) and (b) *L. reuteri* 17(7). The error bars indicate the standard error calculated from three replicates of the data.

The results showed that both the two strains were able to grow to varying extents in the different concentrations of bile tested. *L. reuteri* 17(7) tolerated bile extremely well and showed the same

growth characteristics as the control during growth in both bile concentrations (Fig. 3.8 b). *L. gasseri* 7(3), however, also showed slightly decreased growth in the presence of bile (Fig. 3.8 a).

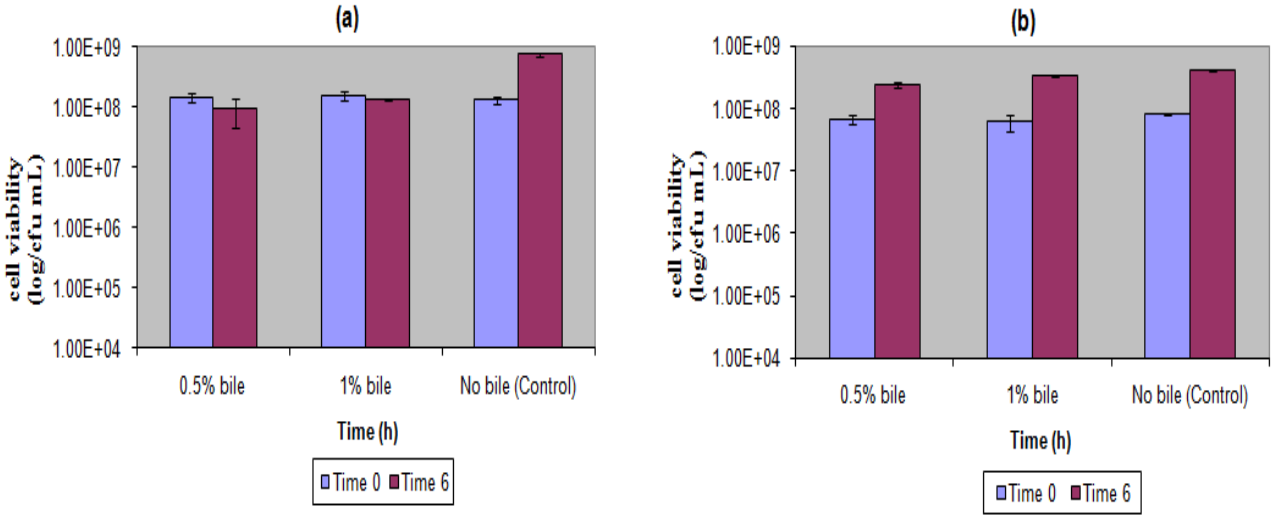

Figure 3.9. The effect of bile salts on the viability of the *Lactobacillus* strains.
(a) *L. gasseri* 7(3) and (b) *L. reuteri* 17(7) at concentrations of 0.5 and 1% of bile. The error bars indicate the standard error calculated from three replicates of the data.

It was also interesting to examine the viability of the strains grown in the presence of 0.5 and 1% bile as compared to growth in the control medium (no bile added). Both strains retained their viability after 6 h of incubation under both conditions (Fig. 3.9). At the beginning of the experiment, the viable counts of *L. gasseri* 7(3) and *L. reuteri* 17(7) were 1.48×10^8 and 6.94×10^7 c.f.u. ml^{-1}, respectively. After 6 h of incubation in the presence of 0.5% bile, the cell counts for *L. gasseri* 7(3) reduced slightly to 9.32×10^7, while they increased to 2.43×10^8 c.f.u. ml^{-1} for *L. reuteri* 17(7). A similar trend was also observed in cell viability at a bile concentration of 1%, where cell numbers reduced to 1.33×10^8 and increased to 3.38×10^8 c.f.u. ml^{-1} for *L. gasseri* 7(3) and *L. reuteri* 17(7), respectively.

The results indicated that there was an increase in the OD values for *L. reuteri* 17(7) in the presence of bile, which correlated with a corresponding increase in the number of viable cells. However, for the *L. gasseri* 7(3) strain, there was an increase in the OD values but a slight reduction in cell viability at 0.5 and 1% bile concentrations. This implies that, although the total mass of the culture, as measured spectrophotometrically increased, there was a simultaneous death of a certain number of the population as measured by viable count. The use of a LIVE/DEAD *Bac*Light Bacterial Viability kit could possibly be used to demonstrate what fraction of population survived, when exposed to bile. *Bac*Light staining has been shown to be a promising technique with several advantages (Lahtinen *et al*., 2005). It is reliable, rapid, and easy to use and yields both viable and total counts in one staining step. The live and dead cells are distinguished based on their color. The viable bacteria fluoresce green, while the dead cells fluoresce red color.

Overall, the two strains tolerated bile very well and maintained their viability over time. Thus, these strains may be likely to survive the harsh environment in human gut and retain their viabililty. The resistance of the two strains to bile salts could be due to the presence of BSH enzyme (Bile salt hydrolase), which eases the lethal effect of bile salts (Begley *et al*., 2005; Patel *et al*., 2009). The relationship between bile salts resistance and BSH enzyme activity patterns on these two strains should be investigated in further studies.

3.4. Conclusion

This study has evaluated important criteria that a potential probiotic bacterium should fulfill. The results have shown that the strains were resistant to kanamycin, vancomycin and streptomycin and had reduced susceptibility to chloramphenicol, rifampicin and erythromycin. If these antibiotics were used during antibiotic treatment, then the probiotics could survive and colonise the gut. The strains

were further tested for their antimicrobial activity, and they were able to inhibit the pathogens tested. *L. reuteri* 17(7) and *L. reuteri* 16(9), in particular displayed high inhibition of the growth of pathogens, which indicated that they might provide health benefits to the host to lessen the risk of diseases. The *Lactobacillus* strains also demonstrated adhesion properties which may assist in colonizing the gastrointestinal tract to exert benefical effects (Charteris *et al*., 1998a). *L. reuteri* 17(7) was one strain, that exhibited both good yeast agglutination and autoaggregation properties indicating that it would possibly adhere efficiently to colonic cells.

The strains also showed the ability to degrade oxalate, with *L. gasseri* 7(3) possessing the highest oxalate degrading activity, which may be exploited further to reduce hyperoxaluria. The two oxalate degrading strains, *L. gasseri* 7(3) and *L. reuteri* 17(7), were selected for studies on their acid and bile tolerance. The strains were sensitive at pH 2, but tolerated pH 4 conditions well. They both also survived in the presence of 0.5% and 1% bile. Several studies have reported that the use of food ingredients enhances the survival of probiotic strain in the gastrointestinal tract (Charteris *et al*., 1998b; Gardiner *et al*., 1999). It has also recently been documented that, the *Lactobacillus* strains could sequester metabolizable sugars to enhance their survival in an acidic environment (Corcoran *et al*., 2005). This suggests the possibility that these strains might adapt to pH 2 conditions during gastric transit by generating resistance mechanisms which enable their survival. The strains were further investigated for their genetic characteristics to determine their mechanism of oxalate degradation and this study is reported in Chapter 4.

CHAPTER 4

GENETIC STUDIES ON THE OXALATE DEGRADATION IN CANDIDATE STRAIN

4.0. Summary

Four *Lactobacillus* strains were screened by PCR for the presence of *oxc* genes using gene specific primers. The *oxc* gene was found to be present in all the 4 strains and this was confirmed by DNA sequencing of the PCR products. *L. gasseri* 7(3) was selected for further investigation of the transcription of the *oxc* gene in the presence or absence of 10 mM ammonium oxalate at pH 6.8 and 5.5. Dot blot analysis of *L. gasseri* 7(3) mRNA transcripts revealed the gene was constitutively transcribed under both pH conditions regardless of whether oxalate was present or absent. This suggests that *L. gasseri* 7(3) may be a suitable probiotic candidate for the regulation of oxalate levels for the prevention of kidney stone disease.

4.1. Introduction

As reviewed in Chapter 1, oxalate-degrading bacteria generally use two enzymes to degrade oxalate. These are oxalyl-CoA decarboxylase (encoded by the *oxc* gene) and formyl-CoA transferase (encoded by the *frc* gene). The significance of these enzymes is that they make up approximately 20% of total protein in the bacteria (Baetz and Allison, 1992) and have been shown to assist *O. formigenes* in degrading oxalate. The aforementioned enzymes are not as common in LAB, although they have been identified in *L. gasseri Gasser* AM63^T (Lewanika *et al*., 2007) and *L. acidophilus* NCFM (Azcarate-Peril *et al*., 2006). Several studies have recently reported that pH has a possible effect on the expression of *oxc* and *frc* genes. For instance, Azcarate-Peril *et al*. (2006), used RT-qPCR and microarray experiments to show that the *oxc* and *frc* genes were not expressed at pH above 5.8. Conversely, when the *L. acidophilus* cells were pre-incubated in MRS broth (pH 5.5) supplemented with noninhibitory concentrations of ammonium oxalate, the transcription levels of the *oxc* and *frc* genes were increased. This is also in agreement with a study by Lewanika *et al*. (2007), who confirmed that the *oxc* gene of *L. gasseri* Gasser AM63^T was induced by oxalate under mildly acidic conditions (pH 5.5). The ability of the lactobacilli to utilize oxalate has, however, been shown to be strain and species specific (Turroni *et al*., 2007).

Understanding the transcriptional regulation of gene expression is, therefore, an essential aspect of molecular biology studies. It plays a vital role in gaining insight into the signaling and metabolic pathways, which trigger the cellular processes, and it is, therefore, important to measure gene expression to determine the effect that several factors, including nutritional and environmental, might have in regulating gene expression (Trayhurn, 1996). There are numerous methods available that have been used to measure transcriptional regulation of genes (Reaue, 1998; Streit *et al*., 2009). These include northern blot analysis, DNA microarray evaluation, the ribonuclease protection assay

and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) among others. Northern blotting is a simple and specific method which has traditionally been used in studying gene expression. It is based on the separation of RNA according to size by agarose gel electrophoresis. The mRNA of interest is detected on the membrane using a hybridization probe with a base sequence complementary to all or a part of the sequence of the target gene (Trayhurn, 1996). Northern blotting is regarded as the only technique that provides detailed information about mRNA transcript size that cannot be obtained by the alternative methods (Bustin, 2000). However, it has some major drawbacks such as RNA degradation and low sensitivity (Streit *et al*., 2009). There are varieties of northern blotting techniques (dot, slot and fast blots) that have been developed to shorten the time taken for blot preparation and to improve semi-quantitative aspects (Reue, 1998).

The ribonuclease protection assay can also be used to study gene expression. It also involves the hybridization of a labelled probe to a target mRNA, but differs from the northern blot in the respect that it is more sensitive, reliable and specific, and the hybridization occurs in a solution that contains both labelled antisense RNA probe and the target mRNA (Reau, 1998; Einspanier and Plath, 1998). DNA microarray analysis is a sensitive technique that simultaneously monitors the expression of thousands of genes in a single hybridization assay (Harrington *et al*., 2000). The microarray technology is based on the hybridization of RNA samples to either cDNA or oligonucleotides immobilized on a glass chip or nylon membrane (Weeraratna and Taub, 2007). The disadvantage of this technique is that it needs to be repeated numerous times to ensure reproducibility. Quantitative RT-PCR is the most sensitive method used to study gene expression and is useful in detecting low-levels of mRNA transcripts (Bustin, 2000). It can be used to characterise patterns of mRNA expression, discriminate between closely related mRNAs and analyse the RNA structure (Bustin, 2000).

The aim of the present study was to determine whether the *oxc* gene is present in 4 *Lactobacillus* strains which have previously been shown to degrade oxalate (Chapter 3) and to determine whether there was increased transcriptional regulation of the *oxc* genes at pH 6.8 and 5.5 in the presence of 10 mM ammonium oxalate.

For this preliminary study, northern blotting (dot blot assay) was selected to study the transcriptional regulation of the *oxc* gene because it is semi-quantitative, robust, sensitive and reliable (Yadetie *et al*., 2004).

4.2. Materials and methods

4.2.1. PCR identification for *oxc* genes

Genomic DNA was extracted from all the *Lactobacillus* strains under investigation using the Genomic DNA Purification kit (Fermentas Life Science), and this was used as a DNA template for the 50 µl PCR reaction. The reaction mixture for the two *L. reuteri* strains contained; 200 ng of genomic DNA, 1.25 µl of each set of LRoxc (F) and LRoxc (R) primers at 10 µM (Table 4.1), 12.5 µl of Kapa Ready Mix (Kapa Biosystems) and 9 µl of sterile distilled water. Amplification was performed on a thermocycler (GeneAmp® PCR system 9700, Applied Biosystems) with initial denaturation at 94^{o}C for 5 mins, followed by 30 cycles of denaturation at 94^{o}C for 30 sec, annealing at 53^{o}C for 30 sec and elongation at 72^{o}C for 30 sec, with a final elongation at 72^{o}C for 5 mins. The reaction mixture for the two *L. gasseri* strains contained, 200 ng of genomic DNA, 1.25 µl of LGoxc (F) and LGoxc (R) primers (Table 4.1) at 10 µM, 5 U of Kapa Taq DNA polymerase (Kapa Biosystems), 0.5 µl of dNTPs, 1 µl of $MgCl_2$ at 2.5 mM, 10× buffer A (Kapa Biosystems) and 16.25 µl of sterile distilled water. The PCR amplification was performed with initial denaturation at 95^{o}C for 5 mins, followed by 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at 50^{o}C for 30 sec and elongation at 72^{o}C for 30 sec, with a final elongation at 72^{o}C for 7 mins. The PCR products were

visualised on a 0.8% agarose gel stained with ethidium bromide to detect the bands of correct molecular size. The PCR products were further purified using the Biospin PCR purification kit (BioFlux) and directly sequenced. The nucleotide sequencing was carried by the fluorescent dideoxynucleotide chain termination method (Sanger *et al*., 1977) using the MegaBACE 500 DNA sequencer (Amersham Pharmacia Biotech, Buckinghamshire, UK). The nucleotide and amino acid sequences were analysed and edited using the DNAMAN program package (Lynnon Corporation, Quebec, Canada) and further analysed on-line using the BLAST server at the NCBI sequence database (http://www.ncbi.nlm.nih.gov).

Table 4.1. Description of primers used

Name	**Primer sequences (5′-3′)**	**PCR fragment size (bp)**	**References**
LGoxc (F) LGoxc (R)	GACTGGATCCTAAAGTATTATGGCTTTCG GACTGAATTCTCATAAGCAGCACCTTACC	400	This study
LRoxc (F) LRoxc (R)	TCGGACAGCAGTTTCAGGC TGATTAGGAACAACTTGGCCG	1020	This study
16S (F) 16S (R)	ACTCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCA	200	Nadkarni *et al*., 2002

4.2.2. Expression of *oxc* genes

4.2.2.1. *Oxc* induction and RNA extraction

The method used was a modification of that of Azcarate-Peril *et al*. (2006). The *L. gasseri* 7(3) strain was grown anaerobically at 37^{o}C for 20 h in either ½ strength MRS broth (control) or ½ strength MRS broth supplemented with 10 mM of ammonium oxalate. The cultures were grown over 2 consecutive days to induce the expression of the *L. gasseri* 7(3) *oxc* gene. The cells were transferred to either ½ strength MRS broth (pH 6.8) or ½ strength MRS broth (pH 6.8) supplemented with 10

mM ammonium oxalate, and incubated at 37^{o}C under anaerobic conditions. The cell growth of the *L. gasseri* 7(3) strain was monitored spectrophotometrically (OD_{600}) for 24 h using the S1000 Diode Array Spectrophotometer (Labotec). The cell cultures were collected after 8 h growth corresponding to the mid-log phase for both ½ strength MRS broth (pH 6.8) and ½ strength MRS broth (pH 6.8) supplemented with 10 mM of ammonium oxalate. In a separate experiment, the above procedure was repeated, except that the cells were transferred to ½ strength MRS or ½ strength MRS with oxalate, adjusted to pH 5.5 with lactic acid. Thereafter, total RNA was extracted using the method of Aiba *et al*. (1981), and purified using the RNeasy Mini Kit (Qiagen). The quality of RNA was assessed by visualizing it on an ethidium bromide stained denaturing formaldehyde agarose gel. The RNA concentration was determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies).

4.2.2.2. Dot blot hybridization

DNA probes for hybridization to the RNA were prepared by PCR of a 400 bp, internal fragment of the *Lactobacillus gasseri oxc* gene amplified with the primers LGox (F) and LGox (R) (Table 4.1). As a control, a probe for the *L. gasseri* 16S rRNA gene was prepared using the universal primers denoted as 16S (F) and 16S (R) (Table 4.1). Equal amounts of the total RNA extracts from ½ strength MRS and ½ strength MRS supplemented with 10 mM of ammonium oxalate were blotted on to a nylon membrane (Hybond-N+, Amersham Biosciences) and fixed to the membrane by exposure to UV for 5 mins. This was followed by hybridization with the *oxc* and 16S rRNA gene specific probes and chemiluminiscent detection with CSPD® substrate was performed according to the manufacture's guidelines (Roche Diagnostics GmbH Mannheim, Germany). The hybridization signals of the experimental samples were visualised using a Molecular Imager® ChemiDoc™ XRS Imaging system, and compared to the 16S rRNA gene control.

4.3. Results and Discussions

4.3.1. PCR identification of *oxc* genes

In Chapter 3, it was reported that all the 4 *Lactobacillus* strains evaluated had the capacity to degrade oxalate to varying degrees. These strains were natural isolates with unknown genome sequences, and, therefore, it was important to investigate whether this oxalate degradation was occurring via the *oxc* mediated oxalate degradation pathway. The presence of the *oxc* gene was detected by carrying out a PCR using *oxc* gene specific primers of the *L. gasseri* and *L. reuteri oxc* genes (Table 4.1). The *oxc* primers were designed using the DNAMAN program package (Lynnon Corporation, Quebec, Canada) by aligning the *L. gasseri* and *L. reuteri oxc* gene sequences obtained from the NCBI GenBank. The specificity of these primers to bind specifically to the *oxc* gene was confirmed by BLAST analysis (Fig. 4.1).

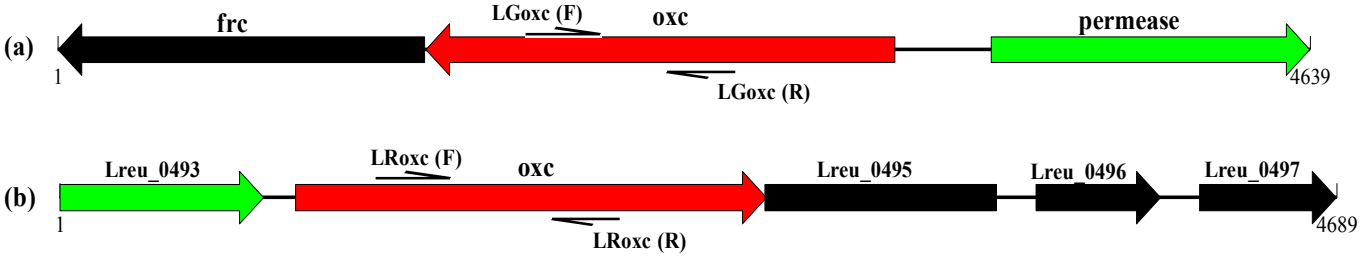

Figure 4.1. Clusters of genes encoding putative oxalate degrading enzymes (a) *L. gasseri* (NC_008530.1) and (b) *L. reuteri* (NC_009513.1). The arrows indicate the positions where the primers bind to the *oxc* gene.

The results gave the expected PCR product sizes (Fig. 4.2) and the DNA sequencing results confirmed that these were internal fragments of the *oxc* gene as compared to the *oxc* sequences found in the NCBI.

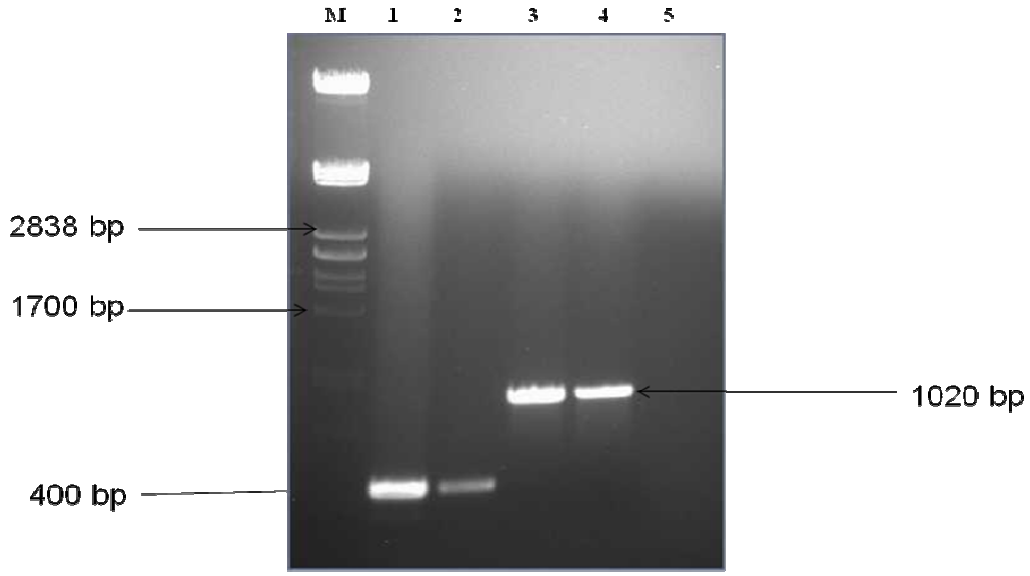

Figure 4.2. Agarose-gel electrophoresis of amplified *oxc* genes from genomic DNA of the 4 strains *Lactobacillus*. Lane M, (λ DNA digested with P*st*I); Lane 1, *L. gasseri* 7(3); Lane 2, *L. gasseri* 17(4); Lane 3, *L. reuteri* 17(7); Lane 4, *L. reuteri* 16(9) and Lane 5, Negative control (no DNA).

The *oxc* gene involved in oxalate degradation has been found in other *Lactobacillus* species such as, *L. acidophilus* (Azcarate-Peril *et al*., 2006) and *L. gasseri* (Lewanika *et al*., 2007). The identity of the internal *oxc* gene fragment of the 4 *Lactobacillus* strains was compared to the *oxc* gene of *L. gasseri* (CP000413.1) and *L. reuteri* strains (CP000705.1) at the amino acid level. The internal fragment of *L. gasseri* 7(3) and *L. gasseri* 17(4) had 91.71% and 100% amino acid identity to the *L. gasseri oxc* gene (Lgas_0248), while *L. reuteri* 17(7) and *L. reuteri* 16(9) had 100% amino acid identity to the *L. reuteri oxc* gene (Lreu_0494), respectively.

Turroni *et al*. (2007) investigated the oxalate degrading ability of a number of *L. reuteri* strains, and found that they had no oxalate degrading activity. Nevertheless, the genome sequences of *L. reuteri* JCM 1112 (NC_010609) and *L. reuteri* DSM 20016 (NC_009513.1) available in GenBank databases have been shown to include *oxc* genes. The lack of oxalate degrading capacity in the *L. reuteri*

studies may, therefore, depend on the in *vitro* assay conditions, or on the functionality of the *oxc* gene in these strains. The *L. reuteri* 17(7) and *L. reuteri* 16(9) strains used in this study also possessed an *oxc* gene and these strains both showed moderate oxalate degrading ability (Chapter 3). In summary, the four strains have been shown to posses an *oxc* gene which encodes the oxalyl-CoA decarboxylase enzyme that participates in oxalate catabolism.

4.3.2. Investigating the expression of *oxc* genes in *L. gasseri* 7(3)

Among the 4 *Lactobacillus* strains, *L. gasseri* 7(3) was selected to investigate the expression of *oxc* genes, because it had high oxalate degrading activity (approximately 33%) and possessed very good probiotic characteristics. These included good antimicrobial activity against pathogenic bacteria, adhesion properties, as well as tolerance of low pH and high concentration of bile salts.

In order to evaluate the effect of oxalate and pH on the growth of *L. gasseri* 7(3), the strain was grown in ½ strength MRS broth (pH 6.8 or 5.5) and ½ strength MRS broth (pH 6.8 or 5.5) supplemented with 10 mM of ammonium oxalate. The growth curve of preadapted *L. gasseri* 7(3) cells in the presence of 10 mM ammonium oxalate as compared to uninduced cells (Fig. 4.3) demonstrated that there was very little difference between the growth of *L. gasseri* 7(3) under these various conditions, although the presence of oxalate did result in slightly slower growth in the exponential phase. Nevertheless, by 24 h, both cultures reached the same final OD_{600} under both pH conditions.

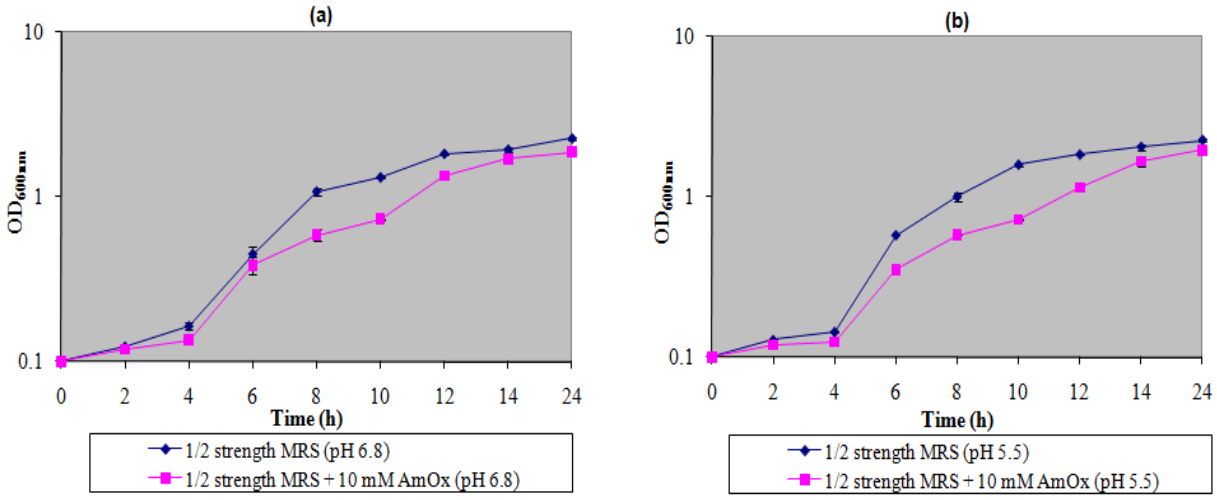

Figure 4.3. The effect of oxalate and pH on the growth of *L. gasseri* 7(3). Cells were grown in ½ strength MRS broth and ½ strength MRS broth supplemented with 10 mM of ammonium oxalate (AmOx) at (a) pH 6.8 or (b) pH 5.5. The error bars indicate the standard error calculated from three biological experiments.

RNA dot blot analysis was performed to investigate the transcription of the *oxc* gene in the *L. gasseri* 7(3) strain in the presence or absence of 10 mM ammonium oxalate at pH 6.8 and pH 5.5. Under these conditions, transcripts were obtained when LGoxc specific probe was hybridized with total RNA isolated from cells at mid-log phase at time 8 h (Fig. 4.3). The 16S rRNA gene probe was used as a control to normalize the signal obtained from the experimental samples.

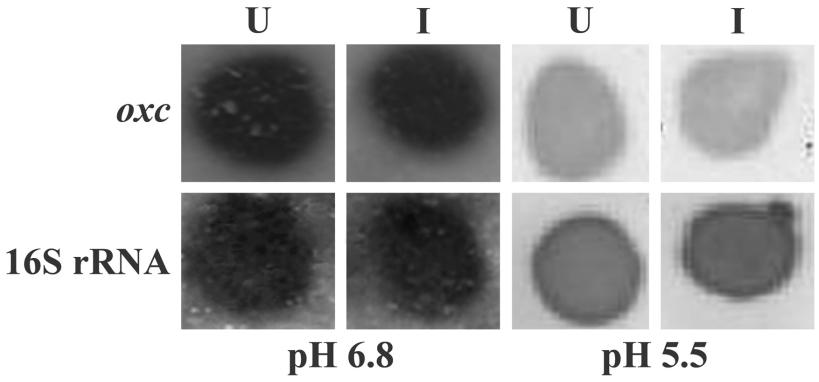

Figure 4.4. Transcriptional analysis to assess *oxc* gene expression in pre-adapted *L. gasseri* 7(3) at pH 6.8 and 5.5. RNA was isolated at mid-log phase. U: Uninduced ½ strength MRS broth; I: Induced ½ strength MRS broth at 10mM of ammonium oxalate. The experiment was done in 3 biological and 2 technical experiments.

The dot blots showed that there was no difference in the levels of transcription of the *oxc* gene at either pH or in the presence or absence of oxalate (Fig. 4.4). The ability of *L. gasseri* 7(3) to express the *oxc* gene in the absence of oxalate indicates that the strain can transcribe the gene constitutively and that the gene product would be available for oxalate degradation even in the presence of alternate carbohydrate substrates. Interestingly, these results are different from the findings of Lewanika *et al*. (2007), who observed that *oxc* gene of *L. gasseri* Gasser AM63^T was not expressed in the absence of oxalate at mid-log phase. This suggested that the presence of oxalate in the medium could have induced the expression of putative *oxc* gene under mildly acidic conditions (pH 5.5), although, the transcriptional analysis of *L. gasseri* Gasser AM63^T strain was not studied under pH 6.8 conditions. However, it should be emphasised here that Lewanika *et al*. (2007) used strain *L. gasseri* Gasser AM63^T and in this study the natural isolate, *L. gasseri* 7(3) was used. The *L. gasseri* 7(3) strain might have a mutation in a regulatory protein or DNA regulatory sequence, which might allow the *oxc* gene to be transcribed even in the absence of oxalate.

The findings of Azcarate-Peril *et al*. (2006) were, however in some ways similar to our findings, although *L. acidophilus* NCFM was used to study the transcriptional analysis. The *L. acidophilus oxc* gene was found to be transcribed in the absence of oxalate under acidic conditions (pH 5.5). However, the genes showed markedly increased expression in the presence of 35 mM of ammonium oxalate at pH 5.5. In their study, however, when 70 mM of ammonium oxalate was added at pH 6.8, transcriptional of the gene was not induced and this could possibly be due to the dissociation of the oxalic acid.

This study suggests that the *L. gasseri* 7(3) strain has a potential role in degrading oxalate, even in the presence of other possible carbon sources. *L. gasseri* 7(3) can, therefore, be considered as a

"generalist" oxalotroph, because it is able to utilize alternative substrates as well as oxalate. They are able to thrive in a wide variety of environmental conditions and use different resources as their source of energy. This finding is clinically important with respect to intestinal reduction of oxalate, because *L. gasseri* 7(3) will be able to degrade oxalate in the presence of alternative energy source. This is different from the "specialist" oxalotroph, *O. formigenes*, which utilizes oxalate as its sole source of energy (Sahin, 2003).

4.4. Conclusion

The four *Lactobacillus* strains have been shown to possess *oxc* genes, which encode oxalyl-CoA decarboxylase. This is significant because of its potential contribution to the detoxification of oxalate present in human and animal intestinal tracts (Sidhu *et al*., 1997). The present study provided an insight into transcriptional regulation of *L. gasseri* 7(3) in the presence or absence of oxalate under different pH conditions. Variation in pH did not influence the expression of *oxc* gene negatively, suggesting that the strain could possibly adapt to the physiological conditions. In addition, this strain was shown to possess a "generalist" character, indicating that it utilizes oxalate as well as alternative carbon sources for energy. Further analysis of *oxc* gene expression is, however, required either using microarray or RT-qPCR in order to validate the results of the dot blot analysis as these techniques have been shown to be more sensitive and specific. Similarly, it would be useful to screen for the *frc* gene in the four *Lactobacillus* strains. In particular, the transcriptional regulation of *frc* gene in *L. gasseri* 7(3) is an interesting area for future research.

CHAPTER 5

GENERAL CONCLUSION AND FUTURE STUDIES

In this study four *Lactobacillus* strains isolated from faecal samples of healthy human individuals were identified to the species level. It was hypothesised that these strains might have the ability to degrade oxalate and could be useful in the development of probiotics for the treatment of kidney stone disease. The identification of these strains was important as this helps to assess, which of the human intestinal microbiota may eventually be used as probiotics. The strains were identified as *L. gasseri* 7(3), *L. gasseri* 17(4), *L. reuteri* 17(7) and *L. reuteri* 16(9) and were autochothonous strains, defined as natural isolates. The probiotic potential of the *Lactobacillus* strains was also assessed by determining their antibiotic resistance characteristics, their antimicrobial activity against pathogens, their adhesion properties, their tolerance to low pH and bile salts, and their oxalate degrading activity. The strains displayed good probiotic properties and had good oxalate degrading activity (Table 4.1).

Table 4.1: Summary of the criteria recommended for selecting potential probiotic strains

Strains	Antimicrobial activity (out of 7 pathogens)	Yeast agglutination	Auto aggregatation	Acid survival	Bile surival	Oxalate utilization
L. gasseri 7(3)	5	++	++	pH 2: NG pH 4: G	0.5%: G 1%: G	++++
L. gasseri 17(4)	7	++	++	Not determined	Not determined	++
L. reuteri 17(7)	7	+++	+++	pH 2: NG pH 4: G	0.5%: G 1%: G	+
L. reuteri 16(9)	7	++	++	Not determined	Not determined	++

(G) growth, (NG) no growth

Overall, *L. gasseri* 7(3) was shown to be the best potential probiotic bacterium of the study. It displayed antimicrobial activity against most of the pathogens tested, good yeast agglutination and autoaggregation properties, acid (pH 2 and pH 4) and bile (0.5% and 1%) tolerance and had the highest oxalate utilization ability.

Further *in vivo* study is, however, needed to confirm the probiotic properties of these *Lactobacillus* strains. This highlights the need for animal studies and double blind placebo controlled clinical trials using a large cohort of human participants, to evaluate the safety and efficiacy of the probiotics and the ability to reduce the urinary oxalate excretion in participants with kidney stone disease. The strains were also screened for the presence of the *oxc* gene and they were shown to possess these genes, which encode oxalyl-CoA decarboxylase that is considered crucial in the detoxification of oxalate present in human and animal intestinal tracts. Of the 4 strains tested, *L. gasseri* 7(3) possessed good probiotic characteristics with excellent oxalate degrading activity. It was therefore, selected to study the transcriptional regulation of the *oxc* gene in the presence and absence of 10 mM of ammonium oxalate under mildly acidic and no acidic conditions. The *oxc* gene was constitutively transcribed under both pH conditions in the presence and absence of 10 mM ammonium oxalate. This observation suggested that, it could possibly be due to several transcriptional factors or possibly mutation. Future work is needed to further characterise the regulatory mechanisms controlling the *oxc* gene expression.

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