PROBIOTICS FOR THE MANAGEMENT OF KIDNEY STONE DISEASE

by

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CERTIFICATION OF SUPERVISOR

In terms of paragraph GP 7 of "General Rules for the degree of Doctor of Philosophy (Ph.D.)" we, as supervisors of the candidate Thokozile R Lewanika, certify that we approve of the incorporation in this thesis of material that has already been submitted for publication.

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CONTENTS

ABSTRACT

Kidney stones contain various combinations of chemicals, however, 80% consist of calcium oxalate. Oxalate in humans is either absorbed into the urinary tract and excreted in urine or degraded by gut bacteria. Oxalate-degrading gut bacteria play a critical role in human oxalate homeostasis, evidenced by increased research interest in them as potential probiotics in the management of kidney stone disease. In South Africa, kidney stones are more prevalent in the white than in the black population, despite the latter having a diet that puts them at greater risk of developing kidney stones. It was, therefore, postulated in this study that differences in oxalate-degrading bacteria contributes to the observed South African kidney stone statistics. The major aims of this study were, consequently, to investigate the faecal microbiota of black and white South Africans, with respect to oxalate-degrading bacteria; and to identify and characterise novel oxalate-degrading probiotic candidates.

The study population comprised twenty stone-free black and white South African males on a normal diet. Results obtained using PCR detection and denaturing gradient gel electrophoresis (DGGE) analyses showed differences in the oxalate-degrading bacteria between the two populations, with the black population recording a higher incidence of known oxalate-degrading bacterial species (70%) vs. the 30% recorded in the white population. Furthermore, culturable faecal bacteria isolated from the black population had greater oxalate-degrading capacities than those isolated from their white counterparts. Oxalate-degrading gut bacteria could, therefore, contribute to the lower incidence of kidney stones in the black South African population, relative to the white one. Two novel oxalate-degrading *Escherichia coli* and *Clostridium innocuum* strains were isolated from the faecal microbiota of a black test subject and physiologically characterised. Both species grew in

oxalate-enriched media and the *E. coli* isolate degraded oxalate under both aerobic and anaerobic conditions. *In silico* genome screening of *Lactobacillus* genomes identified an oxalate-degrading strain of *Lactobacillus gasseri*. Its oxalate-degrading mechanism was physiologically and transcriptionally characterised using *in vitro* growth studies coupled with RNA hybridisation analyses and reverse transcriptase PCR. In addition, the bacterium had significant oxalate-degrading ability under simulated *in situ* conditions in a continuous culture simulator of the human colonic microbiota. This bacterium is a viable candidate for use in the therapeutic management of kidney stone disease.

ABBREVIATIONS

A_{600}	:	absorbance measured at a wavelength of 600 nm
aa	:	amino acids
ADP	:	adenosine diphosphate
ATP	:	adenosine triphosphate
bp	:	base pair(s)
CCF	:	continuous culture fermentor
cDNA	:	complementary DNA
CFU	:	colony forming units
DGGE	:	denaturing gradient gel electrophoresis
DIG	:	digoxygenin
DNA	:	deoxyribonucleic acid
DNAse	:	deoxyribonuclease
dNTP	:	deoxynucleotide triphosphate
EDTA	:	ethylenediaminetetra-acetic acid
g	:	gram
h	:	hour(s)
IPTG	:	isopropyl β-D-thiogalactopyranoside
kb	:	kilobase pair(s)
kDa	:	kilodalton(s)
log	:	logarithmic
M	:	molar
mg	:	milligram
MIC	:	minimum inhibitory concentration
min	:	minute(s)
mM	:	millimolar
mRNA	:	messenger RNA
MW	:	molecular weight
NCBI	:	National Centre for Biotechnology Information

nm	:	nanometers
OD_{600nm}	:	optical density measured at wavelength of 600 nm
ORF	:	open reading frame
p	:	plasmid
P_i	:	inorganic phosphate
PCR	:	polymerase chain reaction
qRT-PCR	:	quantitative real-time PCR
RNA	:	ribonucleic acid
RNAse	:	ribonuclease
rRNA	:	ribosomal RNA
RT-PCR	:	reverse transriptase PCR
s	:	seconds
SDS	:	sodium dodecyl sulphate
SDS-PAGE	:	SDS polyacrylamide gel electrophoresis
spp.	:	species
Tris	:	tris(hydroxymethyl)aminomethane
U	:	units
w/v	:	weight per volume (in grams per 100 ml)
X-gal	:	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
α	:	alpha
β	:	beta
λ	:	lambda
μ	:	micro

CHAPTER 1

General Introduction

Contents

1.1. PROBIOTICS AND HUMAN HEALTH

The human body is host to millions of bacteria, and its gastrointestinal tract (GIT) has been described as "an integrated unit composed of numerous microbial habitats" (Conway, 1995). The large intestine (colon) hosts the largest number of bacteria ($>10^{11} - 10^{12}$ per gram of faeces), comprising several hundred species (Finegold *et al.*, 1983). The importance of microbes and bacteria-host interactions for the maintenance of good health has been recognised since the early 1900s (Metchnikoff, 1908). Metchnikoff proposed that the longevity of the Bulgarian population was partly due to their consumption of large amounts of fermented milk products that contained live bacterial cultures. Probiotics have since been generally defined as non-pathogenic, live microbial feed supplements that have a beneficial effect on the host animal when administered in adequate amounts (Fuller, 1989; Steer *et al.*, 2000). These beneficial effects may either be an alteration in the composition (pathogen exclusion) or metabolic activities (contribution to host nutrition) of the gut microbiota; or modulation of the immune system reactivity in a way that benefits host health. Historically, lactic acid bacteria such as *Lactobacillus* and *Lactococcus* species of dairy product origin were consumed as probiotics (Heller, 2001). In more recent times, however, probiotics are isolated naturally from a representative member of the host animal of their intended use. This measure may have been enforced to increase the safety and efficacy of probiotics administration. Probiotics have been used widely over the last century and numerous benefits of their consumption have been reported (reviewed in Ouwehand *et al.*, 2002). Some of these reported benefits are outlined in Table 1.1.

Table 1.1 Reported health benefits of probiotics consumption by humans.

BACTERIA	REPORTED BENEFIT
Bifidobacterium bifidum	- Improved lactose tolerance (Savaiona *et al.*, 1984). - Repression of *Human rotavirus*-associated diarrhoea in infants, and stimulation of a *rotavirus* specific antibody response (Saavendra *et al.*, 1994) - Synthesis of vitamins (Arunachulum, 2000)
Bifidobacterium lactis	- Stimulation of the immune system (Link-Master *et al.*, 1994).
Escherichia coli strain Nissle 1917	- Repression of diarrhoea (Boudeau *et al.*, 2003)
Lactobacillus rhamnosus GR-1	- Prevention of urinary tract infections in women (Bruce *et al.*, 1992)
Lactobacillus acidophilus *Bifidobacterium infantis* *Streptococcus thermophilus* *Oxalobacter formigenes*	- Reduction of urinary oxalate levels (Campieri *et al.*, 2001; Duncan *et al.*, 2002)

The exact modes of action by which probiotics are able to exert their reported health benefits are not always clearly understood. It is, however, generally accepted that they may provide benefits in the following ways:

- Direct competition for bacterial growth substrates with pathogens, thereby repressing pathogenic populations (Bezkorovainy & Kot, 1998)
- Competition for adhesion receptors with pathogens, effectively supplanting, or preventing colonisation by pathogens (Boudeau *et al.*, 2003)
- Production of antimicrobial substances (Yildrim & Johnson, 1998)
- Stimulation of specific and non-specific immunity (Kailasapathy & Chin, 2000)
- Digestion of complex substrates to simpler sugars (Savaiona *et al.*, 1984)
- Digestion of indigestible, potentially harmful substrates such as oxalate (Campieri *et al.*, 2001, Duncan *et al.*, 2002)

The suitability of a bacterial strain for probiotic use is dependent on various factors, some of which are described below (Collins & Gibson, 1999; Heller, 2001). The probiotic preparation should:

- Be isolated naturally from the species of its intended use
- Be non- pathogenic and non- toxic
- Contain a large number of viable cells
- Remain viable during storage and use
- Have good colonisation potential in the GIT
- Survive the gastric acidic conditions, resist degradation by digestive enzymes and be resistant to the action of bile salts, thus remaining metabolically active in the GIT
- Have the ability to exert a beneficial effect on the host

The numerous reported benefits of probiotic consumption have opened up several possibilities in various areas in which probiotics can be administered. Probiotic administration is no longer confined to its traditional application of treating and/or preventing intestinal disorders (Savaiona *et al.*, 1984; Saavendra *et al.*, 1994; Boudeau *et al.*, 2003) and now encompasses other areas including urinary-genital diseases such as candidiasis and kidney stone disease (Bruce *et al.*, 1992; Campieri *et al.*, 2001; Duncan *et al.*, 2002).

1.2. OXALATE AND KIDNEY STONE DISEASE

Oxalic acid is a strong organic acid that is widely distributed in nature, occurring in both plants and animals. This highly oxidised molecule has the ability to form bonds with various minerals and act as a chelator of cations, especially calcium and magnesium. The compounds subsequently formed are referred to as oxalate salts. Oxalic acid generally accumulates in

plants as a metabolic end product, occurring as the free acid, as soluble sodium or potassium oxalate, or precipitated as an insoluble salt, mainly calcium oxalate (Holmes & Assimos, 1998). Calcium oxalate deposits occur as microscopic crystals in the tissues of a wide variety of plant taxa; accumulated amounts can comprise as much as 85% of the dry weight of some plants (Webb, 1999). Oxalic acid and its oxalate salts occur in the blood (plasma) and urine of animals and humans. The majority of oxalate in animals originates from ingested oxalate-containing plant material, while a small percentage is formed endogenously in the liver via the metabolism of glycine, glyoxylate and ascorbic acid (Holmes & Assimos, 1998). In a study on the effect of dietary oxalate on calcium oxalate formation, Massey *et al.* (1993) showed that ingestion of the oxalate-rich foods spinach, rhubarb, beets, nuts, chocolate, wheat bran and strawberries significantly enhanced excretion of urinary oxalate in healthy individuals.

Humans lack the enzymes required to metabolise oxalate. Consequently, oxalate in humans is dealt with in two principal ways: it is either degraded by microorganisms present in the gastrointestinal tract, or absorbed into the urinary tract and excreted in urine. Alternatively, oxalate can combine with calcium in the gut, forming insoluble salts that are eliminated in faeces. The colon is the major site of oxalate absorption and the relative amounts of calcium and oxalate are important determinants of the rate of oxalate absorption and urinary excretion (Campieri *et al.,* 2001). Although the precipitation of calcium oxalate in a urine sample is dependent on its saturation with both calcium and oxalate ions, it has been argued that, in a metastable state, oxalate ion concentration is more significant in the formation of urinary calcium oxalate stones (Lung *et al.,* 1994). Hyper absorption, or abnormal synthesis of oxalate can cause death in animals and humans due to its corrosive effects. In smaller amounts it causes a variety of pathological disorders including hyperoxaluria, calcium oxalate

urolithiasis, cardiomyopathy, and renal failure (Campieri *et al.*, 2001; Schwille & Hermann, 1992; Scott *et al.*, 1977).

Hyperoxaluria describes excessive urinary oxalate and this condition often leads to urolithiasis, which is a medical term used to describe stone formation. The stones formed are commonly known as kidney stones although they may occur anywhere in the urinary tract (Duncan *et al.*, 2002). Kidney stones are formed when crystals separate from urine and accumulate on the inner surfaces of the kidneys and other surfaces along the urinary tract. They contain various combinations of chemicals, including calcium phosphate, uric acid and cystine, but up to 80% are calcium oxalate stones (Liebman & Costa, 2000). The majority of calcium oxalate urinary stones are idiopathic; there is no clinical explanation for this disease. Small stones may be excreted undetected in urine, but larger stones cause extreme discomfort and even blockage of the urinary tract. Jejuno-ileal bypass surgery and pathological conditions such as Crohn's disease (Scott *et al.*, 1977) and cystic fibrosis (Sidhu *et al.*, 1998) are associated with enteric hyperoxaluria due to enhanced absorption of oxalate in the colon.

1.3. EPIDEMIOLOGY AND TREATMENT OF KIDNEY STONE DISEASE

The prevalence of stone formation varies in different parts of the world (Ramello *et al.*, 2000). The reported incidence of stone disease in Saudi Arabia is approximately 20% (Robertson & Hughes, 1994), and the risk of developing stone disease in other parts of the world has been estimated as 12-13% in North America, 5-9% in Europe and 1-5% in Asia (reviewed in Ramello *et al.*, 2000).

1.3.1. Risk factors associated with kidney stone disease

Idiopathic kidney stone disease is relatively common and various risk factors, both individual and environmental, are reported to influence its epidemiology. These factors include race (Wise & Kark, 1961), sex (Scott *et al.,* 1977), genetics (Curhan *et al.,* 1997; Scott *et al.,* 1999), affluence (Robertson *et al.,* 1981), diet (Taylor & Curhan, 2004), climate (Schwille & Hermann, 1992) and occupation (Borghi *et al.,* 1993).

Race, sex and familial recurrence

Worldwide statistics report a higher incidence of idiopathic stones in white populations than in black populations. The findings of studies conducted in the USA, Brazil, Italy, Saudi Arabia and South Africa (Ramello *et al.,* 2000; Rodgers, 1991; Wise & Kark, 1961; Whalley *et al.,* 1998) have all reflected this trend. Nonetheless, no inborn racial factors have currently been identified as being responsible for this phenomenon. Kidney stones generally, and in all studies on white populations, occur more frequently in males than in females (Serio & Fraioli, 1999; Scott *et al.,* 1977; Whalley *et al.,* 1998). It has been reported that androgens (male hormones) increase urinary oxalate secretion and calcium oxalate precipitation while oestrogens (female hormones) decrease the same (Fan *et al.,* 1999). However, a study in Illinois, USA reported that kidney stone disease was more prevalent amongst female black American and Hispanic females than in their male counterparts (Michaels *et al.,* 1994).

Increased urinary calcium secretion (hypercalciuria) is a suggested risk factor for idiopathic stone formation (Robertson & Peacock, 1980), and familial recurrence has been reported for hypercalciuria (Coe *et al.,* 1979). While it has been suggested that environmental factors may play a major role in familial recurrence of kidney stone disease, Curhan *et al.* (1997) reported an increased risk of stone formation within families even after data had been normalized for

known environmental factors. Nonetheless, to date, no form of genetic predictive assessment of stone formation risk is available for idiopathic urolithiasis (Ramello *et al.*, 2000).

Climate, season and occupation

Kidney stones are more prevalent in hot climates and in warmer seasons (Ramello *et al.*, 2000; Schwille & Herman, 1992). It has been postulated that dehydration leading to decreased urinary volume and a subsequent increase in urinary concentration of oxalate and calcium is responsible for this phenomenon. Additionally, it has been suggested that prolonged exposure to the sun in hot climates as well as in sunny seasons (summer) increases Vitamin D plasma levels (Robertson & Peacock, 1981). Vitamin D reportedly enhances intestinal absorption of calcium and increases oxalate secretion, thus increasing the risk of stone formation (Broadus *et al.*, 1984; Robertson & Peacock, 1980). Occupations that involve continuous exposure to hot environments and resultant substantial sweating have also been implicated in increased risk of stone formation (Borghi *et al.*, 1993).

Diet and socio-economic conditions

As mentioned earlier, most of the oxalate found in humans comes from dietary sources. There is general consensus amongst researchers that dietary oxalate influences urinary oxalate levels (Hesse *et al.*, 1993; Kasidas & Rose, 1980; Holmes & Kennedy, 2000). In addition, high animal protein diets are associated with increased risk of stone formation (Robertson *et al.*, 1981; Robertson & Hughes, 1994; Curhan, 1993). Increased animal protein diets reportedly increase urinary calcium and uric acid, as well as decrease urinary citrate, thereby increasing the risk of stone formation (Curhan *et al.*, 1993; Robertson & Hughes, 1994).

For many centuries kidney stone disease has been viewed as a disease of the aristocracy (Ramello *et al.,* 2000). The incidence of kidney stone disease is highest in more affluent countries such as Saudi Arabia (Robertson & Hughes, 1994) and the United States of America (USA) (Ramello *et al.,* 2000). It was initially suggested that this trend merely reflective better diagnostic procedures in more affluent nations. However, within a single affluent nation such as Great Britain it has been shown that kidney stones are more prevalent amongst members of the higher socio-economic class (Robertson & Peacock, 1980). These results suggest that diagnostic procedures alone cannot account for the observed phenomenon.

1.3.2. Treatment options

The treatment of kidney stone disease usually begins with improved dietary habits, in which patients are encouraged to avoid oxalate-rich foods and beverages. However, they are encouraged to increase their calcium intake so that calcium oxalate crystals are formed in their gut and egested, thereby reducing the amount of oxalate available for absorption in the colon. In a large (>45, 000 male subjects) prospective epidemiological study on the effect of dietary calcium on the risk of kidney stone formation, Curhan *et al.* (1993) concluded that a high calcium intake is associated with a decreased risk of symptomatic kidney stones. Increased fluid (water) intake is also encouraged to help in dissolution of small stones. Certain kidney stones, but not calcium oxalate stones, can also be dissolved with medication. Stones that are too large to pass undetected in urine can be treated with Extra-corporeal Shock Wave Lithotripsy (ESWL) (Lingeman *et al.,* 1986). This non-surgical treatment involves the use of high-energy shock waves to fragmentize kidney stones into pieces that can be passed in urine. In extreme cases, however, surgery is performed to remove large kidney stones).

Calcium oxalate stones recur frequently (Robertson, 1999; Sutherland *et al.,* 1985) and their management can be extremely costly. Estimated annual expenditure on the treatment of

calcium oxalate stones was $1.83 billion in the USA (Chandokhe, 2002) and £111.333 million in the United Kingdom (Robertson, 1999) alone. To this end, numerous pharmaceutical companies have, over the years, dedicated large sums of money to the development of drugs to treat and/or prevent kidney stones. In addition, the burgeoning field of probiotics development, coupled with society increasingly leaning towards "natural" medicine, has led to increased research interest oxalate-degrading gut bacteria as potential probiotics for the management of kidney stone disease.

1.4. OXALATE- DEGRADING BACTERIA OF HUMAN ORIGIN

A large, dynamic consortium of microorganisms, comprising several hundred species, is resident in the human body (Macfarlane & Macfarlane, 2004). This number of microorganisms is so large that humans have been numerically estimated to comprise 90% prokaryotic cells and only 10% eukaryotic cells (Hooper *et al.,* 2002), with the largest number of microorganisms resident in the gastrointestinal tract (GIT). Although the exact composition of GIT bacteria is particular to an individual, and known to vary over time within individuals, numerous studies have reported that the major bacterial populations in the adult gut remain relatively stable (Finegold *et al.*, 1983; Holdeman *et al.*, 1976; Moore & Moore, 1995). GIT bacteria conduct numerous biochemical reactions, and this metabolic activity is of great importance to human health and nutrition (Hooper *et al.,* 2002). The metabolic activity of GIT bacteria includes the degradation of numerous dietary substances that are indigestible to humans, such as oxalate.

Until recently, very little was known about intestinal bacteria that breakdown oxalate. *Oxalobacter formigenes* was the first described oxalate-degrading bacterium isolated from the

rumen (Allison *et al.,* 1985), and is the only oxalate-degrading faecal bacterium on which extensive physiological and genetic analysis has been done. Other types of human oxalate-degrading faecal bacteria that have been described are *Enterococcus faecalis* (Hokama *et al.,* 2000), *Eubacterium lentum* (Ito *et al.,* 1996b), *Providencia rettgeri* (Hokama *et al.,* 2005), lactobacilli (Campieri *et al.,* 2001), and bifidobacteria (Campieri *et al.,* 2001; Federici *et al.,* 2004).

1.4.1. *Oxalobacter formigenes*

Oxalobacter formigenes relies on oxalate as an energy source to the exclusion of all other substrates (Allison *et al.,* 1985), and has consequently been described as a "specialist oxalotroph" (Sahin, 2003). This organism is an obligately anaerobic, non-motile, non-spore forming Gram-negative rod with typical cell dimensions of 0.4 – 0.6 μm x 1.2 – 2.5 μm, as estimated from scanning electron micrographs. The rods are often curved and form occasional chains of spiral or coiled filaments. Strains of this species are differentiated by cellular fatty acid composition (Allison *et al.,* 1985). Humans have a wide variation in the extent of colonisation by *O. formigenes*, from a complete absence of the organism (undetectable levels) to 10^8 cfu/gram-wet weight of faeces (Sidhu *et al.,* 1997b). *Oxalobacter formigenes* has been detected in 100% of children tested and 60 to 80% of adults. The therapeutic use of antibiotics and other drugs is thought to contribute to the loss of *O. formigenes* in the guts of adults and cystic fibrosis patients (Scott *et al.,* 1977; Sidhu *et al.,* 1997b; Sidhu *et al.,* 1998).

1.4.2. *Enterococcus faecalis*

An oxalate-degrading *E. faecalis* strain, isolated from human faeces under anaerobic conditions, was the first described oxalate-degrading facultative anaerobe of faecal origin

(Hokama *et al.*, 2000). This Gram-positive bacterium, formerly known as *Streptococcus faecalis*, occurs as single cocci or in chains of various lengths. *E. faecalis* is frequently isolated from the intestinal tracts of humans and animals, and although it is an intestinal commensal, it is also an opportunistic pathogen causing urinary tract and wound infections.

1.4.3. *Providencia rettgeri*

An oxalate-degrading strain of *Providencia rettgeri*, isolated from human faeces, has recently been described (Hokama *et al.*, 2005). This species comprises rod-shaped, Gram-negative facultative anaerobes and together with *E. faecalis*, is the only other described oxalate-degrading facultative anaerobe of faecal origin. *Providencia* species are generally recognized urinary tract pathogens. *P. rettgeri* has a reported weak virulence and causes infections in urinary tracts, wounds and burns (Hokama *et al.*, 2005).

1.4.4. *Eubacterium lentum*

Studies on oxalate-degradation by intestinal microorganisms identified *Eubacterium lentum* as an oxalate-degrading species (Ito *et al.*, 1996a cited in Hokama *et al.*, 2005; Ito *et al.*, 1996b). The genus *Eubacterium* comprises a diverse range of anaerobic, non-spore forming Gram-positive rods and is the second most common genus found in the human GIT after *Bacteroides*, occurring even more commonly than bifidobacteria (Schwiertz *et al.*, 2000). The phenotypic identification of *Eubacterium* species requires great experience and is extremely time consuming, thus the majority of studies done on faecal bacteria have not dealt with this genus in much detail. Nevertheless, *E. lentum* WHY-1, isolated from the faeces of a Japanese male, has been successfully used to reduce the oxalate content of tea (Ito *et al.*, 1996b).

1.4.5. Lactobacilli and bifidobacteria

The genus *Lactobacillus* consists of generally anaerobic species of bacteria that are often tolerant to aerobic conditions, whereas bifidobacteria are obligately anaerobic. Lactobacilli and bifidobacteria are Gram-positive non-spore forming rods that occur in the intestines of humans and other animals. Lactobacilli, and other lactic acid bacteria, also occur in large numbers in milk and fermented milk products. Lactobacilli have enjoyed widespread application in the food production industry (reviewed in Tannock, 2004), and as probiotics for many decades (reviewed in Reid, 1999; Collins & Gibson, 1999). Like lactobacilli, bifidobacteria have also enjoyed widespread use as probiotics and continue to be major research candidates in probiotics and prebiotics development (Pool-Zobel *et al.*, 2005; Trindade *et al.*, 2003; Gibson & Wang, 1994; Wolf *et al.*, 1998; reviewed in Collins & Gibson, 1999). With several species that are generally regarded as safe (GRAS) organisms, lactobacilli and bifidobacteria are ideal candidates as probiotics to manage kidney stone disease. However, few reports exist concerning oxalate-degrading lactobacilli and bifidobacteria (Campieri *et al.* 2001; Federici *et al.*, 2004; Lieske *et al.*, 2005). Table 1.2. gives a summary of reported oxalate-degrading strains of *Lactobacillus* and *Bifidobacterium*.

Table 1.2. Reported oxalate-degrading lactobacilli and bifidobacteria

SPECIES	REFERENCE
L. plantarum *L. brevis* *L. acidophilus* *B. infantis*	Campieri *et al.* (2001)
B. lactis DSM 10140 *B. animalis* ATCC 27536 *B. breve* MB 283 *B. longum* MB 282 *B. infantis* MB 57 *B. adolescentis* MB 238	Federici *et al.* (2004)
Oxadrop® - a patented preparation comprising *L. acidophilus, B. breve, S. thermophilus* and *B. infantis* strains.	Lieske *et al.* (2005)

The *in vitro* experiments of Campieri *et al.* (2001) showed that the bacterial strains tested were unable to utilise oxalate as a principal carbon source. The strains only degraded oxalate when the growth media contained glucose or lactose as well as oxalate. Oxalate-degraders that utilise carbon sources other than oxalate as energy sources are referred to as "generalist" oxalotrophs (Sahin, 2003).

1.5. OXALATE METABOLISM IN ANAEROBIC GUT BACTERIA

Mechanisms of oxalate metabolism have been described mostly for environmental isolates including: *Ralstonia oxalatica* (Quayle & Keech, 1960), previously known as *Pseudomonas oxalaticus*, and the acetogenic bacteria *Clostridium thermoaceticum* (Daniel & Drake, 1993) and Moorella thermoacetica (Daniel *et al.*, 2004). Studies on the mechanism(s) of oxalate degradation by faecal bacteria have focused on the specialist oxalotroph *O. formigenes* as a paradigm organism.

The generation of energy from oxalate degradation by bacteria occurs via both aerobic and anaerobic respiratory pathways. Oxalate catabolism begins with the transport of the oxalate substrate into the cell, followed by its decarboxylation to CO_2 and the corresponding monocarboxylate (Abe *et al.*, 1996; Harold & Mahoney, 1996; Kuhner *et al.*, 1996). In some aerobic bacteria, such as *R. oxalaticus* and *Bacillus oxalophilus*, formate is the monocarboxylate produced. In these bacteria, formate oxidation is then coupled to energy (ATP) generation by electron transport phosphorylation with O_2 as the terminal electron acceptor (Kuhner *et al.*, 1996). In fermenting bacteria such as *O. formigenes* and *C. thermoaceticum*, however, the free energy change of a decarboxylation reaction (between -17 to -25 kJ per mol) represents only a fraction of the energy required for ATP synthesis via ADP and inorganic phosphate (70 – 75 kJ per mol) (Thauer *et al.*, 1977). This makes

substrate-linked phosphorylation impossible for energy conservation in these bacteria. Studies which showed that decarboxylation of dicarboxylic acids, including oxalate, malonate and succinate, served as a sole energy source for the growth of fermentative bacteria such as *O. formigenes* (Allison *et al.,* 1985) and *C. thermoaceticum* (Daniel & Drake, 1993), suggested that the generation of metabolic energy by these bacteria was coupled to either substrate transport into the cell, or to the decarboxylation reactions themselves (Anantharam *et al.,* 1989; Harold & Maloney, 1996).

1.5.1. Mechanism of oxalate metabolism in *Oxalobacter formigenes*

In most species of oxalate-degrading bacteria, carbon derived from oxalate is incorporated into biosynthetic pathways via the glycerate pathway (Cornick & Allison, 1996). In this mechanism, oxalyl-coenzyme A is reduced to 3-phosphoglycerate, which is then shunted into common biosynthetic pathways. In *O. formigenes*, however, this reaction only represents approximately 1% of the carbon from oxalate since most of it (≅ 99%) is channeled towards conversion to CO_2 and formate.

In *O. formigenes*, formate is produced as an end product of oxalate metabolism because this bacterium has a limited capacity to oxidise formate (Anantharam *et al.,* 1989). It was initially hypothesised that oxalate decarboxylation was coupled to the generation of a sodium gradient and subsequent ATP production, as seen in *Klebsiella aerogenes*. In such a mechanism, the decarboxylation energy is directly converted into an energy rich electrochemical gradient of sodium ions across the membrane. This hypothesis was dispelled by experiments that showed that growth of *O. formigenes* was not impaired in the absence of sodium.

Subsequent experiments have shown that generation of energy in *O. formigenes* is coupled to oxalate transport, mediated by the oxalate transport membrane protein OxlT (Anantharam *et al.,* 1989; Harold & Maloney, 1996; Kuhner *et al.,* 1996, Ruan *et al.,* 1992). OxlT, together with cytosolic oxalyl-coenzyme A decarboxylase (Oxc), function as a combination of a vectorial and a scalar element, forming a type of virtual proton pump (Harold & Maloney, 1996). Experiments conducted using reconstitution of oxalate transport in liposomes showed that OxlT catalyses the electrogenic exchange of divalent oxalate (precursor imported into cell), for formate (product exported out of cell) (Anantharam *et al.,* 1989). Inward movement of the divalent anion and subsequent efflux of a monovalent anion, results in an accumulated single net negative charge within the cell for each completed transport cycle. The concomitant disappearance of a single internal proton during decarboxylation of oxalate to formate establishes the thermodynamic equivalent of an outwardly directed proton pump, with stoichiometry of one H^+ per cycle. Kuhner *et al.* (1996) reported that the exchange of oxalate for formate across membranes that generated an internally negative membrane potential was quantitatively the most important component of this proton motive force (pmf). Three such transport cycles would effectively extrude three H^+ and establish a proton motive gradient sufficient to drive ATP synthesis via a membrane-bound F_oF_1-ATPase (Abe *et al.,* 1996; Anantharam *et al.,* 1989). This mechanism of energy conservation, which is widely used by various species of anaerobic bacteria, is termed "decarboxylation phosphorylation" (Dimroth, 1997; Dimroth & Schink, 1998).

In summary, three proteins involved in oxalate metabolism in *O. formigenes* have been identified: the antiport protein OxlT, formyl-coenzyme A transferase and oxalyl-coenzyme A decarboxylase. OxlT is the oxalate:formate exchange protein that represents the vectorial component of the proton-motive oxalate cycle (Abe *et al.,* 1996; Anantharam *et al.,* 1989;

Ruan *et al.*, 1992). Formyl-coenzyme A transferase (Frc) transfers a coenzyme A moiety to activate oxalic acid (Baetz & Allison, 1990; Sidhu *et al.*, 1997b), and oxalyl-coenzyme A decarboxylase (Oxc) catalyses the thiamine PP_i-dependent decarboxylation of oxalyl-CoA to formate and carbon dioxide (Baetz & Allison, 1989; Lung *et al.*, 1994). These events are summarised in Figure 1.1.

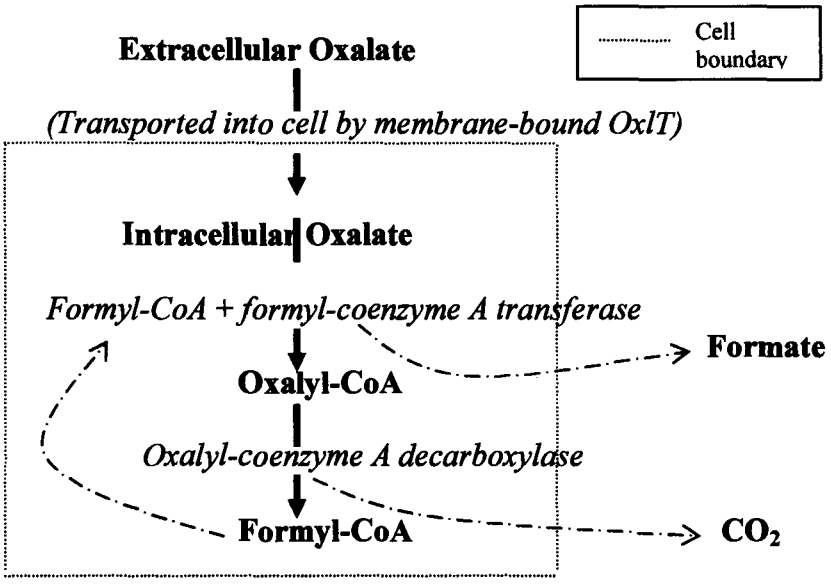


Figure 1.1. Oxalate degradation pathway in *O. formigenes*

1.5.2. Mechanism of oxalate metabolism in *Enterococcus faecalis*

The oxalate-degrading *E. faecalis* strain described earlier, required a limited nutritional environment and repeated sub-culturing on oxalate-containing media to maintain its oxalate-degrading capability (Hokama *et al.*, 2000). SDS-PAGE analyses of protein lysates of oxalate degrading *E. faecalis* strains showed the presence of three proteins, which were absent in non-oxalate-degrading strains of this bacterium. Furthermore, these proteins were not expressed under conditions where the oxalate-degrading strains of *E. faecalis* failed to degrade oxalate. This suggested the presence of an inducible oxalate-degrading system that may be used as a survival mechanism when preferred substrates become scarce. Western blot analyses of two of the putative *E. faecalis* oxalate-degrading proteins showed reactions with antibodies raised

against *O. formigenes* oxalyl-coenzyme A decarboxylase and formyl-coenzyme transferase proteins. These results suggested that the oxalate-degrading enzymes of *E. faecalis* were similar to those found in *O. formigenes*. The third putative *E. faecalis* oxalate-degrading protein could possibly be involved in oxalate transport but Hokama *et al.* (2000) did not investigate this, and the exact mechanism of oxalate degradation in *E. faecalis* has not been reported. This bacterium is, however, a recognized opportunistic pathogen, making it unsuitable as a probiotic research candidate in the management of kidney stone disease.

1.5.3. Mechanisms of oxalate metabolism in *Eubacterium lentum* and *Providencia rettgeri*

The oxalate-degrading proteins oxalyl-CoA decarboxylase and formyl-CoA transferase were reportedly isolated from oxalate-degrading strains of *E. lentum* (Ito *et al.*, 1996a cited in Hokama *et al.*, 2005). The exact mechanism of oxalate metabolism in this bacterium, however, remains unreported.

Two putative oxalate-degrading proteins have been identified in oxalate-degrading strains of *P. rettgeri* (Hokama *et al.*, 2005). These two proteins are not produced by strains of this bacterium that are unable to degrade oxalate. The two identified proteins cross-reacted with the *O. formigenes* oxalate-degrading Oxc and Frc proteins, suggesting a similar mechanism of oxalate degradation in these two species. The exact mechanism of oxalate metabolism in *P. rettgeri*, however, is yet to be reported.

1.5.4. Mechanisms of oxalate metabolism in bifidobacteria and lactobacilli

A PCR investigation for the presence of orthologs of the *O. formigenes* genes *oxlT*, *frc* and *oxc*, failed to detect these genes in any of the oxalate-degrading *Lactobacillus* and *Bifidobacterium* species reported by Campieri *et al.* (2000). More recently, however, Federici

et al. (2004) reported the presence of an *oxc* gene in an oxalate-degrading strain of *B. lactis.* The *oxc* gene encoded a functional oxalyl-CoA decarboxylase enzyme when the recombinant protein was expressed in *E. coli.* Information on the regulation of this gene, or related oxalate-degrading genes, in bifidobacteria is still lacking and warrants further investigation.

Genes encoding functional oxalate-degrading Frc and Oxc enzymes have recently been identified in *L. acidophilus* NCFM (Azcarate-Peril *et al.*, 2006). This is the first report on oxalate-degrading genes in a *Lactobacillus* species. Mildly acidic conditions (pH 5.5) have been reported as a prerequisite for expression of these genes. The exact regulation of these genes, however, has yet to be reported.

1.6. ROLE OF OXALATE-DEGRADING BACTERIA IN KIDNEY STONE DISEASE

As mentioned earlier, oxalate is indigestible to humans and may be absorbed from the colon into the urinary tract, where it has the potential to form stones. Oxalate-degrading bacteria in the gut play a vital role in reducing the amount of oxalate that is available for absorption into the urinary tract, thereby reducing the risk of stone formation. In addition, removal of colonic oxalate by oxalate-degrading bacteria reportedly creates a transepithelial gradient favourable for secretion of oxalate from blood plasma (Hatch *et al.*, 1999). Accordingly, the role of oxalate-degrading bacteria in the treatment of kidney stone disease has become a major research interest in probiotics development (Lung *et al.*, 1991; Duncan *et al.*, 2002; Stewart *et al.*, 2004). This is evidenced by the increasing number of experiments conducted thus far with oxalate-degrading gut bacteria using rat models (Sidhu *et al.*, 2001), continuous culture fermentors (Duncan *et al.*, 2002) and in humans (Campieri *et al.*, 2001; Sidhu *et al.*, 1999b; Lieske *et al.*, 2005) to investigate the suitability of these bacteria as probiotics in the prophylactic treatment of kidney stone disease.

1.6.1. *Oxalobacter formigenes*

O. formigenes and its relevant enzymes have been used in the probiotic treatment of hyperoxaluria and hyperoxaluria-simulated conditions, in *in vivo* studies with rat models (Sidhu *et al.*, 1999b; Sidhu *et al.*, 2001), in continuous-culture fermentors, and in humans (Duncan *et al.*, 2002).

Sidhu *et al.* (1999b) reported reduced urinary oxalate excretions in laboratory rats fed a high oxalate diet (2% ammonium oxalate) supplemented with oxalate-degrading enzyme replacement therapy. Enzyme replacement therapy involved feeding the rats a crude protein lysate of two *O. formigenes* oxalate-degrading proteins: formyl-coenzyme A transferase and oxalyl-coenzyme A decarboxylase. The rats were fed 3 mg of protein per day with an oxalate-degrading capacity of 180 mg/min per mg of protein. By day fifteen of feeding, the rats receiving the protein lysate showed significantly reduced urinary oxalate excretions (3.71 ± 0.9 mmol/L), when compared to the control group of rats, who were fed the high oxalate diet without enzyme replacement therapy (6.99 ± 1.1 mmol/L). In the experiments of Sidhu *et al.* (2001), a group of rats were fed a probiotic preparation of *O. formigenes* and placed on a high oxalate diet for fourteen days. Urinary oxalate was measured as an indication of the oxalate-degrading ability of the administered *O. formigenes*. Results showed that rats fed *O. formigenes* were able to maintain lower levels of urinary oxalate than the control group, which was not fed *O. formigenes*. Furthermore, higher concentrations of *O. formigenes* supplement kept urinary oxalate levels at baseline values.

Duncan *et al.* (2002) tested the oxalate-degrading ability of *O. formigenes* in a single-stage continuous culture fermentor, as well as in four human subjects. The former experiment

involved inoculation of the fermentor with a faecal sample from a human subject who had no oxalate degrading ability detectable prior to the study. Results obtained showed that *O. formigenes* strain OxB was able to colonise the fermentor and degrade all of the oxalate supplied in the culture medium (20 mM oxalate) in two days. The latter study involved ingestion of an oxalate load as well as 500 mg of an *O. formigenes* preparation in a sandwich by four human subjects. Six hours after administration, all four individuals showed reduced urinary oxalate levels when the oxalate load was co-administered with the *O. formigenes* preparation (mean results = 3.0 ± 0.6 mg $^{h-1}$ reduced to 1.9 ± 0.1 mg $^{h-1}$), rather than alone (experimental control).

1.6.2. Lactobacilli and bifidobacteria

In *in vitro* experiments with pure bacterial cultures, Campieri *et al.* (2001) supplemented the growth media with 10 mM oxalate, and found that the lactobacilli and bifidobacteria did not degrade a significant amount of oxalate after three days growth. *Lactobacillus acidophilus* showed the highest percentage breakdown of 11.8%, and *L. plantarum* the lowest at 1.4%, after three days growth. However, an orally administered freeze-dried preparation comprising all of the strains tested (> 2 x 10^{11} CFU/g), significantly reduced urinary oxalate in six patients with a history of idiopathic calcium oxalate urolithiasis and mild hyperoxaluria (55.5 ± 19.6 mg/24 h reduced to 35.5 ± 15.9 mg/ 24 h). These individuals were fed the probiotic-candidate preparation over a four-week period, and continued to show lower than baseline urinary oxalate levels one month after feeding had been discontinued. Additionally, four weeks into the treatment, faecal concentrations of oxalate dropped in two of the patients (743 ± 95 reduced to 443 ±77, and 1,400 ± 135 reduced to 1,072 ± 115 mg oxalate/g wet faeces).

Federici *et al.* (2004) worked only with strains of bifidobacteria and the results of their pure culture *in vitro* experiments showed much higher levels of oxalate degradation than Campieri *et al.* (2001). However, Federici *et al.* (2004) included only 5 mM oxalate in their growth medium and measured oxalate degradation after five days. Oxalate is a highly oxidising molecule and increased concentrations of it in growth medium could be relatively toxic to "generalist" oxalotrophs. Federici *et al.* (2004) reported that *B. lactis* DSM 10140 showed the highest level of oxalate degradation at 61% and that oxalate-degrading ability was strain specific. For instance, *B. breve* MB 283 degraded 37.8% of oxalate, while *B. breve* MB 151 degraded only 1% of the same. These results suggest that whilst "generalist" oxalotrophs may metabolise sugars preferentially, they too could play a significant role in intestinal degradation of oxalate.

Lieske *et al.* (2005) recently reported decreased urinary oxalate levels in ten Inflammatory Bowel Disease (IBD) patients with a history of calcium oxalate urolithiasis, after administration of Oxadrop®, a preparation comprising oxalate-degrading strains of *L. acidophilus, B. breve, S. thermophilus* and *B. infantis*. In this study, seven out of ten patients showed decreased urinary oxalate secretion after one month of a single daily dose of Oxadrop®. The mean decrease reported was 19%, which remained relatively stable in the following month (24% average) when dosage was increased to two doses per day. During the washout period of the study, when Oxadrop® feeding was discontinued, five of the ten patients recorded decreased urinary oxalate levels (20% average) when compared to baseline values.

1.6.3. Comparative efficacy of candidate oxalate-degrading probiotics

The wide distribution of oxalate in the human diet, the pathological conditions associated with a high absorption of oxalate in the colon as well as the earlier mentioned suffering associated

with kidney stone disease, have made research into oxalate degrading bacteria a necessary endeavour. The high cost of management of this disease further reinforces this necessity. While the majority of calcium oxalate stones remain idiopathic, the results of the numerous *in vitro* as well as *in vivo* experiments conducted on oxalate-degrading bacteria to date clearly indicate that oxalate-degrading gut bacteria have a vital role in the homeostasis of oxalate in humans (Allison *et al.*, 1986; Sidhu *et al.*, 1998, Sidhu *et al.*, 1999b, Sidhu *et al.*, 2001; Kumar *et al.*, 2002; Campieri et al, 2000; Duncan *et al.*, 2002; Lieske *et al.*, 2005).

While most research has concentrated on the "specialist" oxalotroph *O. formigenes* as the probiotic candidate of choice in kidney stone disease, the possibility that this organism has emerged as the major oxalate-scavenging bacteria in the gut purely due to selective pressure exerted by high oxalate diets should not be discounted. Although experiments have shown that *O. formigenes* has been successfully used to treat hyperoxaluria, this bacterium requires the presence of relatively high amounts of oxalate to survive in the gut, and remains extremely sensitive to antibiotic therapy (Sidhu *et al.*, 1997b; Sidhu *et al.*, 1999b; Sidhu *et al.*, 2001). Additionally, its absolute dependency on oxalate as an energy source limits the use of *O. formigenes* as a probiotic in preventive therapy where diets are low in oxalate. Notwithstanding, its application as a probiotic would more than likely require a co-administration of oxalate as a prebiotic to enable it to colonise the gut. This additional oxalate may exacerbate prevailing hyperoxaluria and/or urolithiasis in the patients being treated.

The increasing number of reports of "generalist" oxalotrophs, particularly lactobacilli and bifidobacteria species (Campieri *et al.* 2001; Federici *et al.*, 2004; Lieske *et al.*, 2005) that are able to reduce urinary oxalate levels in human trials are evidence that "generalist" oxalotrophs are credible probiotic candidates in kidney stone disease, and warrant further investigation.

As mentioned earlier, prolonged antibiotic therapy, necessitated by conditions such as cystic fibrosis, results in rapid decolonisation of *O. formigenes* thus making the use of oxalotrophs with better demonstrated antibiotic resistance, a worthy alternative approach. Furthermore, *O. formigenes* remains a very fastidious organism to culture using standard laboratory techniques, further compounding the problems associated with its manipulation for probiotic administration.

1.7. UROLITHIASIS IN SOUTH AFRICA AND THE AIMS OF THIS STUDY

The incidence of kidney stone disease in the white South African population is comparable to that of other Caucasians in westernized countries, ranging between 12 and 15% (Meyers, 1994). South African statistics show that kidney stones occur in 15% of white men and 5% of white women, while less than 1% of black people ever develop them (Whalley *et al.,* 1998). This is despite the latter having a diet that is traditionally high in oxalate (large spinach intake) and low in calcium due to low intake of milk products (widespread lactose intolerance) (Viljoen & Gericke, 2001). High oxalate intake and low calcium intake are reported risk factors for kidney stone formation. The ability of South African black people to maintain normal urinary oxalate levels, despite having a diet that puts them at a higher risk of stone formation than their white counterparts, suggested that this population had a greater "oxalate handling" mechanism than the white population (Lewandowski, 2003).

In the study reported in this thesis, it was hypothesized that the black South African population harboured potentially novel oxalate-degrading species that were absent from their white counterparts. The main objectives of this study were:

- To determine the prevalence of known and putative oxalate-degrading bacteria in the faecal microbiota of a test population comprising 20 non-stone forming black and white male subjects,
- To determine the oxalate-degrading activities and biodiversity of lactic acid bacteria and bifidobacteria in the two test groups,
- To identify and characterise novel oxalate-degrading species from the black population with probiotic potential for use in the management of kidney stone disease.
- To test the oxalate-degrading efficacy of any identified probiotic candidates under simulated *in vivo* conditions.

CHAPTER 2

Oxalate-degrading Bacteria in Black and White South African Male Populations, and Identification of Novel Oxalate-degrading Strains

Contents

2.1. SUMMARY

South African statistics show that kidney stones are much more prevalent in the white population than in the black population. Urinary oxalate concentration in humans is a critical factor in kidney stone formation, and gastrointestinal oxalate-degrading bacteria have been shown to play a key role in host oxalate homeostasis. A study of faecal microbiota, with respect to oxalate-degrading bacteria, was undertaken in two groups of subjects, comprising ten black and ten white stone-free South African males. PCR detection of the oxalate-dependent bacterium *O. formigenes* showed that it was present in 70% of the black test subjects, and in 30% of the white test subjects. PCR detection of *Lactobacillus* group and bifidobacteria showed that these bacteria were present in 100% of the black and white test subjects. Additionally, the culturable lactic acid bacteria and bifidobacteria isolated from the black subjects had greater oxalate-degrading capacities than those isolated from the white subjects. DGGE analysis of the culturable lactic acid bacteria showed differences in the predominant lactic acid bacteria populations present in the black vs. white test subjects. Two putative oxalate-degrading species were isolated from a black test subject under anaerobic conditions, and identified as *Escherichia coli* and *Clostridium innocuum* based on 16S rRNA gene analysis and biochemical tests. Both of these isolates grew in oxalate-enriched media, and the *E. coli* isolate degraded oxalate under both aerobic and anaerobic conditions. The *E. coli* isolate, however, lost its ability to degrade oxalate upon sub-culturing on non-oxalate containing media. This is the first report on the isolation of oxalate-degrading *Escherichia coli* and putatively oxalate-degrading *Clostridium innocuum* from the human gastrointestinal tract.

2.2. INTRODUCTION

Kidney stone disease is much more prevalent in the white South African population (15%) than it is in the black population (1%), as described in Chapter 1 (Meyers *et al.*, 1994; Whalley *et al.*, 1998). Earlier studies proposed that the reason for the low incidence of kidney stone disease in the South African black population was the dietary habits of this race group; specifically, low consumption of calcium and animal protein (Muskat *et al.*, 1951; Modlin, 1967). More recent studies, however, report that the black population has a higher consumption of oxalate than the white population (Whalley *et al.*, 1998; Lewandowski, 2003), which puts the black population at a higher risk of developing kidney stones. A diet low in calcium but high in oxalate represents a high risk for the formation of urinary calcium oxalate stones. As described earlier, dietary oxalate can combine with dietary calcium and be subsequently excreted in faeces, or absorbed into the urinary tract and excreted in urine. Additionally, oxalate present in the gut can be degraded by oxalate-utilising gut bacteria.

A dietary analysis of 20 (10 white, 10 black) South African males on a normal diet showed that the black population had a higher oxalate intake, but lower calcium and total protein intake, than their white counterparts (Table 2.1, adapted from Lewandowski, 2003). Although the black population consumed significantly higher amounts of oxalate than the white population in the afore-mentioned study (Table 2.1), urinary analysis showed that urinary oxalate levels in the two race groups were almost identical (0.16 mmol/24h vs. 0.15 mmol/24h), respectively (Lewandowski, 2003).

Table 2.1. Nutrient intake of black and white subjects from dietary questionnaires on normal diet[a]

Dietary substance	Black population	White population	p-value
Calcium (mg/day)	663.11 ± 99.78	1080.10 ± 105.55	0.011*
Total protein (g/day)	79.56 ± 9.41	120.15 ± 12.71	0.022*
Oxalate (mg/day)	297.89 ± 68.94	128.30 ± 21.44	0.025*

[a]Adapted from Lewandowski (2003)
*Significance at $p<0.05$; ± std error

Furthermore, when both race groups were challenged with dietary oxalate, urinary oxalate levels increased significantly in the white population, but remained unchanged in the black population (Lewandowski, 2003). Lewandowski (2003) postulated that the black and white populations differed in their oxalate-handling mechanisms, with the black population possibly having a higher number of oxalate-degrading bacteria that could be responsible for the observed phenomenon. The current study, however, aims to investigate whether the oxalate-degrading bacteria in black and white South African populations are qualitatively, rather than quantitatively, different.

Several reports correlate the absence of *Oxalobacter formigenes* in the gut microbiota with the formation of calcium oxalate stones (Han *et al.*, 1985; Sidhu *et al.*, 1997b; Sidhu *et al.*, 1998; Sidhu *et al.*, 1999a; Kumar *et al.*, 2002). Conventional PCR, with species-specific primers designed to amplify the *oxc* gene, has been the method of choice for detection of *O. formigenes* in faecal samples (Sidhu *et al.*, 1997a; Sidhu *et al.*, 1998; Sidhu *et al.*, 1999a; Kumar *et al.*, 2002; Kodama *et al.*, 2002). For example, in a study involving 63 calcium oxalate stone formers and 40 normal individuals from North India, Kumar *et al.* (2002) reported that *O. formigenes* was detected in 65% of the normal individuals; however, only 30% of the stone formers had detectable levels. From this data, Kumar *et al.* (2002) concluded that the absence of

O. formigenes increased the risk of intestinal oxalate absorption and resultant calcium oxalate urolithiasis.

Cystic fibrosis (CF) patients are reportedly at high risk of developing hyperoxaluria and consequential calcium oxalate urolithiasis (Sidhu *et al.*, 1998). In a study involving 43 CF patients and 21 healthy control subjects, Sidhu *et al.* (1998) correlated the absence of *O. formigenes* with an increased risk of hyperoxaluria. Using conventional PCR detection of the *O. formigenes oxc* gene, Sidhu *et al.* (1998) reported that *O. formigenes* was present in 71% of the 21 healthy control group subjects; but only 16% of the 43 CF patients harboured the bacterium. Prolonged antibiotic use necessitated by CF has been proposed as the reason for undetectable *O. formigenes* in the gut microbiota of CF patients (Sidhu *et al.*, 1998). The lack of *O. formigenes* has been further postulated to contribute to hyperoxaluria in CF patients, with resultant calcium oxalate urolithiasis. The exact reasons for hyperoxaluria in CF patients, however, remain unknown.

The absence of *O. formigenes* is a widely recognised risk factor for the formation of calcium oxalate stones (Duncan *et al.*, 2002). Numerous studies in various parts of the world, including the USA, India and Germany report a higher incidence of *O. formigenes* in stone-free individuals (60 – 80%), when compared to the incidence in kidney stone formers (Goldkin *et al.*, 1986; Sidhu *et al.*, 1997b; Han *et al.*, 1985; Kleinschmidt *et al.*, 1993; Kumar *et al.*, 2002). Accordingly, it is possible that *O. formigenes* could be responsible for the efficient oxalate-handling mechanism noted in the black South African population.

The increasing reports of oxalate-degrading lactobacilli discussed in Chapter 1 suggest that this genus could also play a vital role in oxalate metabolism in humans (reviewed in sections 1.4.5 and 1.6.2). Lactobacilli occur in significant numbers in the human gut, approximating 10^{11} organisms per gram dry weight of faeces, as estimated by culturing techniques (Finegold *et al.*, 1983). In a study involving 20 (10 white, 10 black) healthy South African males, Lewandowski (2003) reported that the black population had significantly higher amounts of culturable lactobacilli than the white population (5.84×10^9 vs. 1.16×10^9, respectively). In view of that, one of the aims of the work in the present study was to investigate whether oxalate degradation by lactic acid bacteria, particularly lactobacilli, contributes to the low urinary oxalate levels, and resultant lower occurrence of kidney stone disease, in the black South African population.

Ecological studies on the gut microbiota have routinely employed selective and non-selective culture-dependent microbiological methods (Macfarlane & Macfarlane, 2004). Two limitations of these methods have been the underestimation of bacterial population sizes and the genetic diversity of the organisms being studied. Molecular techniques, however, have provided alternative culture-independent methods for the compositional analysis of mixed microbial populations. Sequence variation in the rRNA gene has traditionally allowed the detection of bacterial taxa from mixed microbial populations using PCR amplification of rRNA genes, coupled with cloning and nucleotide sequencing (Muyzer *et al.*, 1993). In this approach, PCR amplifications are carried out using genus- or species-specific primers and the DNA amplicons are cloned and sequenced individually. This approach is both labour intensive and costly. In recent times, however, molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) have allowed the rapid evaluation of the genetic diversity and stability of complex microbial populations.

In DGGE, DNA fragments of equal length are separated on the basis of their base-pair sequence (Muyzer et al., 1993). PCR amplification is used to generate DNA fragments that are then separated based on their electrophoretic mobilities in a polyacrylamide gel containing a gradient of increasing DNA denaturants. A GC- clamp is incorporated onto the 5′ end of one of the PCR amplification primers to avoid the complete denaturation of PCR amplicons during electrophoresis (reviewed in Macfarlane & Macfarlane, 2004). The electrophoretic pattern obtained on a DGGE gel represents a 'fingerprint' for a particular microbial community, which can be analysed further by sequence analysis. DGGE has been used extensively used to study the profiles and dynamics of complex microbial communities (Muyzer *et al.*, 1993; Heilig *et al.*, 2002; Satokari *et al.*, 2001). In the present study, DGGE was used for a comparative study of the genetic diversity of the culturable lactic acid bacteria in the black and white test populations.

The work presented in this chapter involved an investigation of the faecal microbiota of a pilot group of black and white South African populations with respect to oxalate-degrading bacteria. PCR detection of *O. formigenes,* lactic acid bacteria and bifidobacteria is reported for black and white South African populations. Additionally, the oxalate-degrading capacities of culturable lactic acid bacteria and bifidobacteria in these two populations are described. The biodiversity of culturable lactic acid bacteria in the afore-mentioned populations is also reported. Two novel oxalate-degrading species, isolated from a black test subject, are identified and physiologically characterised.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains, plasmids and culture conditions

All bacterial strains were propagated anaerobically at 37°C in an anaerobic chamber (Forma Scientific, Model 1024) in an atmosphere of 5% H_2, 10% CO_2 and 85% N_2. The bacterial isolates SF1 and SF2 were cultured on an oxalate-enriched medium, Medium A, agar (lacking Na_2S) (Allison *et al.*, 1985); and Brain Heart Infusion Supplemented broth or 1.5% (w/v) agar (BHIS) (Holdeman *et al.*, 1977). The *E. coli* strain isolated from the faecal sample was propagated aerobically at 37°C with shaking; and cultured on BHIS and Medium A. The *E. coli* DH5α and JM109 strains used in the cloning experiments were grown aerobically in Luria-Bertani (LB) broth or 1.5% (w/v) agar (Sambrook *et al.*, 1989) at 37°C. The plasmid used in cloning experiments was the M13-derived plasmid pBluescript SK (pSK) (Stratagene, La Jolla, California, USA). This plasmid was chosen because PCR primers for cloning purposes were designed with *Hin*dIII and *Eco*RI cloning sites that occur within the multiple-cloning site of this plasmid. All positive clones were cultured on LB agar supplemented with 100 μg/ml ampicillin.

2.3.2. General recombinant DNA procedures

All DNA procedures and manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). Competent *E. coli* DH5α and JM109 cells were prepared using the rubidium chloride method (Armitage *et al.*, 1988) and plasmid DNA was isolated using the alkali lysis method of Ish-Horowicz & Burke (1981). Restriction enzymes and T4 ligase were used according to the manufacturer's instructions (Roche). Gel electrophoresis was conducted in 0.8%

w/v agarose gels in Tris-Acetate-EDTA (TAE) buffer as previously described (Sambrook *et al.*, 1989).

2.3.3. Study population

The study population comprised 20 (10 black, 10 white) healthy South African males on a normal diet. The inclusion criteria for the study participants were the absence of any history of kidney, gastrointestinal or kidney stone disease and antibiotics treatment at least six months prior to collection of faecal samples. All participants completed informed consent forms, as well as dietary and medical history questionnaires. The 20 subjects in this study were part of a larger collaborative study, comprising 90 individuals, being conducted by the Department of Molecular and Cell Biology and the Kidney Stone Research Unit at the University of Cape Town. Dr Sonja Lewandowski, of the Kidney Stone Research Unit, handled all consent forms and dietary and medical history questionnaires.

2.3.4. Collection of faecal samples and extraction of faecal DNA

All study subjects received sterile containers and an instruction sheet on how to collect faecal samples (Lewandowski, 2003). All faecal samples were processed within an hour of collection under strictly anaerobic conditions in the anaerobic chamber described in section 2.3.1. Faecal DNA was extracted using the QIAamp® DNA Stool Mini Kit according to the manufacturer's instructions, and all faecal and DNA samples were stored at -20°C.

2.3.5. PCR detection of oxalate-degrading species in faecal DNA of study population

Previously described genus- and group-specific primers were used to detect *O. formigenes*, bifidobacteria and the *Lactobacillus* group in the faecal DNA samples obtained from the black and white test subjects (Table 2.2).

Table 2.2. Primer sets used for PCR detection of oxalate-degrading species.

Target gene	Forward (F) and reverse (R) primer sequences (5′- 3′)	Product length (bp)	T (°C)[a]	References
*O. formigenes oxc**	**F:** AATGTAGAGTTGACTGA **R:** TTGATGCTGTTGATACG	416	60	Sidhu *et al.*, 1999a
Bifidobacterium 16S rRNA	**F:** GGGTGGTAATGCCGGATG **R:** CCACCGTTACACCGGGAA	510	66	Langendijk *et al*, 1995. Kok *et al.*, 1996.
Lactobacillus group 16S rRNA**	**F:** CCTACGGGAGGCAGCAG **R:** CACCGCTACACATGGAG	190	60	Muyzer *et al.*, 1993. Hielig *et al.*, 2002.

[a]PCR annealing temperature.
* PCR amplification consisted of 35 cycles and the annealing temperature was 60°C for 1 s with a gradual decrease of 0.1°C/s to 55°C.
** Reverse primer specific to *Lactobacillus, Leuconostoc, Pediococcus* and *Weissella* species.

Faecal DNA templates used in PCR amplifications ranged in concentration from 100 (minimum) – 400 ng (maximum). For each PCR amplification cycle, faecal DNA in a final volume of 50 µl containing 2.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of the four deoxy-nucleotides and 0.5 U Supertherm polymerase (Southern Cross Biotechnology) were used. The PCR amplification consisted of 30 cycles of 94°C for five minutes, 94°C for one minute, respective annealing temperature (Table 2.2) for thirty seconds, 72°C for ninety seconds and a final elongation step of 72°C for three minutes. PCR amplification products were purified using the High Pure PCR Product Purification Kit according to the manufacturer's instructions (Roche). All PCR amplifications were performed using a Gene Amp9700 machine (Applied Biosystems).

2.3.6. Generation of standardised MRS pools from study population

Freshly voided faecal samples were collected (0.1 g of faeces suspended in 1 ml of anaerobic saline diluent), and serially diluted in 10-fold stages in the same solution. Thereafter, 100 µl aliquots of each dilution were plated in triplicate onto MRS selective media, and incubated anaerobically at 37°C for forty-eight hours. Standardised MRS bacterial pools were then generated using plates with approximately 100 colonies (colonies scraped off using 1 ml anaerobic MRS broth) and stored as frozen 30% glycerol stocks in 100 µl aliquots at -70°C.

2.3.7. Oxalate-degrading capacities of bacterial MRS pools generated from the study population

The Federici *et al.* (2004) protocol, used to determine oxalate-degrading ability of bifidobacteria, was used in this study to determine the oxalate-degrading capacities of the lactic acid bacteria and bifidobacteria-containing MRS pools. One aliquot (100 µl) of each MRS bacterial pool was inoculated into 5 ml MRS supplemented with 5 mM sodium oxalate (MRS-OX), and incubated anaerobically for five days at 37°C (Federici *et al.*, 2004). Uninoculated MRS-OX was used as the experimental control. Residual oxalate in MRS-OX culture and control broths was measured using an oxalate enzymatic kit assay (Sigma), with sample inactivation and purification steps as previously described (Federici *et al.*, 2004). Results were expressed as the percentage of oxalate-degrading capacity of the respective MRS pool, standardized per 100 µl of initial bacterial inoculum.

2.3.8. Extraction of bacterial chromosomal DNA

Chromosomal DNA from type-culture strains and the generated MRS pools was extracted using the Genomic DNA Purification Kit (Fermentas). DNA was eluted in water and routinely stored at -20°C.

2.3.9. DGGE analysis of the biodiversity of lactic acid bacteria in black and white test subjects

A set of previously described primers, specific for lactic acid bacteria 16S rRNA genes, was used to generate PCR amplification products from the faecal microbiota of the test population for Denaturing Gradient Gel Electrophoresis (DGGE) analysis. The forward primer, Uni341F, had sequence 5′ CCT ACG GGA GGC AGC AG 3′ (Muyzer *et al.*, 1993); and the reverse primer, Lab0677R, had sequence 5′ CAC CGC TAC ACA TGG AG 3′ (Heilig *et al.*, 2002). The first round of PCR amplification used Uni341F and Lab0677R primers and the PCR amplification was performed as described in section 2.3.5, with a PCR annealing temperature of 60°C. Thereafter, a 40 bp GC clamp (5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3′) was attached to Uni341F, generating Uni341F-GC, and a second round of PCR amplification was performed using Uni341F-GC and Lab0677R. The amplification products from the second round PCR were then analysed by DGGE.

DGGE was performed using the Dcode™ Mutation Detection System (Biorad), according to the manufacturer's instructions. PCR amplification products were loaded onto 8% (w/v) polyacrylamide gels (40% bisacrylamide gel stock solution, 37:1; Sigma) in 1X TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA at pH 8.0); and the polyacrylamide gels

were made with denaturing gradients from 30 to 60% urea. All gels were electrophoresed at 60°C for sixteen hours at 60V. Following electrophoresis, the gels were stained in a 50 µg/ml ethidium bromide solution for thirty minutes, destained in 1X TAE buffer for ten minutes, and photographed using the Gel Doc™ XR (Biorad). All DGGE experiments were performed in triplicate.

2.3.10. Identification of putative oxalate-degrading strains by random colony selection on an oxalate-enriched medium

A stool specimen was collected from a black test subject and placed in the anaerobic chamber described in section 2.3.1. The faecal sample was serially diluted in 10-fold stages using anaerobic saline solution. Thereafter, 100 µl aliquots of each dilution were plated in triplicate onto 20 mM sodium oxalate-containing Medium A agar (lacking Na_2S) (Allison *et al.*, 1985) and incubated anaerobically at 37°C for forty-eight hours. Individual colonies were propagated aerobically and anaerobically at 37°C on Medium A agar.

2.3.11. Identification of SF1 and SF2

A set of universal primers, F27 (5 ′ CGC CAG GGT TTT CCC AGT CAC GAC AGA GTT TGA TCC GGC TCAG 3 ′), and R1512 (5 ′ CAG GAA ACA GCT ATG AC ACG GIT ACC TTG TTA CGA CTT 3 ′) was used to amplify the 16S rRNA genes from strains SF1 and SF2. Underlined sequence represents polymerase chain reaction (PCR) primers that are homologous to highly conserved regions in the bacterial 16S rRNA gene; while non-underlined sequence represents sequence homologous to the primers used for cycle sequencing. For each PCR amplification cycle, 100 ng of genomic DNA in a final volume of 50 µl containing 2.5 mM

$MgCl_2$, 0.5 μM of each primer, 0.2 mM of the four deoxy-nucleotides and 0.5 U Supertherm polymerase (Southern Cross Biotechnology) were used. The PCR amplification consisted of 25 cycles of 96°C for two minutes, 96°C for forty-five seconds, 55°C for thirty seconds, 72°C for ninety seconds and a final elongation step of 72°C for three minutes. PCR amplification products were purified as described in section 2.3.5.

For carbohydrate utilisation experiments on SF1, 1.0% (w/v) of each carbohydrate, with the exception of esculin, which was added at a concentration of 0.5% (w/v), was added to Peptone Yeast (PY) broth (Jousimies-Somer *et al.*, 2002). The pH of the starting media was adjusted to pH 6.9 with the exception of esculin, which was at pH 6.6. A standard inoculum that gave an initial OD_{600nm} reading of 0.1 was added to each broth. Final OD_{600nm} and pH readings were determined after twenty-four hours of anaerobic growth at 37°C. All pH readings were done using the ϕ70 pH meter (Beckman).

2.3.12. Identification of a putative *E. coli* isolate

The universal primers F27 and R1512, described in section 2.3.11 were used to amplify the 16S rRNA gene from the putative *E. coli* isolate by means of PCR. PCR amplification and cycle conditions as well as PCR amplification product analysis and purification were as described for SF1 and SF2. Lactose utilisation of the putative *E. coli* strain was tested on MacConkey agar (Biolab), and Eosin Methylene Blue (EMB) agar (Oxoid). An API 20E test for identification of *Enterobacteriaceae* was also performed (Biomérieux).

2.3.13. Growth in oxalate media and oxalate-degrading capacities of isolates

The *E. coli* isolate was grown aerobically and anaerobically in 10 ml culture broths of Campieri Base Medium (CAMP) (Campieri *et al.*, 2001), CAMP supplemented with 12 mM ammonium oxalate (CAMP-OX) (Merck), and CAMP-OX supplemented with 1% (w/v) 0.20 μm filter sterilised glucose (CAMP-GLU-OX). Culture broths were inoculated with an overnight culture grown in BHIS to give initial OD_{600nm} readings of 0.01, and incubated for two days at 37°C. OD_{600nm} readings were determined after incubation. SF1 was grown anaerobically in 10 ml culture broths of Medium A containing 12 and 18 mM ammonium oxalate. Culture broths were inoculated with an overnight culture grown in BHIS, to give initial OD_{600nm} of 0.08, and incubated for four days at 37°C. Absorbance readings at 600 nm were determined after incubation. All growth experiments were repeated using equivalent amounts of sodium oxalate instead of ammonium oxalate.

The residual amount of oxalate in culture broths was determined using a modification of the Dawson *et al.* (1980) calcium precipitation assay. The enzymatic oxalate kit assay described in section 2.3.7 was not used because it had not been described for use with microbiological media at the time of completion of these experiments in 2003. In the Dawson *et al.* (1980) method, residual oxalate is precipitated using calcium chloride and the resultant calcium oxalate is then measured using gas chromatography. In the present study, however, gas chromatography was not available. Consequently, calcium chloride precipitation of residual oxalate was followed by turbidity measurements at OD_{600nm} (Lewandowski, 2003). Oxalate concentrations were then extrapolated from a standard curve prepared with oxalate standards of known concentration. All concentrations reported are the means of eight samples.

2.3.14. Nucleotide sequencing and analysis

All nucleotide sequencing was performed using the DYEnamic ET Dye Terminator Cycle sequencing Kit from MegaBACE (Molecular Dynamics), which is based on previously described dideoxynucleotide chain termination (Sanger *et al.*, 1977). All reactions performed were as per the manufacturer's specifications and cycle sequenced on a GeneAmp PCR system 9700 using the respective PCR primers required (Perkin Elmer, Applied Biosystems). The sequencing reaction products were separated using the MegaBACE 500 Sequence system (Amersham Pharmacia Biotech). Nucleotide sequences were analysed using the MegaBACE 500 Sequence Analyser v2.4 and DNAMAN software packages. All nucleotide and amino acid homology searches were performed using the BLAST algorithms of NCBI databases (Altschul *et al.*, 1997).

2.4. RESULTS AND DISCUSSION

2.4.1. PCR detection of *O. formigenes*, bifidobacteria and lactobacilli in faecal DNA from study population

There are two major limitations of the inferences made from studies on bacterial gut populations based on molecular analyses of faecal DNA. These are cell lysis efficiency and the quality of extracted DNA (Li *et al.*, 2003), since these two parameters greatly affect the sensitivity and efficacy of subsequent analyses, including PCR and DGGE. The QIAamp® DNA Stool Mini Kit used to extract faecal DNA from the study population has been previously evaluated and found to be comparable to the widely used bead beating method (Li *et al.*, 2003). The QIAamp® DNA

Stool Mini Kit has the advantages of being quicker and more amenable for use with larger samples than the bead beating method. Moreover, in a study that compared the QIAamp® kit with four other commercial kits, McOrist *et al.* (2002) reported that the QIAamp® kit was the most effective for extraction of DNA from *Lactobacillus* and *Bacteroides* species. The QIAamp® kit was, therefore, used in the present study and PCR amplification of the 16S rRNA gene with F27 and R1512 universal primers was routinely used to test the quality of the extracted faecal DNA.

As mentioned earlier, various studies have correlated the absence of the oxalate-degrading bacterium, *O. formigenes*, with a higher risk of forming calcium oxalate stones (Goldkin *et al.*, 1986; Sidhu *et al.*, 1997b; Han *et al.*, 1985; Kleinschmidt *et al.*, 1993; Kumar *et al.*, 2002). In the present study, the occurrence of *O. formigenes* was tested in healthy black and white South African males to ascertain if this bacterium is a factor in the lower incidence of kidney stone disease reported for the black South African population. PCR amplifications of faecal DNA, using primers specific to the *O. formigenes oxc* gene, were successful and the expected 416 bp PCR amplicons were obtained. Figure 2.1 illustrates an example of the results obtained from four subjects from each of the test groups.

Non-specific amplifications were observed in 2 black subjects, B14 and B17, which suggested the presence of potentially novel *oxc* gene-containing bacteria in these two individuals (Lanes 8 and 10, Figure 2.1). Of the 10 subjects tested in each group, *O. formigenes* was detected in 70% of the black test subjects, but only 30% of the white test subjects gave positive results (Table 2.3).

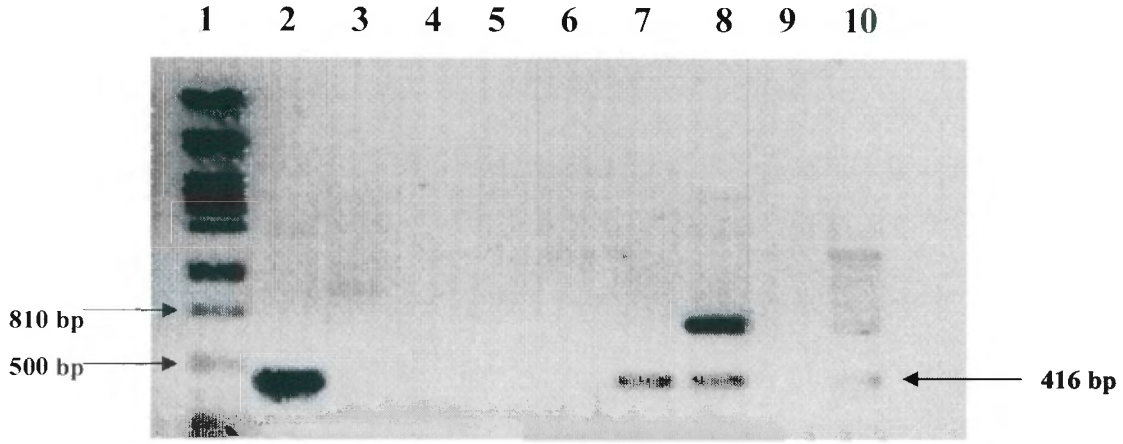


Figure 2.1. Agarose-gel electrophoresis of *O. formigenes oxc* PCR amplification products from faecal DNA samples of test subjects. Lane 1, λ DNA digested with *Pst*I. Lane 2, *O. formigenes* genomic DNA control. Lanes 3-6, faecal DNA samples from white test subjects. Lanes 7-10, faecal DNA samples from black test subjects.

The 70% incidence of *O. formigenes* in the black test population is within the 60 – 80% range reported for other stone-free populations in various parts of the world (reviewed in section 2.2). The 30% occurrence in the white test population is, however, relatively low. These results suggested that *O. formigenes* contributed to the efficient oxalate-handling mechanism observed in the black South African population. The black South African population has a diet that is traditionally rich in oxalate, as discussed in section 1.7, and this dietary oxalate could play a role in enabling this population to maintain high numbers of the oxalate-dependent bacterium *O. formigenes* in the gut microbiota.

Increasing reports of oxalate-degrading bacteria other than *O. formigenes*, particularly lactobacilli and bifidobacteria, impelled us to investigate the presence of the latter two genera in the test populations. The *Lactobacillus* group-specific primers used in this study were designed to

amplify the 16S rRNA gene of lactobacilli and other related lactic acid bacteria: *Leuconostoc*, *Pediococcus* and *Weissella* species (Heilig *et al.*, 2002). Lactobacilli reportedly lack a monophyletic origin, and are very closely related to other lactic acid bacteria. For this reason, 16S rRNA primers that are specific only to lactobacilli remain unreported. The PCR amplifications that targeted the 16S rRNA genes of both bifidobacteria and the *Lactobacillus* group were successful, and Figure 2.2 gives an illustration of the results obtained from the bifidobacteria-specific PCR amplifications.

Bifidobacteria and the *Lactobacillus* group were detected in 100% of both black and white test subjects. These results were expected since these bacterial species reportedly occur in reasonable numbers in the normal gut microbiota (Finegold *et al.*, 1983; Tannock, 1995; Macfarlane & Macfarlane, 2004). In order to determine if the *Lactobacillus* group and bifidobacteria in the test population included oxalate-degrading strains, the oxalate-degrading ability of culturable lactic acid bacteria and bifidobacteria was tested.

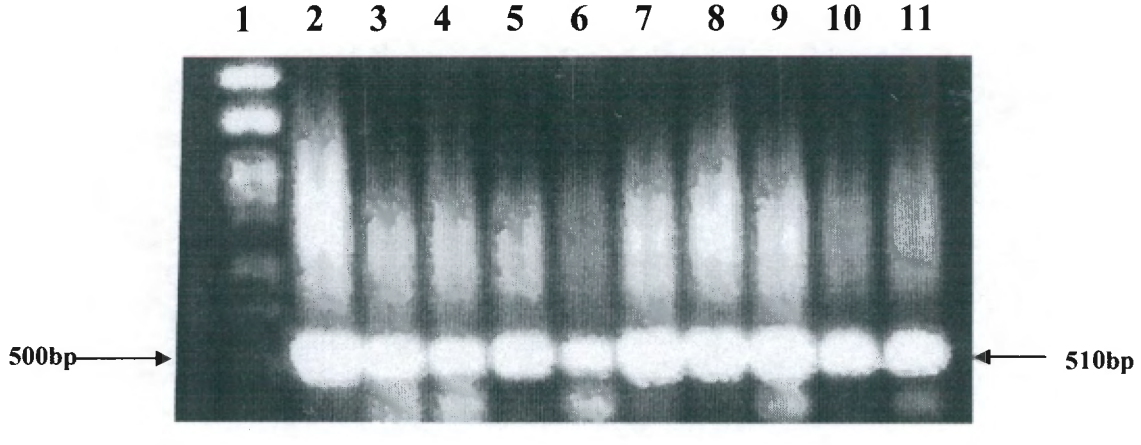


Figure 2.2. Agarose-gel electrophoresis of *Bifidobacterium* genus-specific PCR amplification from faecal DNA samples. Lane 1, λ DNA digested with *Pst*I. Lane 2, *B. lactis* genomic DNA control. Lanes 3-6, faecal DNA samples from white test subjects. Lanes 7-11, faecal DNA samples from black test subjects.

2.4.2. Oxalate-degrading capacities of bacterial MRS pools from study population

Culturable lactic acid bacteria and bifidobacteria from faecal samples were isolated on MRS medium. MRS medium was formulated to favour growth of lactobacilli (De Man *et al.*, 1960), but this medium is not highly selective, and growth of *Streptococcus*, *Leuconostoc* and other lactic acid bacteria has been reported (Dave & Shah, 1996). In addition, anaerobic incubation of MRS supports the growth of bifidobacteria. Nonetheless, MRS medium at pH 5.9 is generally considered as a *Lactobacillus* group-specific medium (Biolab).

The oxalate-degrading capacities of the MRS pools of bacteria generated in the present study were determined using the protocol of Federici *et al.* (2004). The bacterial pools were grown in MRS medium supplemented with 5 mM sodium oxalate, and residual oxalate in the culture broths was measured after five days of anaerobic incubation. The results obtained showed that the organisms isolated from the black population generally had higher oxalate-degrading capacities than those from their white counterparts (Table 2.3).

The MRS pools of bacteria from 40% of the black test subjects had oxalate-degrading capacities higher than 50%; while only 10% of those from the white test subjects had similar levels (Table 2.3). Additionally, the MRS pools from 3 black test subjects (B8, B16, B17) had oxalate-degrading capacities >95%, while only 1 of the white test subjects (W15) had a comparable level of oxalate degradation of 98% (Table 2.3). The MRS pool isolated from black subject B8 recorded the highest oxalate-degrading capacity of 100%.

Table 2.3. Correlation between the oxalate-degrading capacities of the MRS pools of bacteria and the presence of *O. formigenes* in the study population

SAMPLE[a]	% oxalate-degrading capacity of MRS pool[b]	*O. formigenes oxc* PCR amplification result
B8	100 ± 0	+
B9	10 ± 0	+
B12	30 ± 6	-
B13	80 ± 13	+
B14	17 ± 2	+
B16	97 ± 0	-
B17	97 ± 2	+
B18	17 ± 0	+
B19	0 ± 0	+
B20	10 ± 4	-
W7	13 ± 3	-
W8	4 ± 3	-
W12	2 ± 1	-
W13	38 ± 2	+
W14	11 ± 7	-
W15	98 ± 2	-
W16	17 ± 4	-
W17	8 ± 5	-
W18	18 ± 7	+
W20	19 ± 9	+

[a]B – black test subject, W – white test subject
[b]Results represent mean ± SD values, n = 3

O. formigenes is a specialist oxalotroph and it was, therefore, not isolated on the MRS medium used to generate the MRS bacterial pools. Consequently, only bifidobacteria and *Lactobacillus* group species were responsible for the oxalate-degrading capacities reported in Table 2.3. As a specialist oxalotroph, *O. formigenes* is considered the ultimate scavenger of dietary oxalate (Allison *et al.*, 1985; Duncan *et al.*, 2002). For that reason, an investigation into the correlation between the presence of *O. formigenes* in the faecal microbiotas and the oxalate-degrading capacities of MRS pools of the test population was of particular interest in the present study.

O. formigenes was detected in 2 of the black test subjects (B13 and B17) whose MRS pools had high oxalate-degrading capacities of 80 and 97%, respectively (Table 2.3). These results suggested that bacteria other than *O. formigenes* could also contribute significantly to the

degradation of oxalate within individuals who harbour *O. formigenes*. Accordingly, the presence of *O. formigenes* in the faecal microbiotas of a test population should not preclude investigations into alternative oxalate-degrading species within that population. Furthermore, *O. formigenes* was not detected in two individuals (B16 and W15); but the MRS pools of these individuals had oxalate-degrading capacities of 97 and 98%, respectively (Table 2.3). These results suggested that generalist oxalate-degrading species could be the primary oxalate-degraders within these individuals; and that the absence of *O. formigenes* would have been an inaccurate benchmark for the oxalate-degrading potential of the gut microbiota in these individuals. The high oxalate-degrading capacities of the MRS pools, particularly from the black test subjects, prompted an investigation into their biodiversity. DGGE was used to investigate the biodiversity of the *Lactobacillus* group in the isolated MRS pools.

2.4.3. DGGE analysis of the *Lactobacillus* group biodiversity in the MRS pools of the test population

The majority of studies on the diversity of faecal microbiota have focused on numerically predominant bacterial populations, and have not dealt with the biodiversity of less populous groups, such as lactobacilli (Heilig *et al.*, 2002). The importance of lactobacilli gut populations to human health has been discussed earlier, but this genus represents only approximately 1% of the adult faecal microbiota (Sghir *et al.*, 2000). Nevertheless, DGGE has proved a useful culture-independent tool in studying the diversity of lactobacilli from various gut populations (Heilig *et al.*, 2002; Guan *et al.*, 2003; Walter *et al.*, 2000; Walter *et al.*, 2001). In this study, differences in the oxalate-degrading capacities of the standardized MRS pools isolated from the black and white test subjects suggested a qualitative difference in the bacterial species isolated from the two test groups. Accordingly, PCR amplifications were performed on the MRS pools generated from the

study population using *Lactobacillus* group-specific primers described by Heilig *et al.* (2002). The PCR amplicons obtained were then subjected to DGGE analysis. The results obtained showed that the predominant *Lactobacillus*-group organisms isolated from the black test subjects were relatively different from those isolated from the white test subjects, with greater diversity being present within the black population (Figure 2.3).

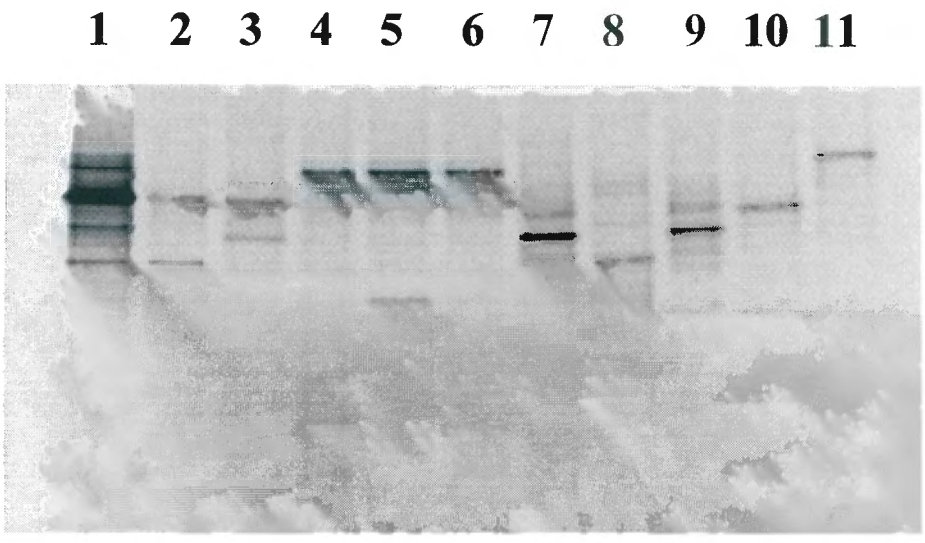


Figure 2.3. DGGE analysis of the *Lactobacillus*-group communities in the bacterial MRS pools generated from the sample population. DGGE analysis of amplicons generated by PCR with Uni341F-GC and Lab0677R primers.Lane 1, *Lactobacillus* species ladder comprising in descending order: *Lactobacillus gasseri*, *Lactobacillus brevis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. Lanes 2-6, MRS pools from white test subjects. Lanes 7-11, MRS pools from black test subjects.

Figure 2.3 illustrates the differences that were observed in the predominant *Lactobacillus*-group organisms in the black vs. the white subjects, but differences within the individual populations are also apparent. In the white test subjects, for instance, the PCR amplicon migration patterns shown in Lanes 3 and 4 (Figure 2.3) indicate that the MRS pools generated from these three individuals had different predominant *Lactobacillus*-group species. On the other hand, the

migration patterns shown in Lanes 4, 5 and 6, suggested that these individuals had the same predominant *Lactobacillus*-group organisms. Equal migration distances on DGGE analyses have, however, been reported for different species of *Lactobacillus*, including *L. gasseri* and *L. johnsonii* (Walter *et al.*, 2000).

As mentioned earlier, the sample population of twenty individuals (ten each) used in the present pilot study is relatively small. Nonetheless, the preliminary findings of an ongoing related study with forty test subjects (twenty each), of which the twenty individuals in the present study are members, have also reported differences in the predominant *Lactobacillus*-group organisms in black vs. white South African male populations as determined by DGGE analyses (Cliff Magwira, unpublished results, Cape Town). Therefore, the findings of the current study comprising twenty test subjects have been correlated by a larger study comprising forty individuals.

In the present study, species identification of the predominant *Lactobacillus*-group organisms isolated from the test subjects was not attempted because the possible contribution of bifidobacteria to the oxalate-degrading capacities of the MRS pools could not be separated from that of the lactic acid bacteria. However, an ongoing related study consisting of forty individuals, of which the twenty test subjects of the present study are members, has reported differences in the bifidobacterial populations of white vs. black South African males, also determined by DGGE analyses (Brian Kullin, unpublished results, Cape Town). The presence of bifidobacteria in all of the MRS pools genenerated from the sample population of the current study was confirmed using genus-specific primers (results not shown). It was, therefore, concluded that qualitative differences in bifidobacterial populations could also contribute to the differences in the oxalate-

degrading capacities of the MRS pools of bacteria isolated from the black and white test subjects, as exemplified by the differences in predominant Lactobacillus-group organisms (Figure 2.3).

Not all bacteria are culturable; therefore selective culture medium such as MRS may lead to the enrichment and isolation of a very limited number of species. Accordingly, DGGE analysis was used to determine if the dominant *Lactobacillus*-group communities in the MRS pools were comparable to those in the faecal microbiotas of the study population. The MRS pools that had high oxalate-degrading capacities were of particular interest, and four of these pools (B8, B13, B16 and B17) were used in this comparative DGGE analysis. The results obtained showed that the dominant *Lactobacillus*-group communities in the MRS pools differed from those of the faecal microbiotas in all but one (B17) of the subjects analysed (Figure 2.4).

In some instances, culturing on MRS medium saw the emergence of *Lactobacillus*-group species in the MRS pools that were undetectable in the faecal microbiotas of the test subjects (B13 faecal vs. B13 MRS pool; B16 faecal vs. B16 MRS pool, Figure 2.4). In another case, there was a shift in which species predominated in the MRS pool vs. the faecal microbiota (B8 faecal vs. B8 MRS pool, Figure 2.4). It was only in test subject B17 that the dominant *Lactobacillus*-group species in the faecal microbiota and generated MRS pool remained unchanged (Lanes 8 and 9, Figure 2.4).

The most frequently encountered *Lactobacillus*-group species in the MRS bacterial pools (encircled on Figure 2.4) were *Lactobacillus mucosae* (B8M, 97% identity); *Lactobacillus paracasei* (B13M and B16M, 99% identity) and *Lactobacillus acidophilus* (B17M, 99% identity). The *L. acidophilus* (B17F and B17M, Figure 2.4) and *L. paracasei* (B13M and B16M,

Figure 2.4) natural isolates aligned with the equivalent strains used in the *Lactobacillus* species ladder (Lane 1, Figure 2.4). However, two additional strains of *L. acidophilus* were isolated from the MRS pools that had different migration patterns (data not shown).

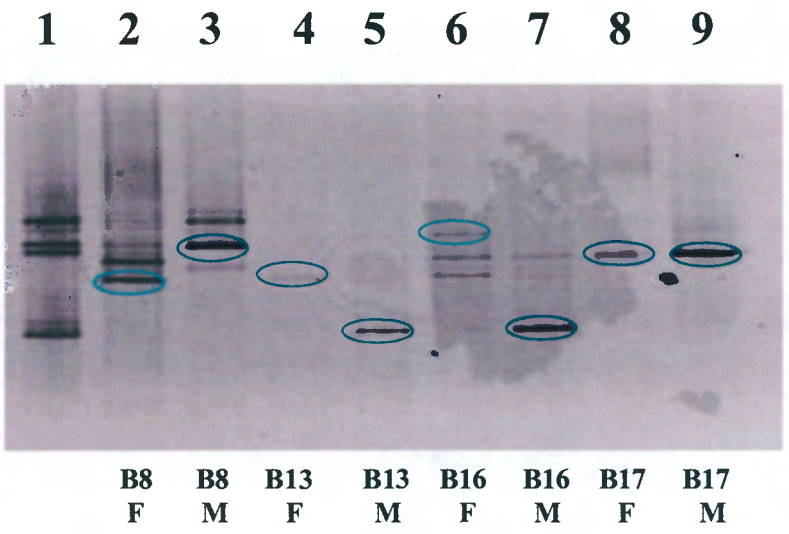


Figure 2.4. Comparative investigation of the dominant *Lactobacillus*-group communities in the bacterial MRS pools and faecal microbiotas of the sample population. DGGE analysis of amplicons generated by PCR using Uni341F-GC and Lab0677R primers. Lane 1, *Lactobacillus* species ladder comprising in descending order: *Lactobacillus gasseri*, *Lactobacillus salivarius*, *Lactobacillus acidophilus* and *Lactobacillus paracasei*. Lane 2, B8 faecal DNA (B8F). Lane 3, B8 MRS pool (B8M). Lane 4, B13 faecal DNA (B13F). Lane 5, B13 MRS pool (B13M). Lane 6, B16 faecal DNA (B16F). Lane 7, B16 MRS pool (B16M). Lane 8, B17 faecal DNA (B17F). Lane 9, B17 MRS pool (B17M). PCR amplicons that were excised and sequenced are encircled in blue.

Sequencing analysis of the dominant *Lactobacillus*-group species in the B8F, B13F, and B16F faecal microbiotas (encircled bands on Figure 2.4) resulted in sequences with significant identity (96 to 99%) to the 16S rRNA genes of uncultured *Eubacterium* clones. This result was not unexpected because overamplification of *Eubacterium biforme*-like species from faecal samples has been reported for the Lab0677 primer used for DGGE analysis in the current study (Heilig *et*

al., 2002). The sequencing results obtained from DGGE analysis suggested that the MRS medium used in this study favoured the growth of lactobacilli above other *Lactobacillus*-group organisms. However, the possibility that this synthetic culture medium (MRS) enriched the growth of only a limited number of *Lactobacillus* species cannot be discounted. Nevertheless, in studies that aim to identify potential probiotic candidates, such as the present study, isolation of culturable bacteria is essential.

2.4.4. Selection of putative oxalate-degrading colonies from Medium A

Medium A was formulated to facilitate the detection of oxalate-degrading species (Allison *et al.*, 1985). It is an opaque medium owing to the presence of calcium oxalate, and colonies of oxalate-degrading bacteria are identified by the formation of clear zones around them. The anaerobic culture method of choice in identifying oxalate-degrading colonies on Medium A is the roll tube method (Holdemann *et al.*, 1977 cited in Allison *et al.*, 1985). The thin layer of agar used in the roll tube culture technique facilitates the formation and observation of clear zones around colonies of oxalate-degrading species growing on Medium A. Medium A and modifications thereof are routinely used in conjunction with the roll tube culture technique to isolate oxalate-degrading bacteria (Dawson *et al.*, 1980; Allison *et al.*, 1985; Hokama *et al.*, 2000).

In the present study, however, the roll tube culture technique was not used to detect oxalate-degrading strains from the faecal microbiota of a black test subject. Instead, Medium A plates were prepared using standard petri dishes and hence no clear zones were observed around any of the colonies growing on this medium. Consequently, all predominant colonies growing on Medium A were considered putative oxalate-degrading species. Three distinctive colonies were identified on the Medium A plates, two of which were strictly anaerobic species and one was a

facultative anaerobe. Preliminary Gram-stain analysis showed that the two anaerobic species were Gram-positive spore-bearing rods. These two putative oxalate-degrading strains were then designated Spore Former (SF) 1 and 2, and subjected to further analysis (sections 2.4.5 and 2.4.6). Gram-stain analysis of the facultative anaerobe showed Gram-negative rods similar to *Escherichia coli*. This putative *E. coli* isolate was also characterized further as described in section 2.4.7.

2.4.5. Identification of SF1

SF1 did not grow aerobically; therefore it is an obligate anaerobe. A Gram stain preparation of SF1 showed Gram-variable bacilli bearing oval terminal spores. Younger cultures (eighteen-hour-old) stained Gram-positive while older cultures (twenty-four hours and older) were Gram-variable. The colony morphology observed on BHIS and PYG agars was of raised colonies of about 5 mm diameter with distinctive scalloped edges. Sequencing data of the 16S rRNA gene over 1,440 bp demonstrated 98% identity to *Clostridium innocuum* CM970.

Three species of *Clostridium*, viz, *Clostridium ramosum*, *Clostridium innocuum* and *Clostridium clostridioforme* are collectively referred to as the RIC group. This group of bacteria is notoriously difficult to identify positively due to Gram variability, a lack of spores in certain strains and unusual colony morphology that is not typical of *Clostridium* species (Alexander *et al.*, 1995). Consequently, specialised diagnostic kits have been devised to positively identify these bacteria, but in the absence of these specialised kits a carbohydrate fermentation profile may be performed. Such a profile was performed on the putative *C. innocuum* isolate and the results obtained are shown in Figure 2.1.

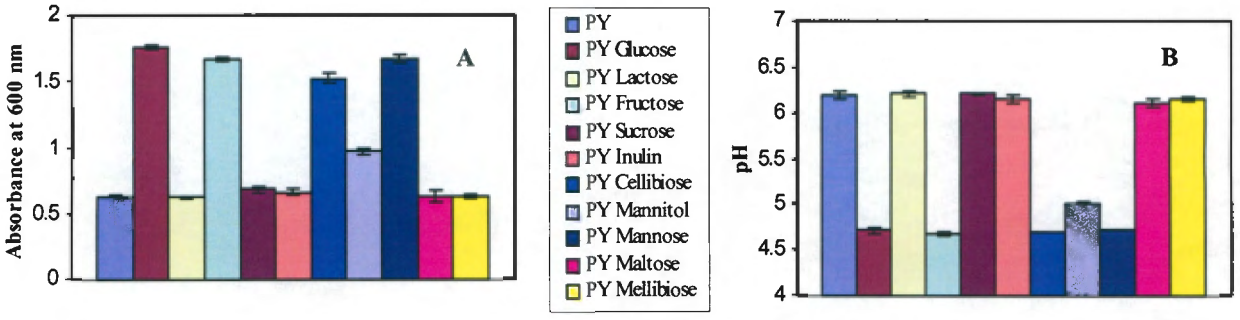


Figure 2.1. Carbohydrate fermentation profile of the putative *C. innocuum* isolate. (A) Anaerobic growth in PY-substituted media after twenty-four hours. (B) pH values of PY-substituted media after twenty-four-hour growth of SF1. Results reflected are the means of duplicates of two independent experiments. Error bars reflect SD values.

The fermentation profile obtained for SF1 was identical to that reported for *C. innocuum* (Holdeman *et al.*, 1977; Jousimies-Somer *et al.*, 2002). This species is reportedly differentiated from all other saccharolytic clostridia by its ability to ferment mannitol, its failure to ferment lactose (Figure 2.1) and the presence of terminal spores (Holdeman *et al.*, 1977). Of the clostridia, *C. innocuum* has the highest recorded resistance to vancomycin (16 µg/ml MIC) and in recent times vancomycin susceptibility tests are routinely used to confirm its identity (Alexander *et al.*, 1995; Mory *et al.*, 1998). Preliminary investigations of the putative *C. innocuum* strain isolated in this study showed vancomycin resistance up to 10 µg/ml MIC. The afore-mentioned results positively identified SF1 as a strain of *Clostridium innocuum*, and the isolate was designated *C. innocuum*-OX.

2.4.6. Identification of SF2

The SF2 isolate was unable to grow under aerobic conditions and is, therefore, also an obligate anaerobe. Gram stain analysis showed that SF2 is a spore-bearing Gram-positive bacillus.

Preliminary sequencing data of the 16S rRNA gene over 500 bp demonstrated 97% identity to *Clostridium botulinum* type B, a well-known pathogen. SF2 was not conclusively identified as *C. botulinum,* nonetheless, the sequencing results were deemed relevant enough for SF2 to be considered a highly unfavourable candidate in this research that centred on probiotic development. Similar to the reported oxalate-degrading pathogen *Enterococcus faecalis* (reviewed in section 1.4.2), an oxalate-degrading *C. botulinum* would be irrelevant to probiotic research. No further experiments were carried out on the SF2 isolate.

2.4.7. Identification of the putative *E. coli* isolate

The putative *E. coli* isolate was able to grow under both aerobic and anaerobic conditions and it is, therefore, a facultative anaerobe. Gram stain analysis showed non-spore bearing Gram-negative rods. Sequencing data of the 16S rRNA gene over 940 bp demonstrated 99% identity to *E. coli* CFT073 (NC_004431). Pink colonies were obtained on MacConkey agar confirming that the putative *E. coli* isolate was indeed Gram-negative, and also able to ferment lactose. Dark colonies with diameters of approximately 2 mm, surrounded by a green metallic sheen, were observed on EMB agar. The metallic green sheen observed on EMB agar is characteristic of *E. coli* strains growing on this medium.

Results obtained from the API 20E tests showed that the *E. coli* isolate most closely resembled *E. coli* and *Enterobacter agglomerans* profiles. Some biotypes of *E. coli* are known to be difficult to differentiate from *E. agglomerans* (Holt *et al.*, 1994), however, all the results obtained for the isolate in the API 20E tests were consistent with the standard profile of all *E. coli* strains. Furthermore, *Enterobacter* species grow as grey-brown colonies of 4-6 mm diameter on EMB agar without a metallic sheen. On the basis of the 16S rRNA sequencing and the biochemical

tests results, the putative *E. coli* isolate was confirmed as a strain of *E. coli* and designated *E. coli*-OX.

2.4.8. Growth in oxalate media and oxalate-degrading capacity of *E. coli*-OX

Figure 2.2 shows that *E. coli*-OX was able to utilise oxalate in CAMP-O and CAMP-GO media both aerobically and anaerobically; and typically of *E. coli*, aerobic growth was favoured over anaerobic growth. Growth studies in medium containing both glucose and oxalate (CAMP-GO) indicated that the isolate utilised both glucose and oxalate, and maximal growth was obtained in the presence of both substrates. In order to ascertain that the enhanced growth seen in CAMP-O and CAMP-GO media was due to the addition of the oxalate and not the ammonium present in ammonium oxalate, growth studies in CAMP-substituted media were repeated using sodium oxalate instead of ammonium oxalate. The results obtained with sodium oxalate-substituted CAMP media were identical to those obtained with ammonium oxalate-substituted media. These results confirmed that the enhanced growth seen in CAMP-O and CAMP-GO media was oxalate-dependent.

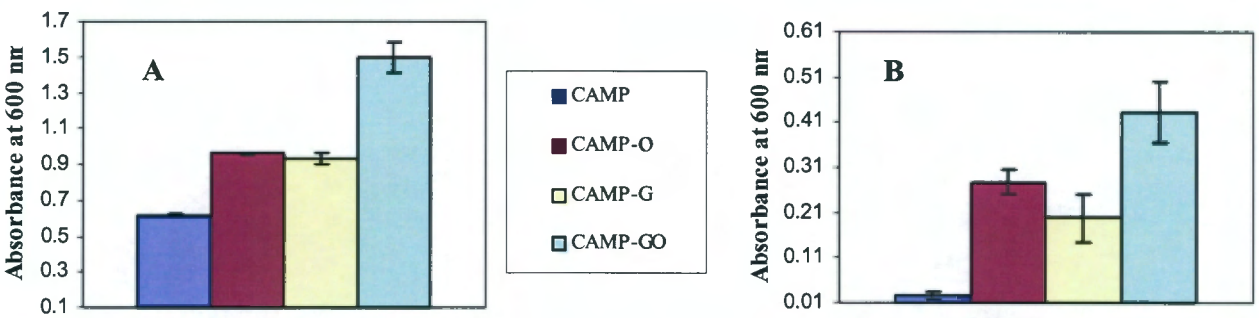


Figure 2.2. Aerobic (A) and anaerobic (B) growth of *E. coli*-OX in CAMP-substituted media after forty-eight hours. Results reflected are the means of duplicate samples of two independent experiments and error bars reflect SD values. CAMP – Campieri basal medium. CAMP-O – Campieri basal medium with 12 mM oxalate. CAMP-G – Campieri basal medium with 1% glucose. CAMP-GO – Campieri basal medium with 1% glucose + 12 mM oxalate.

The results from the oxalate degradation assays showed that *E. coli*-OX degraded oxalate in CAMP-O and CAMP-GO media both aerobically and anaerobically (Table 2.4). Oxalate degradation in CAMP-GO was subject to glucose repression, indicated by the reduced levels in CAMP-GO (Table 2.4).

Table 2.4. Oxalate-degradation by *E. coli*-OX in CAMP-substituted media.

Growth Medium	Growth conditions	% Oxalate degraded per OD unit of growth[a]
CAMP-O	Aerobic	11.8 ± 0.99
CAMP-GO	Aerobic	5.47 ± 0.99
CAMP-O	Anaerobic	13.5 ± 0.94
CAMP-GO	Anaerobic	2.17 ± 0.98

[a]Reported results represent duplicate samples of two independent experiments ± SD.

Although *E. coli*-OX grew much better aerobically than it did anaerobically (Figure 2.2), the levels of oxalate degradation under the two growth conditions were approximately the same when results were standardized per OD unit of growth (Table 2.4). There is no published data on oxalate-degrading *E. coli* strains. However, an oxalate-degrading gene ortholog (*frc*, discussed in section 1.5.1) from *E. coli* has been cloned and its protein structurally characterised (Gruez *et al.*, 2003. This gene has been confirmed as an acyl CoA transferase that is able to utilise oxalate as a substrate.

When *E. coli*-OX was subcultured on rich media (without oxalate) and subjected to growth studies in oxalate-substituted CAMP and minimal media, the isolate lost its ability to degrade oxalate aerobically and anaerobically (results not shown). Subculturing on Medium A agar produced smaller colonies than those obtained from freshly isolated *E. coli*-OX, but the oxalate-degrading phenotype was not recovered. As mentioned in sections 1.5.2 and 1.5.3, the reported

oxalate-degrading *E. faecalis* and *P. rettgeri* strains required a poor nutritional environment and repeated sub-culturing on oxalate-containing media to maintain their oxalate-degrading capabilities (Hokama *et al.*, 2000). These results suggested that the oxalate metabolism systems in the three facultative anaerobes were inducible systems.

E. coli DH5α and JM109 laboratory strains were not able to grow on Medium A agar aerobically, but weak growth was observed anaerobically after forty-eight-hour incubation. The colonies of these *E. coli* strains were, however, much smaller than those of freshly isolated *E. coli*-OX after twenty-four-hour incubation (either aerobically or anaerobically). This suggested that the oxalate degradation pathway in the DH5α and JM109 strains was possibly only weakly expressed under anaerobic conditions. *E. coli* DH5α and JM109 strains are laboratory strains that are traditionally propagated on rich non-oxalate-containing media and may not be expressing optimal oxalate-degrading ability.

2.4.9. Growth in oxalate media and oxalate-degrading capacity of *C. innocuum*-OX.

The *C. innocuum*-OX isolate was unable to grow in CAMP-substituted media; therefore, its growth was tested in Medium A broth. Uninoculated Medium A was used as the experimental control. Figure 2.3 shows that the isolate's growth in 12 mM ammonium oxalate was minimal, reaching a final OD_{600nm} reading of approximately 0.12 after four days. Growth in 18 mM ammonium oxalate was inhibited further (Figure 2.3), showing that oxalate was toxic to the bacterial cells.

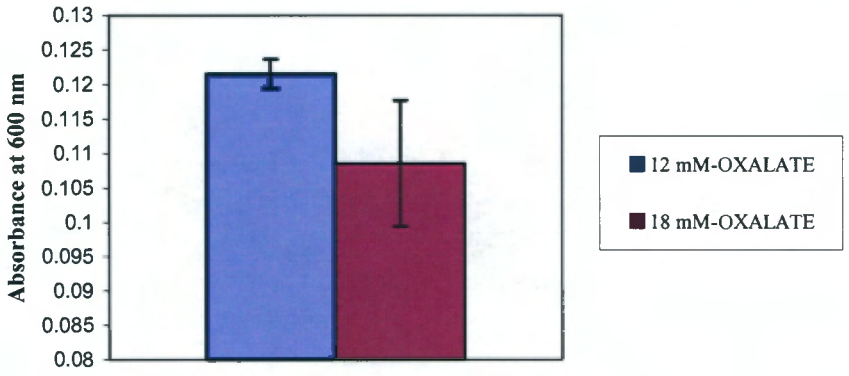


Figure 2.3. Anaerobic growth of *C. innocuum*-OX in Medium A broth after four days. Results shown are the means of duplicate samples from two independent experiments, and error bars reflect SD values.

Poor growth in Medium A was not an unexpected result since Medium A is a stringent oxalate-enriched medium (20mM sodium oxalate) containing 2 mM sodium acetate and 0.05% yeast as the only other alternative carbon sources. CAMP basal medium, on the other hand, contains 1% yeast and 2% protease peptone in addition to 2 mM sodium acetate as alternative carbon sources. The modified $CaCl_2$ assay used in this study was unable to detect any oxalate-degrading activity when *C. innocuum*-OX was grown in Medium A containing either 12- or 18 mM ammonium oxalate. However, undetectable oxalate degradation was not an unexpected result as the level of growth recorded (Figure 2.3), was very low. The OD_{600nm} readings, which followed calcium precipitation of residual oxalate in the culture broths, did not represent a very sensitive detection method; and were consequently not expected to detect slight decreases in oxalate concentrations.

There is no published data reporting oxalate utilisation by *C. innocuum*, however, oxalate-dependent growth of soil-dwelling *Clostridium oxalicum* (Dehning & Schink, 1989) and a *Clostridium thermoaceticum* strain originally isolated from horse manure, (Daniel & Drake, 1993) have been reported. When the ninety-six-hour growth of *C. innocuum*-OX in 12 mM-

oxalate Medium A is compared to that obtained for *C. thermoaceticum* after sixty hours of growth in an equally stringent 15 mM-oxalate medium (Daniel & Drake, 1993), the maximum levels of growth attained of both species are comparable. The growth of the *C. innocuum* isolate reached a maximum absorbance at 600 nm of 0.12 while the *C. thermoaceticum* reached a maximum absorbance at 660 nm of 0.175, declining gradually thereafter. Daniel & Drake (1993) used a highly sensitive radiolabelled oxalate assay, with ^{14}C product detection, to confirm oxalate degradation by *C. thermoaceticum* despite its poor growth. Oxalate metabolism in *Clostridium* species is reportedly characterised by low cell biomass yields (Daniel & Drake, 1993; Dehning & Schink, 1989). The oxalate-degrading ability of *C. innocuum* could not be proven in this study. Nonetheless, its comparable growth to *C. thermoaceticum* under similar growth conditions suggested that a more sensitive oxalate detection assay should be employed to detect possible oxalate-degrading activity.

2.5. CONCLUSIONS

South African statistics show that kidney stones occur very rarely amongst black South Africans, despite them having a diet that puts them at a high risk of developing kidney stones (Whalley *et al.*, 1998; Viljoen & Gericke, 2001; Lewandowski, 2003). Lewandowski (2003) suggested that oxalate-degrading bacteria may be responsible for the low incidence of kidney stone disease in black South Africans; and reported higher numbers of culturable oxalate-degrading species in the faecal microbiotas of the black population relative to the white population. As mentioned earlier, numerous studies in various parts of the world have reported a higher incidence of *O. formigenes* in stone-free populations (Goldkin *et al.*, 1986; Sidhu *et al.*, 1997b; Han *et al.*, 1985;

Kleinschmidt *et al.*, 1993; Kumar *et al.*, 2002); however, the role of *O. formigenes* in the incidence of kidney stone disease in black and white South African populations has not been previously described. The present study gives the first account of the possible role of *O. formigenes* in the relative incidence of kidney stone disease in black and white South African male populations.

The absolute dependency of *O. formigenes* on oxalate as a carbon source has attracted great interest in its role in oxalate homeostasis in humans (Sidhu et al., 1998; Sidhu et al., 1999a; Duncan *et al.*, 2002; Stewart *et al.*, 2004. *O. formigenes* is difficult to culture using standard laboratory techniques (Sidhu *et al.*, 1997b), but the development of DNA-based methods for its detection in faecal microbiota has facilitated studies based on this bacterium (Sidhu *et al.*, 1997a; Sidhu *et al.*, 1999a). In the present study, *O. formigenes* was detected in the faecal microbiotas of 70% of the black test subjects; but only 30% of the white subjects had detectable levels of this bacterium. These preliminary results suggested that *O. formigenes* could play a role in the lower incidence of kidney stone disease in black South Africans.

In adult humans, the use of antibiotics reportedly results in the rapid decolonization of *O. formigenes* from the gut (Sidhu *et al.*, 1998; Sidhu *et al.*, 1999b; Duncan *et al.*, 2002). In the present study, all the members of the test population had not consumed antibiotics at least six months prior to the collection of faecal samples. Consequently, antibiotic use was not regarded as a factor in the low incidence of *O. formigenes* in the white test subjects. Nonetheless, studies on the reestablishment of *O. formigenes* after antibiotic-induced decolonization have not been reported and so the possible effect of antibiotic use prior to the six-month exclusion period used in this study cannot be discounted entirely. Environmental and genetic factors have been

postulated to play a role in the colonization of *O. formigenes* in humans (Kumar et al., 2002); and these two factors could also play a role in the occurrence of *O. formigenes* in black and white South Africans.

The human gut contains a relatively stable, vast and genetically diverse microbiota, with lactic acid bacteria and bifidobacteria amongst its autochthonous species (Finegold *et al.*, 1983; Tannock, 1995). Increasing reports of oxalate-degrading lactic acid bacteria and bifidobacteria (Campieri *et al.*, 2001; Federici *et al.*, 2004; Lieske *et al.*, 2005) suggested a possible role for these bacteria in the homeostasis of oxalate in black and white South African populations investigated in the present study. The results obtained showed that culturable lactic acid bacteria and bifidobacteria in the black South African population generally had higher oxalate-degrading capacities than those of their white counterparts. The findings of this preliminary investigation suggested that oxalate-degrading lactic acid bacteria and bifidobacteria could play a role in the lower incidence of kidney stone disease in the black South African population. The sequencing results from DGGE analysis of the culturable lactic bacteria used in the oxalate-degradation studies further suggested that lactobacilli, in particular, contributed to the reported oxalate-degradation. However, the possible contribution of bifidobacteria to the reported oxalate-degradation cannot be discounted and warrants further investigation.

In the present study, the low incidence of kidney stone disease in the black South African population made the faecal microbiota of this population a potential reservoir of novel oxalate-degrading species. Investigations into the oxalate-degrading bacteria in a black test subject yielded one novel oxalate-degrading species and one putative one. This research reports the first

account of a 'generalist' oxalate-degrading *E. coli* isolate and a putative oxalate-degrading *C. innocuum* strain.

Clostridia are a major constituent of anaerobic intestinal flora and some species, including *C. botulinum* and *Clostridium perfringens*, are well known pathogens (Ackermann *et al.*, 2001). *C. innocuum* is part of the normal intestinal flora of human infants and adults and its pathogenicity has not been conclusively established (Holdeman *et al.*, 1977; Alexander *et al.*, 1995). There are a few isolated reports of infections caused by *C. innocuum*, including endocarditis (Cutrona *et al.*, 1995) and intra-abdominal sepsis (Mory *et al.*, 1998). In addition, *C. innocuum* is regularly isolated from clinical samples of patients with *Clostridium difficile* associated diarrhoea (Ackermann *et al.*, 2001). *C. innocuum*, however, does not produce any toxins and has subsequently been classified as a non-pathogenic species (Ackermann *et al.*, 2001; Holdemann *et al.*, 1977).

Clostridia have a general reported resistance to vancomycin that is less than 2 μg/ml minimum inhibitory concentration (MIC) (Mory *et al.*, 1998). The high level of resistance of *C. innocuum* to vancomycin and cephalosporins (Alexander *et al.*, 1995) could explain why it is often a clinical isolate since antibiotic therapy with these antibiotics would select for a *C. innocuum* population. Genetic investigations into the vancomycin resistance displayed by *C. innocuum* have shown the absence of defined enterococci vancomycin resistance genes, and so resistance must either be intrinsic or due to undefined genetic elements (Mory *et al.*, 1998). Popular opinion leans towards intrinsic resistance, as *C. innocuum* isolated from clinical samples is most often associated with *C. difficile* and other clostridial species that remain highly susceptible to

vancomycin. Although *C. innocuum* is classified as a non-pathogenic bacterium, its close phylogenetic relationship with GIT pathogens made it an unsuitable probiotic candidate in this study.

E. coli is the most abundant species of facultative anaerobes in human intestinal microbiota, occurring also in the normal microbiota of most warm-blooded animals (Holt *et al.*, 1994). This species comprises numerous strains, many of which are non-pathogenic; however, certain strains, such as *E. coli* 0157:H7, are recognised epidemiological pathogens. Nonetheless, *E. coli* Nissle 1917 has for many years enjoyed widespread use as a probiotic in the treatment and prevention of intestinal disorders (Boudeau *et al.*, 2003; Stentebjerg-Olesen *et al.*, 1999). Additional reports exist on the probiotic effects of administration of other non-pathogenic strains of *E. coli* (Lodinova-Zadnikova *et al.*, 2003).

The beneficial effects of probiotic *E. coli* strains are largely due to their excellent colonising ability of the GIT, which enables them to effectively displace, or prevent colonisation by, pathogenic bacteria (Boudeau *et al.*, 2003; Stentebjerg-Olesen *et al.*, 1999). In addition, immune-stimulating properties of *E. coli* strains have been reported (Lodinova-Zadnikova *et al.*, 2003). Consequently, some non-pathogenic strains of *E. coli* are administered as probiotics, despite harbouring a pathogenic strain within the species. Unlike *C. innocuum*-OX, *E. coli*-OX was not excluded as a probiotic candidate from this study on the basis of its close phylogenetic relationship with a known GIT pathogen. Instead, its loss of oxalate-degrading ability upon sub-culturing on non-oxalate-containing media made it unreliable for probiotic administration.

The identification of potentially novel oxalate-degrading strains was successful although both *E. coli*-OX and *C. innocuum*-OX were deemed unsuitable for further development as probiotic candidates in the management of kidney stone disease. Nonetheless, an analysis of the genes that could confer oxalate-degrading ability (actual and putative) on these species was undertaken and is reported in Chapter 3. In addition, the high oxalate-degrading capabilities noted in the lactobacilli-containing MRS pools impelled us to identify more suitable probiotic candidates, principally lactobacilli, using the alternative approach of bioinformatic analysis (Chapter 3).

CHAPTER 3

Cloning, Sequencing and Characterisation of Oxalate-Degrading Genes in Novel Oxalate-degrading Bacteria

Contents

3.1. SUMMARY

Molecular analyses of the *E. coli* CFT073 and partially assembled *L. gasseri* ATCC 33323 and *Lactobacillus reuteri* 100-23 genomes demonstrated the existence of clusters of genes encoding putative acyl-CoA transferases and thiamine pyrophosphate-requiring enzymes, possibly involved in oxalate degradation. The presence and arrangements of the gene clusters in *E. coli*-OX and *L. gasseri* DSM 20243^T were confirmed by PCR amplification and sequencing. The ability of *L. gasseri* DSM 20243^T to degrade oxalate was confirmed *in vitro*, and 5 mM oxalate was shown to be inhibitory to its growth. Transcriptional analysis by RNA hybridization of the putative *L. gasseri oxc* gene showed a slight induction of this gene by mildly acidic conditions (pH 5.5). Oxalate-dependent induction of the *L. gasseri oxc* gene was observed when *L. gasseri* cells, pre-adapted by exposure to sub-inhibitory concentrations of sodium oxalate (3.73 mM) at pH 5.5, were exposed to 37.3 mM sodium oxalate at pH 5.5. RT-PCR confirmed that the clustered putative *L. gasseri* DSM 20243^T *oxc* and *frc* genes are regulated as an operon, on a single mRNA transcript.

3.2. INTRODUCTION

Oxalobacter formigenes is the reference organism for oxalate metabolism in gut bacteria (Campieri *et al.*, 2001; Duncan *et al.*, 2002; Federici *et al.*, 2004; Azcarate-Peril *et al.*, 2006) and the three proteins involved in its catabolism of oxalate are OxlT (Abe *et al.*, 1996), formyl-coenzyme A transferase (Baetz & Allison, 1990) and oxalyl-coenzyme A decarboxylase (Baetz & Allison, 1989). These three proteins have been characterized and are routinely used in bioinformatic and molecular analyses of oxalate-degrading species (Allison *et al.*, 1985; Hokama *et al.*, 2000; Campieri *et al.*, 2001; Kodama *et al.*, 2002; Federici *et al.*, 2004).

3.2.1. Characterisation of OxlT in *O. formigenes*

OxlT, the oxalate:formate antiport protein, is a hydrophobic single integral membrane polypeptide of 418 predicted residues, with an approximate size of 38 kDa as determined by SDS PAGE (Ruan *et al.*, 1992). The size of OxlT is comparable to that of other secondary bacterial carrier proteins and amino acid sequencing has shown that OxlT conforms to the general pattern of most membrane carriers, including the presence of twelve hydrophobic probable transmembrane segments (Abe *et al.*, 1996). The orientation of this protein is such that the N and C termini are located within the cytoplasm. OxlT represents approximately 5 – 10% of *O. formigenes* inner membrane proteins (Ruan *et al.*, 1992).

The non-coding DNA promoter sequence upstream of the *oxlT* gene indicates that transcription of the OxlT protein is typical of well-established patterns in bacterial systems (Abet *et al.*, 1996). A 29 bp stretch, approximately 70 nucleotides upstream of the initiating AUG codon, contains putative bacterial –35 and –10 promoter sequences, of TTGAAA and TTCAAT, respectively. It has been postulated that transcriptional termination is mediated by

a 31 bp stem-loop structure that begins 72 nucleotides from the first of two in-frame stop codons (UAA). The *O. formigenes oxlT* gene is highly specialized and to date has not been characterized in other bacterial species. Bacteria in which putative oxalate:formate antiporters were identified by bioinformatic analysis in this study included: *Halobacterium* spp. NRC-1 (NP_279295), *Haloquadratum walsbyi* (CA_J51615) and *Streptococcus thermophilus* CNRZ 1066 (YP_141801).

3.2.2. Characterisation of Frc in *O. formigenes*

Frc catalyses the CoA activation of oxalate prior to its decarboxylation to formate (Baetz & Allison, 1990). By transferring the CoA moiety from formyl-CoA back to oxalate, Frc saves *O. formigenes* activation energy. The *O. formigenes* Frc does not act reversibly, unlike some acyl-CoA transferases, such as the one found in *Clostridium kluyveri* (Sly & Stadtman, 1963). A formyl coenzyme A transferase has also been isolated in the soil bacterium *Pseudomonas oxalaticus* where it uses either oxalate or succinate as acceptors for CoA (Quayle, 1962). The Frc of *O. formigenes* is a monomeric cytosolic protein with 428 deduced amino acids, and an SDS PAGE estimated size of 44 kDa. Frc makes up 0.2% of the soluble protein in *O. formigenes* (Baetz & Allison, 1990).

Sequencing of the *O. formigenes frc* gene has revealed a single open reading frame, (ORF), beginning with an expected methionine codon (Sidhu *et al.,* 1997c). This ORF extends for 1,284 bp ending with a TGA stop codon. A putative Shine-Dalgarno sequence (Shine & Dalgarno, 1974), 5' –GAAATG, was identified 8 bp upstream of the start codon. Located in the regions –45 to –40 bp and –77 to –70 bp are a putative TATA box with sequence 5'-GAATAA, and an RNA polymerase binding site, respectively (Sidhu *et al.*, 1997c). Downstream of the TGA stop codon (position 1284), in the regions 1311 to 1319 bps and

1324 to 1331 bps, is an 8 bp inverted repeat that precedes a 7 bp thymidine stretch. This arrangement is suggestive of a ρ-independent termination sequence, as observed with the *oxlT* gene.

3.2.3. Characterisation of Oxc in *O. formigenes*

The oxalate decarboxylase protein, Oxc, is a predicted 568 amino acid cytosolic protein (Baetz & Allison, 1989). In some bacteria, acyl-CoA decarboxylases are intimately associated with the cytoplasmic membrane because their decarboxylation activity is coupled to the formation of a sodium gradient. These decarboxylases act as pumps for translocation of sodium ions across the membrane (Dimroth, 1982). In *O. formigenes*, however, the cytosolic Oxc decarboxylation reaction is coupled to the anion exchange activity of membrane-bound OxlT for formation of a pmf, as described in Chapter 1. Native protein migration experiments using gradient PAGE coupled with SDS PAGE analyses showed that the functional Oxc enzyme is a tetramer of identical 65 kDa subunits (Baetz & Allison, 1989). Oxc makes up approximately 10% of the soluble protein of the *O. formigenes* bacterial cell, and together with Frc 20% of the total protein of the cell; showing the importance of these proteins, and oxalate degradation, to this bacterium.

Sequencing of the *oxc* gene has shown a 1,704 bp single ORF, beginning with an initiating methionine residue (ATG), and ending with the stop codon TAA at position 1705 (Lung *et al.*, 1994). A putative Shine-Dalgarno sequence, 5'-GAAAGG, is located approximately 10 bp upstream of the initiating methionine (positions –14 and –9). Putative –10 TATA (5'-TTATCA) and –35 Pribnow boxes (5'-TTATCA), occur in the -32 to -27, and -50 to -45 regions, respectively. The putative promoter sequences suggest that transcription is controlled by a σ^{70} mediated mechanism; however, absolute identification of the *oxc* promoter is yet to

be reported. The in-frame TAA stop codon precedes multiple in-frame stop codons and a 12 bp perfect-inverted repeat located between positions 1758 to 1769 and 1772 to 1783. Seven thymidine residues follow this inverted repeat, and this arrangement again suggests a ρ-independent termination sequence, as noted for the *oxlT* and *frc* genes (Baetz & Allison, 1989; Lung *et al.*, 1994). The *O. formigenes oxc* gene was the first gene involved in oxalate degradation to be sequenced, and it has been used repeatedly in the PCR-amplification method for identification and/or quantification of oxalate-degrading bacteria (Allison *et al.*, 1985; Sidhu *et al.*, 1997a; Kodama *et al.*, 2002; Kumar *et al.*, 2002).

3.2.4. Regulation of oxalate-degrading genes in *O. formigenes*

The earlier-described mechanism of oxalate degradation by *O. formigenes* clearly requires coordinated control of the *oxlT*, *frc* and *oxc* genes. Genetic analyses, however, have shown that these genes are not part of a polycistronic operon, and all three genes contain independent promoter and ρ-independent termination sequences (Sidhu *et al.*, 1997c). Restriction mapping experiments of the *frc* and *oxc* genes have shown that these genes are at least 5 kb apart on the bacterial chromosome. Further experiments showed that the *oxc* and *frc* genes may not be part of a regulon since repeat elements that are upstream of the *oxc* gene promoter region are absent from the *frc* gene. The exact regulation of these genes has not been reported.

3.2.5. Bioinformatics and *in silico* genome screening

Over the last few decades, advancements in molecular biology have led to the rapid sequencing of genomes, in whole or in part, of a wide variety of organisms, particularly bacterial taxa. The great majority of these sequences are stored in electronic (computer-based) open source sequence databases, including Genbank and EMBL. In bioinformatic

analysis of genetic sequences, one of the approaches taken is the use of computational search and alignment techniques to compare a new genome against an existing set of known genes (Bansal, 2005). Thereafter, sequence identity and/or similarity between the known genes and the unknown genome are used to annotate the structure, and predict the function, of genes in the newly sequenced genome. It is this approach that was primarily employed in the work outlined in this chapter.

The work presented in this chapter is two-fold. Firstly, it describes the attempts at identifying *O. formigenes oxc* and *frc* orthologs in the novel oxalate degrading isolates reported in Chapter 2. Secondly, it describes the use of the *O. formigenes* oxalate degrading genes, particularly *oxc*, in the bioinformatic identification of a novel oxalate-degrading *Lactobacillus* species. Transcriptional characterisation of the oxalate-degrading genes in this *Lactobacillus* species is also reported.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial strains, plasmids and culture conditions

All strains were propagated anaerobically as described in section 2.3.1. *Lactobacillus gasseri* DSM 20243^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) culture collection, and was routinely grown anaerobically on MRS agar (Biolab), supplemented with 0.05% (w/v) cysteine-HCl. The *E. coli* strains, DH5α and JM109, used in the cloning experiments, were grown aerobically in Luria-Bertani (LB) broth or 1.5% (w/v) agar (Sambrook *et al.*, 1989) at 37°C. The plasmids used in cloning experiments were the M13-derived plasmid pBluescript SK (pSK) (Stratagene, La Jolla,

California, USA) and pGEMTeasy vector (Promega, Southampton, UK). All positive clones were cultured on LB agar supplemented with 100 μg/ml ampicillin.

3.3.2. Bacterial chromosomal DNA extraction

Bacterial cells were grown to late exponential phase and chromosomal DNA was extracted using the High Pure PCR Template Preparation Kit (Roche). DNA was eluted in water.

3.3.3. General recombinant DNA procedures

All DNA procedures and manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). Competent *E. coli* DH5α and JM109 cells were prepared using the rubidium chloride method (Armitage *et al.*, 1988) and plasmid DNA was isolated using the alkali lysis method of Ish-Horowicz & Burke (1981). Restriction enzymes and T4 ligase were used according to the manufacturer's instructions (Roche). Gel electrophoresis was conducted in 0.8% w/v agarose gels in Tris-Acetate-EDTA buffer as previously described (Sambrook *et al.*, 1989).

3.3.4. PCR amplification of *oxc* and *frc* orthologs from *E. coli*-OX

Microbial genomes of *E. coli* CFT073 (NC_004431), *E. coli* K12 (NC_000913) and *E. coli* 0157:H7 (NC_002695) and the *O. formigenes oxc* sequence (AAA53683) were obtained from NCBI (www.NCBI.nlm.nih.gov) and used in bioinformatics analysis. A set of oligonucleotide primers, based on the *E. coli* CFT073 (NC_004431) sequence, was designed to amplify the entire *oxc* ortholog in *E. coli*-OX. The forward primer, designated ECOxc-F, was 5 ′ GAA TTC GGA TAT TAA AGC TGC GCC TC 3 ′ and contained a *Hin*dIII restriction site, the reverse primer, designated ECOxc-R, was 5 ′ CCC AAG CTT AAG GGT CAG ACG AGT GTT CCG 3 ′ and contained an *Eco*RI restriction site. The PCR

amplification reactions and purification of PCR amplification products were performed as described in section 2.3.2. The annealing temperature for the *oxc* PCR amplifications was 68°C. The primers designed to amplify the *frc* ortholog were: forward primer, ECFrc-F (5′ CCC AAG CTT ATC GAG TGC CGC TAT GAC G 3′), located within the 5′ end of the *oxc* ortholog; and reverse primer, ECFrc-R (5′ GGG AAT TCA ACG AAT GCC TTC CGC CTG 3′), located in the intergenic region downstream of the *frc* ortholog. This PCR amplification had an annealing temperature of 42.1°C.

3.3.5. PCR amplification of an *oxc* ortholog in *Clostridium innocuum*

Multiple sequence alignments of the *oxc* gene of *O. formigenes* and its orthologs in *E. coli* strains K12 and CFT073 were performed at the amino acid level using the DNAMAN software package (Lynnon Corporation, Quebec, Canada) to identify highly conserved regions within the Oxc protein. Thereafter, a set of oligonucleotide primers was designed based on the identified highly conserved regions. These primers were optimised using a general *Clostridium* sp. codon preference table, compiled on the basis of described codon preferences of three *Clostridium* species: *Clostridium perfringes* (NC_003366), *Clostridium acetobutylicum* (NC_003030) and *Clostridium tetani* (NC_004557) using Glasgow University Bioinformatics resources (http://doolittle.ibls.gla.ac.uk). The primers designed, designated ClOxc-F and ClOxc-R, were located approximately 225 and 1,380 bp downstream of the 5 ′ end of the *O. formigenes oxc* gene, respectively. Primers were: ClOxc-F 5′ TTT AAC TGT ATC TGC TCC 3 ′ and ClOxc-R 5′ TTC CAT TCC AGA AAA TCC 3′. PCR amplification reactions and purification of PCR products were performed as described for *E. coli*-OX. The PCR amplification had an annealing temperature of 42.5°C.

3.3.6. *In silico* identification of putative oxalate-degrading lactobacilli

Complete and partially assembled genomes of lactobacilli were obtained from the NCBI Entrez Genome (http://www.ncbi.nlm.nih.gov), and screened *in silico* for the presence of *O. formigenes oxc* and *frc* orthologs. *Lactobacillus acidophilus* NCFM (NC_006814), *Lactobacillus gasseri* ATCC 33323^T (NZ_AAAO00000000), *Lactobacillus johnsonii* NCC553 (NC_005362), *Lactobacillus plantarum* WCFS1 (NC_004567) and *Lactobacillus reuteri* 100-23 (NZ_AAOV00000000) genomes were analysed.

3.3.7. PCR amplification of *oxc* and *frc* orthologs from *Lactobacillus gasseri* DSM 20243^T

Sequences of the partially sequenced and assembled genome of *L. gasseri* ATCC 33323^T (NZ_AAAO00000000), synonym *L. gasseri* DSM 20243^T, were obtained from NCBI, as described in 3.3.6, and used in bioinformatic analysis. DNA was extracted from the *L. gasseri* type strain using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). PCR amplifications were used to confirm the presence and arrangement of *frc* and *oxc* orthologs in the *L. gasseri* type strain. Primers used to amplify the entire *oxc* ortholog were LGOxc-F (5′ TAC ATG ATG GGC CTG ATT GG 3′) and LGOxc-R (5′ ATC CGC GGC CTT AGA AAG 3′). In order to confirm the positions of the *frc* and *oxc* orthologs in relation to each other, primers were designed flanking the intergenic region of these two genes ; LGOF-F 5′GCT TGA TCC AAC TGG TCG CT 3′, was located within the *frc* ortholog and LGOF-R 5′ GCT GAT CCT GGT GCA ATG TTT TC 3′ was located within *oxc* the ortholog (Figure 3.11). PCR amplification reactions and cycles were as described for *E. coli*-OX. The annealing temperatures for the *oxc* and *frc-oxc* fragment PCR amplifications were 51°C and 53°C, respectively.

3.3.8. Nucleotide sequencing and phylogenetic analysis

DNA sequencing was performed and analysed as described in section 2.3.2. Protein sequences obtained from the Entrez Protein Database at NCBI were aligned and used to generate unrooted phylogram trees using the neighbour-joining method (Clustal X and Mega2.1 software). Conserved domains in the putative proteins encoded by the identified *oxc* and *frc* orthologs were inferred using the Clusters of Orthologous Groups (COG) of Proteins from the NCBI database (http://www.ncbi.nlm.nih.gov/COG/).

3.3.9. Growth in oxalate media and *in vitro* test for oxalate-degrading capacity of *L. gasseri* DSM 20243^T

The protocol of Federici *et al.* (2004), used to determine the oxalate-degrading capacity of the probiotic bacterium *B. lactis* DSM 10140, was used to determine the *in vitro* oxalate-degrading capacity of *L. gasseri* DSM 20243^T. Briefly, the *L. gasseri* type strain was grown anaerobically for five days in triplicate 5 ml MRS broth cultures supplemented with 0.05% (w/v) cysteine-HCl and 5 mM sodium oxalate (MRS-OX). Uninoculated MRS-OX was used as the experimental control. Residual oxalate in *L. gasseri* culture and control broths was measured using an oxalate enzymatic kit assay (Sigma, Poole, Dorset, UK), with sample inactivation and purification steps as previously described (Federici *et al.*, 2004).

Thereafter, growth curves were constructed to determine the effects of 5 mM oxalate on the growth of the *L. gasseri* type strain. *L. gasseri* was grown anaerobically for five days in duplicate 5 ml MRS and MRS-OX broth cultures. Following incubation, absorbance readings of the broth cultures, at 600 nm, were used to construct growth curves. Residual oxalate in the MRS-OX broth cultures, at various time points of the growth curves, was measured as described earlier.

3.3.10. RNA Extraction

The Azcarate-Peril *et al.* (2006) protocol, used to study expression of oxalate-degrading genes in *L. acidophilus* NCFM, was used to investigate the expression of the putative *L. gasseri* DSM 20243^T oxalate-degrading genes in this study. The *L. gasseri* type strain was grown anaerobically in MRS broth for sixteen hours and then transferred twice to MRS broth containing 3.73 mM sodium oxalate (pH 5.5, acidified with lactic acid) for sixteen hours, as described by Azcarate-Peril *et al.* (2006). Thereafter, cells were transferred to (i) MRS broth (pH 5.5) and (ii) MRS broth containing 37.3 mM sodium oxalate (pH 5.5). Following incubation at 37°C, samples were collected at time 2, 4 and 6 hours (MRS); and 4, 6 and 9 hours (MRS + 37.3 mM sodium oxalate), representing early-, mid- and late-log growth samples, respectively. Total RNA was isolated according to the method of Aiba *et al.*, (1981), and the quality of the isolated RNA was confirmed by electrophoresis in 1.5% denaturing formaldehyde agarose gel (Fourney *et al.*, 1988). RNA was quantified using the NanoDrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc).

3.3.11. RNA dot blots

For RNA dot blots, a 249 bp internal fragment of the *L. gasseri oxc* ortholog was used as the experimental probe, and a *L. gasseri* 16S rRNA gene probe was constructed for use as the internal control. The primers used in construction of these probes, and their appropriate PCR amplification annealing temperatures are shown in Table 3.1. The PCR products were purified as earlier-mentioned. Hybridisation and detection of DIG-labelled DNA probes were performed according to the manufacturer's instructions, and chemiluminescent signals were detected using CSPD® (Roche). RNA dot blots were performed in duplicate and equal amounts of RNA, suspended in equal volumes, extracted from *L. gasseri* cells grown in MRS

and MRS + 37.3 mM sodium oxalate were spotted onto nylon membranes (Roche). Hybridisation intensities were measured using a densitometer (Syngene Gel Documentation System), and processed as TIFF images. The *oxc* mRNA autoradiograph signal densities were analysed relative to their corresponding 16S rRNA signals.

Table 3.1. Oligonucleotides used to amplify internal fragments of the *L. gasseri oxc* and 16S rRNA genes

Target gene	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	T (°C)[a]	Fragment size (bp)
oxc	TCGTCATCTGGGACAAGACCT	TCAATGATGACCTTGGCGAA	62	250
16S rRNA	GCCAGCAGCCGCGGTAATAC	CACGAGCTGACGACAICCATGC	55	500

[a]PCR annealing temperature

3.3.12. Reverse transcriptase PCR

RNA (1 μg) was obtained from *L. gasseri* type strain cells grown in MRS supplemented with 37.3 mM sodium oxalate (pH 5.5). The set of primers designed to confirm the positions of the *oxc* and *frc* orthologs in relation to each other, described in section 3.3.7, were used to amplify the intergenic region between the putative *oxc* and *frc* genes in *L. gasseri* DSM 20243^T. The forward primer, LGOF-F, and reverse primer, LGOF-R, were located within the *oxc* and *frc* genes, respectively. The cDNA was synthesized by reverse transcriptase PCR (RT-PCR) from RNA using the LGOF-R oligonucleotide, and the Omniscript® Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. The resultant cDNA was then amplified with the LGOF-F and LGOF-R primers to obtain a fragment containing the intergenic region.

3.4. RESULTS AND DISCUSSION

3.4.1. PCR amplification of *oxc* and *frc* orthologs in *E. coli*-OX

In silico analysis of the *E. coli* genomes available in the NCBI database revealed a highly conserved region, comprising four genes, which contained both *oxc* and *frc* orthologs. In *E. coli* CFT073, the *oxc* ortholog, *c2909*, is annotated as a probable oxalyl-CoA decarboxylase (Figure 3.1).

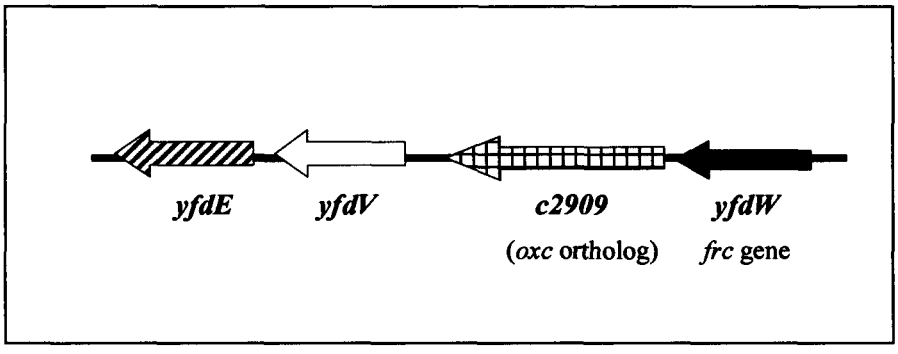


Figure 3.1. Putative oxalate-degrading genes cluster in *E. coli* CFT073 (NC_004431). The arrangement of the *oxc* and *frc* orthologs, *c2909* and *yfdW* respectively, and putative membrane proteins was identical in all the *E. coli* strains analysed. The individual *frc* and *oxc* orthologs in the *E. coli* strains were 99.37% identical at the nucleotide level, but 100% identical at the amino acid level.

The *c2909* deduced amino acid sequence showed a conserved domain, COG0028, present in all TPP- requiring enzymes, as well as the cd02004 conserved domain of the TPP_BZL_OCoD_HPCL subfamily, a TPP-binding module comprising proteins similar to benzaldehyde lyase (BZL), oxalyl-CoA decarboxylase (OCoD) and 2-hydroxyphytanoyl-CoA lyase (2-HPCL). Oxalyl-CoA decarboxylases are TPP-requiring enzymes with a conserved TPP motif within their sequences (Baetz & Allison, 1989; Federici *et al.*, 2004). Both the COG0028 and cd02004 conserved domains, identified in the *c2909* predicted gene product,

are present in the functional *O. formigenes* Oxc protein. The predicted *E. coli c2909* gene product had 53% identity to its *O. formigenes* counterpart and is, therefore, the putative *E. coli oxc* gene.

Located about 50 bp downstream of *c2909* is an *frc* ortholog, *yfdW*, which has been characterised as a functional acyl-CoA transferase that is able to activate oxalate, forming oxalyl-CoA (Gruez *et al.*, 2003). The *yfdW* deduced amino acid sequence has 61% identity to its *O. formigenes* counterpart. Approximately 35 bp upstream of *c2909* is a putative membrane transport protein, *yfdV*, whose deduced amino acid sequence harbours the COG0679 conserved domain, common to all predicted permeases; as well as the pfam03547 domain, which occurs universally in auxin efflux carriers. The *yfdV* gene product could possibly be involved in the transport of oxalate into the bacterial cell, although it has no significant identity to the *O. formigenes* OxlT protein. Interestingly, upstream of *yfdV* (35 bp) is *yfdE*, a predicted bile acid inducible operon protein that harbours COG1804, a conserved domain found in acyl-CoA transferases/carnitine dehydratases, typically involved in energy production and conversion. Unlike the acyl-CoA transferase encoded by *yfdW*, the *yfdE* gene product has not been functionally characterised.

The expected DNA fragment sizes of approximately 2 kb (*oxc*) and 1.6 kb (*oxc-frc*) were obtained during PCR amplifications of the genes from the *E. coli*-OX strain isolated in this study (Figure 3.2). These were verified by DNA sequencing. The arrangement of the oxalate-degrading genes, *oxc* and *frc*, in *E. coli*-OX is, therefore, identical to that found in *E. coli* CFT073.

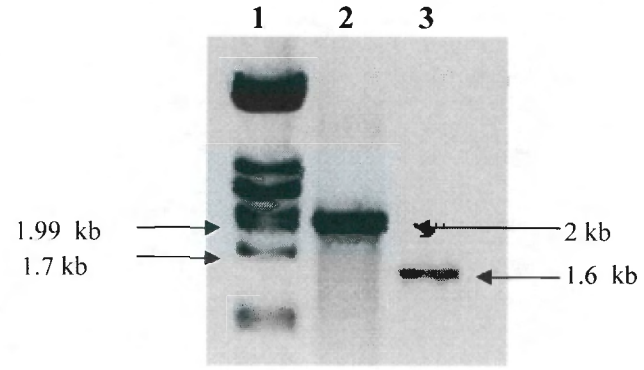


Figure 3.2. Agarose–gel electrophoresis of *E. coli*-OX *oxc* and *frc* PCR amplification products. Lane 1, λ DNA digested with *Pst*I. Lane 2, *oxc* PCR amplification product. Lane 3, *oxc-frc* PCR amplification product.

The presence of *oxc* and *frc* orthologs in *E. coli*-OX suggested that this bacterium might follow a similar oxalate degradation mechanism as seen in *O. formigenes* (outlined in section 1.5.1). The clustering of the *E. coli oxc* and *frc* orthologs suggested the possible existence of an oxalate operon in *E. coli*, in which oxalate utilisation genes are co-regulated by a single promoter. The 35 – 50 bp spaces between the individual genes, however, do not rule out the possibility that these genes are co-regulated as independent cistrons from their own promoters. The loss of oxalate-degrading activity for the *E. coli*-OX isolate, reported in section 2.4.6, made any attempts at transcriptional characterisation of these putative oxalate-degrading genes impossible.

3.4.2. PCR amplification of an *oxc* ortholog in *C. innocuum*

All attempts to isolate an *oxc* ortholog from the *C. innocuum* isolate using optimised degenerate primers were not successful. The PCR amplifications were characterised by non-specific DNA amplifications, and varying PCR amplification parameters, including annealing temperature and $MgCl_2$ concentration, did not improve the results obtained (Figure 3.3).

The expected size of the *oxc* ortholog PCR amplification product was approximately 1.2 kb. A DNA fragment of this size was obtained (Fig. 3.3, Lanes 2 and 3), however, a fragment of similar size was obtained in the CIOxc-F primer-only PCR amplification control (Lane 4). This result showed that CIOxc-F alone amplified a 1.2 kb product. The 1.2 kb DNA fragment in lane 2 was purified and used as DNA template in a subsequent PCR amplification. The annealing temperature was increased to a temperature that did not produce any product in the primer only control. The PCR amplification at the elevated temperature was successful and the 1.2 kb DNA fragment was purified. Sequencing of this product, however, was not successful as the sample contained mixed products.

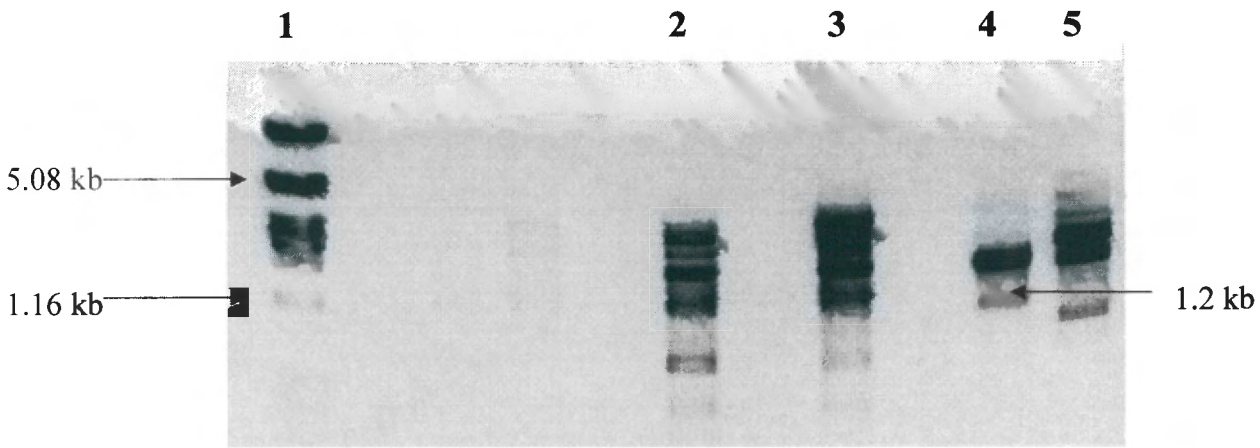


Figure 3.3. Agarose-gel electrophoresis of the *C. innocuum oxc* PCR amplification products using various parameters. Lane 1, λ DNA digested with *Pst*I. Lane 2, 2.5mM $MgCl_2$. Lane 3, 2.0mM $MgCl_2$. Lane 4, 2.5mM $MgCl_2$ with CIOxc-F only primer control. Lane 5, 2.5mM $MgCl_2$ with CIOxc-R only primer control.

C. innocuum has a low mol% G+C of 43-44 (Holdeman *et al.*, 1977), which may have contributed to the non-specific binding of the designed primers. The guanidine and cytosine residues incorporated into the ends of the primers to enhance primer annealing may have exacerbated the non-specific binding (Figure 3.3). Additional primers were not designed as it

was decided that attempting to isolate an *oxc* ortholog in *C. innocuum* by PCR amplification, using degenerate primers based on the *oxc* gene, was not an ideal approach for this genus. In all described anaerobic pathways of oxalate degradation, the decarboxylation of oxalate is dependent on the activation of oxalate. However, the decarboxylation of the activated oxalate is not always by the action of an oxalayl-CoA decarboxylase enzyme (Daniel & Drake, 1993; Sahin, 2003). In the proposed pathways of oxalate degradation by *Clostridium thermoaceticum* (Daniel & Drake, 1993) and the soil-dwelling anaerobic bacterium *Clostridium oxalicum* (Dehning & Schink, 1990), the decarboxylation of activated oxalate is catalysed by water (4 Oxalyl-CoA + 4 H_20 $\longrightarrow$ 4 Formyl-CoA + 4 HCO_3^-). These two *Clostridium* species are, therefore, not expected to contain *oxc* orthologs. If *C. innocuum* utilises oxalate using a similar pathway, it could explain the failure of the *oxc* ortholog PCR amplifications.

3.4.3. *In silico* identification of putative oxalate-degrading lactobacilli

As discussed in Chapter 2, *E. coli* and *C. innocuum* are not ideal probiotic candidates in the management of kidney stone disease. GRAS lactobacilli, on the other hand, have enjoyed widespread use as probiotics for many decades and increasing reports of oxalate-degrading lactic acid bacteria (Campieri *et al.*, 2001; Federici *et al.*, 2004) suggested the existence of uncharacterised oxalate-degrading *Lactobacillus* species. This prompted the *in silico* screening of *Lactobacillus* genomes within this study, for identification of genes possibly involved in oxalate degradation. Lactobacilli bearing oxalate-degrading genes would then be characterised as probiotic candidates in the prophylactic management of kidney stone disease.

Campieri *et al.* (2001) failed to detect *frc* and *oxc* genes in their reported oxalate-degrading *L. acidophilus* strain, as described in Chapter 1. In this study, a search for orthologs of the

O. formigenes oxc and *frc* genes in *Lactobacillus* genomes revealed clusters of genes in *L. gasseri* and *L. reuteri* genomes, possibly encoding enzymes involved in oxalate degradation (Figure 3.4). More recently, Azcarate-Peril *et al.* (2006) have reported an oxalate-degrading *L. acidophilus* NCFM, with functional *oxc* and *frc* genes (Figure 3.4).

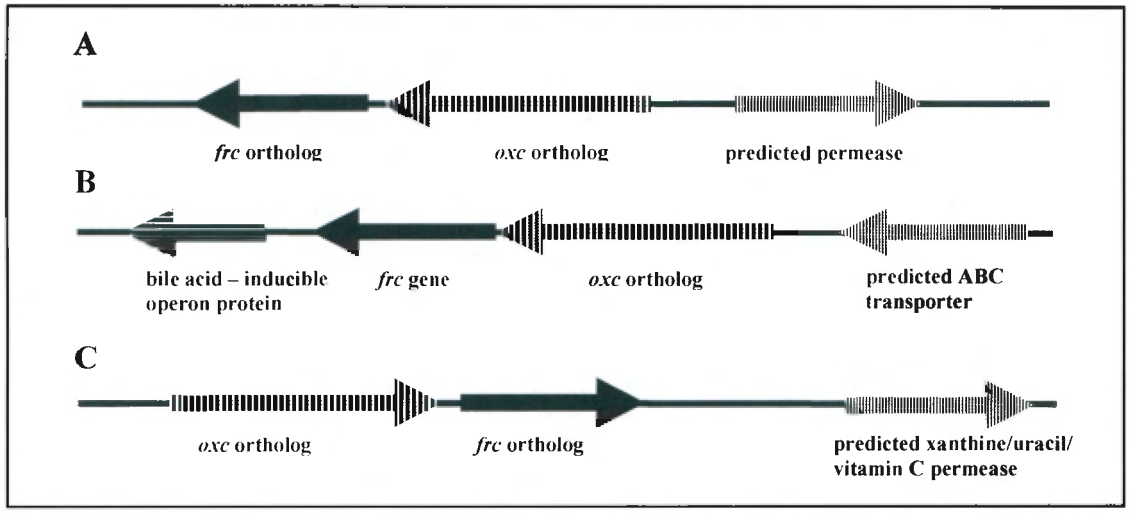


Figure 3.4. Gene clusters encoding putative oxalate-degrading enzymes in (A) *L. gasseri* (NZ_AAAO00000000), (B) *L. acidophilus* NCFM (NC_006814) and (C) *L. reuteri* (NZ_AAOV00000000). In (A), the *frc* and *oxc* orthologs had gene loci tags Lgas02001417 and Lgas02001418, respectively. In (B), the *frc* and *oxc* orthologs had gene loci tags LBA0395 and LBA0396, respectively. In (C), the *frc* and *oxc* orthologs had gene loci tags Lreu23DRAFT_0637 and LreuDRAFT_0636, respectively.

The putative oxalate-degrading genes in *L. gasseri* ATCC 33323, identified at the commencement of this study (2004), have recently been mentioned elsewhere (Azcarate-Peril *et al.*, 2006). Identification of the putative *L. reuteri* oxalate-degrading genes, however, is novel to this study. No putative oxalate-degrading genes were identified in *L. johnsonii* and *L. plantarum* genomes. *In silico* screening of *Lactobacillus* genomes thus identified *L. gasseri* and *L. reuteri* as potentially novel oxalate-degrading lactobacilli.

3.4.3.1. *In silico* analysis and PCR amplification of *frc* and *oxc* orthologs in *L. gasseri* DSM 20243^T

In *L. gasseri* ATCC 33323 (synonym *L. gasseri* DSM 20243^T) the 1358 bp *frc* ortholog encodes a putative 452-amino acid protein annotated as a predicted acyl-CoA transferase/carnitine hydrolase. This protein had 47, 84 and 53% predicted amino acid identity to the *O. formigenes* and *Lactobacillus acidophilus frc* genes, and the *E. coli* CFT073 acyl-CoA transferase, *yfdW*, respectively. A conserved domain (pfam0215), identified in a new family of CoA transferases (type III), including the *L. acidophilus* formyl-CoA transferase, was present in the predicted *L. gasseri frc* ortholog. Reported members of this CoA transferase family include oxalyl-CoA transferase, succinyl-CoA:(R)-benzylsuccinate CoA transferase and butyrobetainyl-CoA:(R)-carnitine CoA transferase (Heider, 2001).

The 1736 bp *oxc* ortholog, located 6 bp downstream of the *frc* ortholog (Figure 3.4), encoded a predicted 578-amino acid protein annotated as a thiamine pyrophosphate (TPP) -requiring enzyme, possibly belonging to the phosphonopyruvate decarboxylase, acetolactate synthase, pyruvate dehydrogenase or glyoxylate carboligase enzyme families. The TPP- requiring enzymes conserved domain, COG0028, as well as the cd02004-conserved domain, found in oxalyl-CoA decarboxylases, was present in the deduced *L. gasseri oxc* amino acid sequence. A typical ribosome binding site (GGAGG; 7 nucleotides from the start codon), similar to the *E. coli* Shine-Dalgarno sequence (Shine & Dalgarno, 1974), was located upstream of the *oxc* ortholog. In a similar manner, Azcarate-Peril *et al.* (2006) identified a typical ribosome binding site 7 nucleotides upstream of the ORF of the *L. acidophilus oxc* gene. The *L. gasseri oxc* ortholog had 49, 73, 49 and 50% predicted amino acid identity to the *O. formigenes, L. acidophilus* NCFM, *Bifidobacterium lactis* DSM 10140 and the putative *E. coli* CFT073 *oxc* genes, respectively.

Located 357 bp downstream of the *L. gasseri oxc* ortholog is a 1181 bp gene, which encodes a predicted permease transcribed in the opposite direction to the *frc* and *oxc* genes. This predicted permease is, therefore, not expected to be transcribed on the same mRNA transcript as the *frc* and *oxc* orthologs. The *L. gasseri* predicted permease had the COG0679 conserved domain common to bacterial permeases, as well as the auxin efflux carrier proteins pfam0347 domain, as noted for the predicted *yfdV* protein in *E. coli* CFT073. The deduced amino acid sequences of the *L. gasseri* and *E. coli* predicted permeases exhibited 53% identity, and the *L. gasseri* putative permease had negligible sequence identity to the *O. formigenes* oxalate:formate antiporter (<13%). In this study, *in silico* screening of the *L. gasseri* genome failed to identify a putative oxalate antiporter, in the same way as Azcarate-Peril *et al.* (2006) failed to identify an oxalate-specific permease in the *L. acidophilus* NCFM genome. The ability of non-dissociated forms of organic acids, such as oxalate, to diffuse through cytoplasmic membranes has been suggested as a possible reason for the inability to detect oxalate-specific transporters in lactobacilli (Azcarate-Peril *et al.*, 2006).

Nonetheless, the clustering of the putative *oxc* and *frc* genes (Figure 3.4) and high degree of deduced amino acid identity between the putative *L. gasseri* DSM 20243^T and *E. coli* and *O. formigenes frc* and *oxc* genes strongly suggested that the *L. gasseri* genes encoded functional proteins that would confer oxalate-degrading ability on this bacterium. PCR amplification of *L. gasseri* DSM 20243^T using primers designed to isolate the putative *oxc* gene gave the expected amplicon size of 2 kb (Figure 3.5). Primers designed to confirm the positions of the *oxc* and *frc* orthologs in relation to each other gave a fragment size of 586 bp. The results of this *frc-oxc* fragment PCR amplification are illustrated in Figure 3.12. DNA

sequencing results confirmed that the arrangement of the putative oxalate-degrading gene cluster in *L. gasseri* DSM 20243^T was identical to the lodged sequence on NCBI.

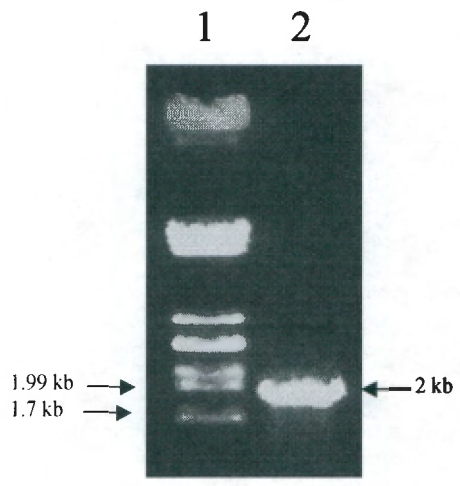


Figure 3.5. Agarose–gel electrophoresis of the *oxc* PCR amplification product from *L. gasseri*. Lane 1, λ DNA digested with *Pst*I. Lane 2, *oxc* PCR amplification product.

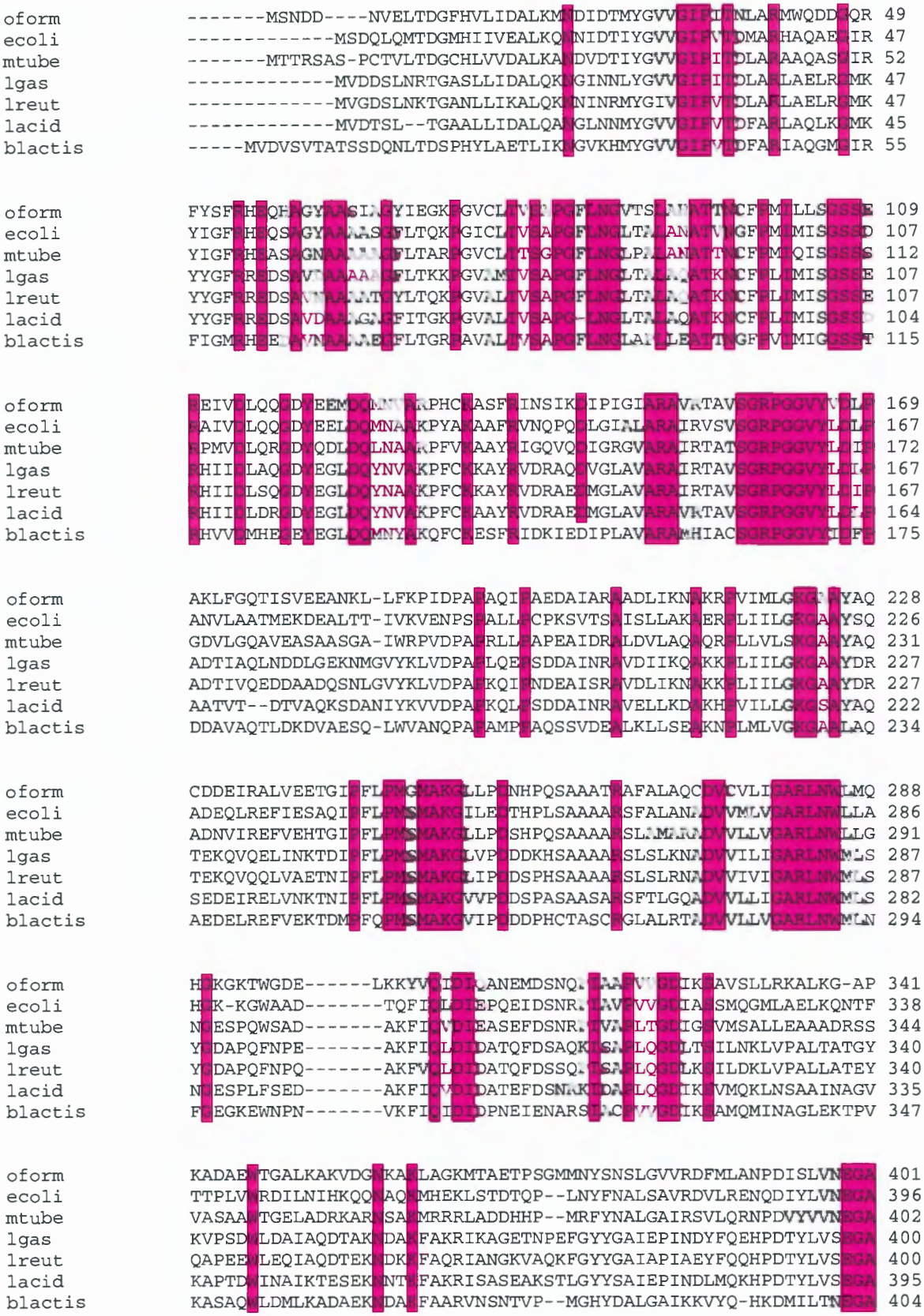
3.4.3.2. *In silico* analysis of *oxc* and *frc* orthologs in *L. reuteri* 100-23

The partially assembled *L. reuteri* genome was only recently released on NCBI (10th April, 2006). Therefore, it was only available for analysis in the latter stages of this study; and its analysis was consequently limited to *in silico* characterisation. The putative *oxc* ortholog in *L. reuteri* encodes a predicted 577-amino acid protein, annotated as a TPP-requiring enzyme (Figure 3.4). The cd02004 and COG0028 conserved domains, noted in the *E. coli* and *L. gasseri oxc* orthologs, were present in the predicted gene product of the *L. reuteri oxc* ortholog. The *L. reuteri*, *L. gasseri* and *L. acidophilus oxc* orthologs exhibited 82% identity at the amino acid level. Located 7 bp upstream of the *oxc* ortholog is an *frc* ortholog (Figure 3.4), annotated as a bile acid inducible carnitine dehydratase, which encodes a predicted 443-amino acid protein. This predicted protein had the COG1804 and CaIB domains found in acyl CoA-transferases, as well as the pfam02515 domain found in the CoA transferase family III described earlier. The deduced amino acid sequences of the *L. reuteri*, *L. gasseri* and

L. acidophilus frc orthologs had 88% identity. A distant 227 bp upstream of the *L. reuteri oxc* ortholog is a putative permease annotated as a xanthine/uracil/vitamin C permease:sulphate transporter. This permease had extremely low identity to the *L. gasseri* and *L. acidophilus* permeases, of 14 and 8%, respectively. Nevertheless, the high degree of deduced amino acid identity between the putative *L. reuteri* 100-23 and functional *L. acidophilus* NCFM *frc* and *oxc* genes again suggested that the *L. reuteri* genes encoded functional proteins that would confer oxalate-degrading ability on this bacterium.

3.4.3.3. Multiple alignment and phylogenetic relationships of the bacterial oxalyl-CoA decarboxylase enzyme, Oxc

The multiple-alignment of the deduced amino acid sequences of bacterial *oxc* genes and orthologs, constructed using CLUSTALX, exhibited overall sequence identity of 61% and identified several conserved regions within this enzyme (Figure 3.6). The Oxc proteins used in the alignment were from the intestinal isolates *O. formigenes* (AAA53683), *L. gasseri* (Lgas02001418), *L. reuteri* (LreuDRAFT_0636), *L. acidophilus* (LBA0396), *Bifidobacterium lactis* (AB163432), *Mycobacterium tuberculosis* (CAA17312), and *Escherichia coli* (AAN81359). Highly conserved regions were identified in the N-terminal binding, and central domains of the Oxc proteins, as well as in the TPP-binding site located in the C-terminal region (Figure 3.6). The TPP-binding domain site-specific amino acids were 100% conserved in all bacterial species (Figure 3.6).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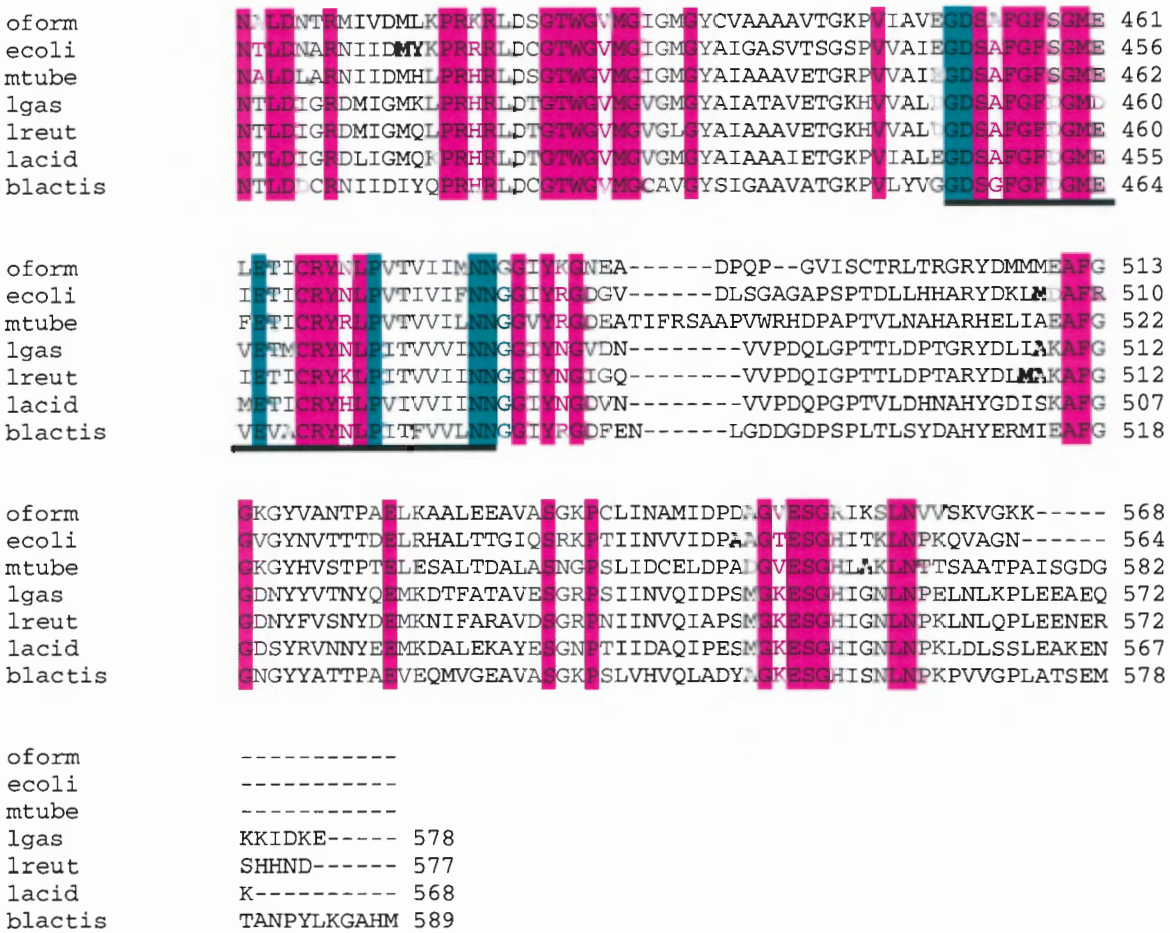

Figure 3.6. Multiple sequence alignment of bacterial Oxc predicted amino acid sequences. The organisms used were *Oxalobacter formigenes* (oform) (Accession number (AAA53683), *Lactobacillus gasseri* (lgas) (Lgas02001418), *Lactobacillus reuteri* (lreut) (LreuDRAFT_0636), *Lactobacillus acidophilus* (lacid) (LBA0396), *Bifidobacterium lactis* (blactis) (AB163432), *Mycobacterium tuberculosis* (mtube) (CAA17312) and *Escherichia coli* (ecoli) (AAN81359). Identical amino acids are shaded in pink. The bold underline indicates the TPP binding site and its site-specific amino acids are shaded blue.

The bootstrapped neighbour-joining phylogram tree constructed to explore the phylogenetic relationships of Oxc protein sequences from environmental and intestinal bacterial isolates is shown in Figure 3.7.

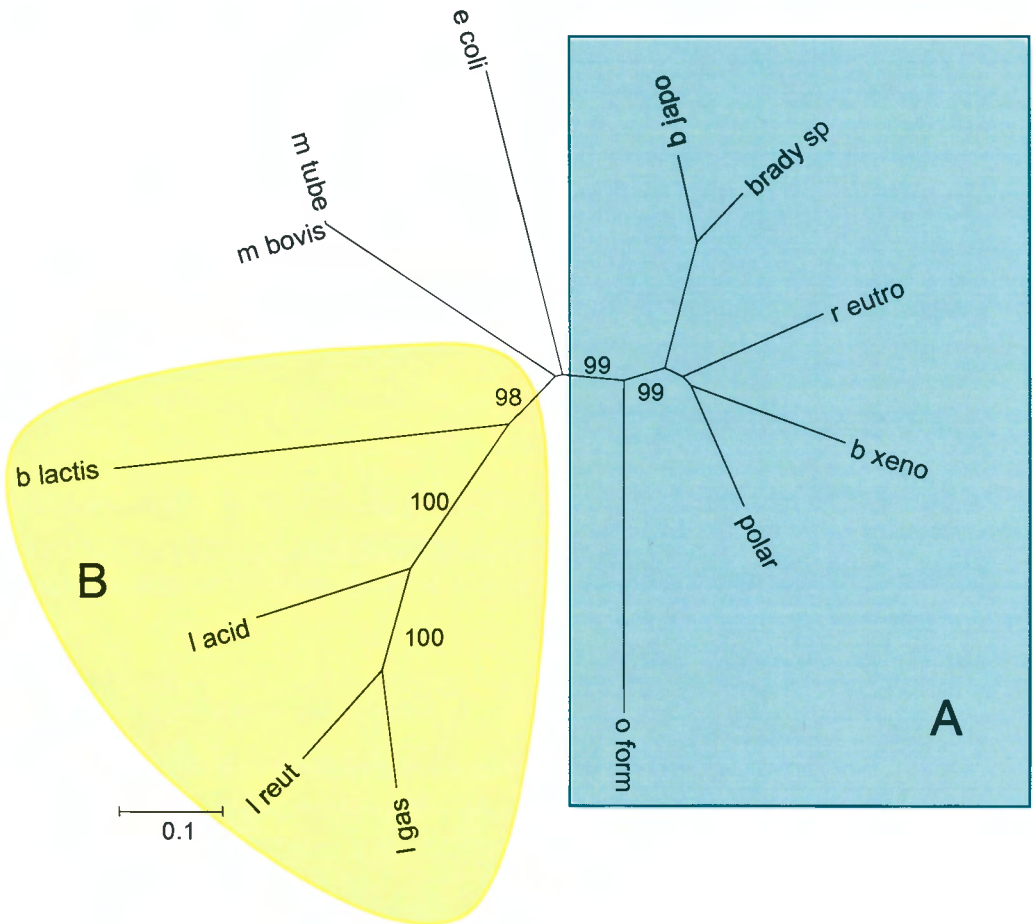

Figure 3.7. Unrooted phylogram tree, derived from 10,000 iterations, of predicted oxalyl-CoA decarboxylase (Oxc) amino acid sequences from diverse organisms. Protein sequences were aligned by CLUSTALX, and used for tree construction using MEGA 2.1. Bootstrap values are shown on the tree branches. The organisms used were *Oxalobacter formigenes* (o form) (Accession number (AAA53683), *Lactobacillus gasseri* (l gas) (Lgas02001418), *Lactobacillus reuteri* (l reut) (LreuDRAFT_0636), *Lactobacillus acidophilus* (l acid) (LBA0396), *Bifidobacterium lactis* (b lactis) (AB163432), *Mycobacterium bovis* (m bovis) (CAD92983), *Mycobacterium tuberculosis* (m tube) (CAA17312), *Escherichia coli* (e coli) (AAN81359), *Bradyrhizobium japonicum* (b japo) (BAC48422), *Bradyrhizobium* sp. BTAil (brady sp) (EAP29421), *Ralstonia eutropha* (r eutro) (YP_298857), *Burkholderia xenovorans* (b xeno) (ABE33236), *Polaromonas* sp. JS666 (polar) (ABE44716). Environmental isolates are grouped in clade (A) and GIT species are grouped in clade (B).

The Oxc protein sequences formed two distinct clusters, A and B, with high bootstrap values of 98 and 99, respectively (Figure 3.7). In clade A, the environmental species *B. japonicum*, *Bradyrhizobium* sp., *R. eutropha*, *B. xenovorans* and *Polaromonas* sp. JS666 clustered together; while clade B consisted of GIT-dwelling *Bifidobacterium* and *Lactobacillus* species. *O. formigenes*, which is frequently isolated from the rumen and large bowel of humans and animals, clustered with the environmental isolates (Clade A). This was unsurprising as *O. formigenes* has also been isolated from lake sediments; and this genus includes other sediment-dwelling species including *Oxalobacter vibrioformis* (Dehning & Schink, 1990). The *E. coli* intestinal isolate, however, had a low bootstrap value of 62 (data not shown), when clustered with the other intestinal isolates. Nonetheless, clustering of predominantly environmental (clade A) and intestinal species (clade B) into distinctive groups supported the hypothesis that oxalate-degrading genes in bifidobacteria and lactobacilli could have arisen from horizontal gene transfer events in the human GIT (Federici *et al.*, 2004; Azcarate-Peril *et al.*, 2006). The lactobacilli clustered closely within Clade B (bootstrap value of 100), with *L. gasseri* and *L. reuteri* clustering more closely to each other than to *L. acidophilus*. However, this clustering represented a protein sequence identity difference of 85% between *L. gasseri* and *L. reuteri*; and 82% between *L. acidophilus* and the former two species.

3.4.4. Growth in oxalate-media and oxalate degrading activity of *L. gasseri* DSM 20243^T

After confirmation of the presence of *oxc* and *frc* gene orthologs in *L. gasseri*, it was necessary to determine if these genes conferred in vitro oxalate-degrading ability on this bacterium. After five days of anaerobic incubation, *L. gasseri* DSM 20243^T degraded 56% of the 5 mM oxalate in MRS-OX (Table 3.2). This result presented the first report of an oxalate-degrading strain of *L. gasseri* that has a gene cluster that could be involved in oxalate metabolism. The 56% *in vitro* oxalate-degrading ability of the *L. gasseri* type strain in 5 mM

MRS-OX was generally comparable to the 61% reported for the oxalate-degrading probiotic bacterium *B. lactis* DSM 10140, grown under identical conditions (Federici *et al.*, 2004).

Table 3.2. Oxalate degradation by *L. gasseri* grown in MRS + 5 mM sodium oxalate for 120 hours

Hours[a]	Oxalate degraded (%)[b]	Growth phase
7	5 ± 2.0	Early-log
24	12 ± 1.8	Early-stationary
96	49 ± 0.3	Late-stationary
120	56 ± 0.01	Late-stationary

[a]Hours of growth in MRS-OX medium.
[b]Results reflected are mean ± SD, n = 4.

The growth curves constructed to determine the effects of 5 mM sodium oxalate on the growth of *L. gasseri* DSM 20243^T indicated that oxalate inhibited its growth (Figure 3.8). The inhibitory effects of oxalate on bacterial growth have also been reported for *L. acidophilus* NCFM (Azcarate-Peril *et al.*, 2006). The growth of the *L. gasseri* type strain in MRS was characterised by a short lag phase of approximately two hours, reaching an OD_{600nm} of approximately 8 within nine hours (Figure 3.8). In MRS + 5 mM sodium oxalate, the growth of *L. gasseri* was characterised by a lag phase of approximately three hours, with a maximum OD_{600nm} of 7 attained after twenty-seven hours. As mentioned in Chapter 1, oxalate is a highly oxidising molecule that is a recognised nutritional stress factor (Sahin, 2003). The 5 mM oxalate may, therefore, have inhibited the growth of the *L. gasseri* type strain in this capacity. Measurements of residual oxalate in the MRS-OX culture broths used to construct growth curves (Figure 3.8) showed that oxalate degradation occurred primarily in the stationary phase of growth (Table 3.2).

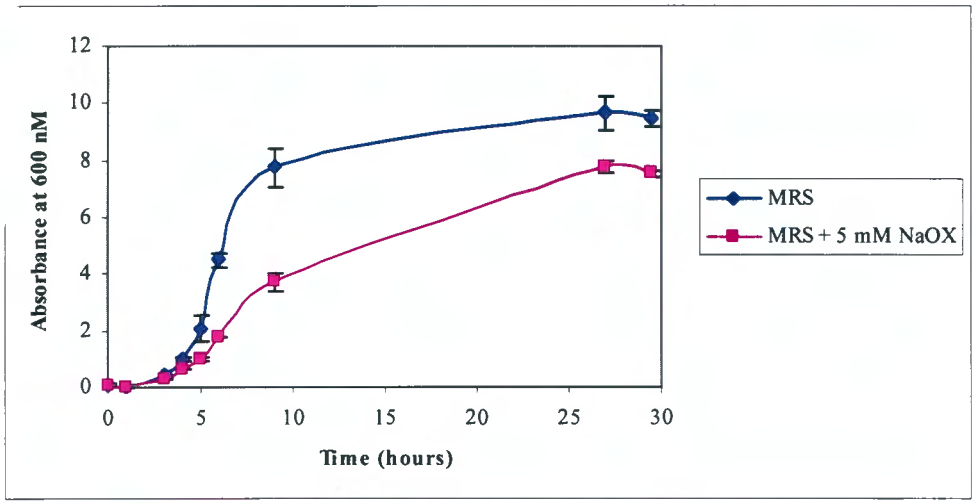

Figure 3.8. Anaerobic growth curves of *L. gasseri* DSM 20243^T in MRS and MRS + 5 mM sodium oxalate (NaOX) media. Results reflected are duplicates of two independent experiments and error bars reflect SD values.

This result suggested that oxalate degradation could possibly be pH-dependent and may have occurred as a consequence of the reduction in culture pH due to fermentation and the production of lactic acid by *L. gasseri*. The MRS-OX media used in the above-mentioned growth curves and oxalate-degradation experiments had an initial pH of 6.8, which dropped to approximately 4.2 after five days. During growth of lactobacilli in MRS medium, culture pH typically drops from 6.5 to less than 4 (Azcarate-Peril *et al.*, 2006). Alternatively, transcription of the oxalate-degrading genes in *L. gasseri* could be induced under stress conditions (stationary-phase growth), when nutrients become scarce, and stress-inducible genes are expressed to scavenge energy from limited resources. These hypotheses were investigated further in the transcriptional characterisation of the putative *L. gasseri oxc* gene.

3.4.5. Transcriptional expression of the *L. gasseri* DSM 20243^T *oxc* ortholog

The conditions used in this study to investigate transcriptional regulation of the putative *L. gasseri oxc* gene were modified from those reported in section 3.4.4 as a result of the work of Azcarate-Peril *et al.* (2006) on *L. acidophilus* NCFM. In their experiments using

quantitative reverse transcriptase Real-Time PCR, Azcarate-Peril *et al.* (2006) reported that the *L. acidophilus oxc* and *frc* genes were not expressed in MRS and MRS containing 32 mM ammonium oxalate (0.5%) media at pH 6.8. Instead, transcription of the *L. acidophilus oxc* and *frc* genes required acid induction at pH 5.5. Furthermore, pre-adapting the *L. acidophilus* NCFM cells with sub-inhibitory amounts of ammonium oxalate (3.5 mM) and then exposing them to MRS containing 32 mM ammonium oxalate, resulted in markedly increased expression of the *oxc* and *frc* genes (Azcarate-Peril *et al.*, 2006).

Consequently, in the present study, transcription of the *L. gasseri oxc* gene was evaluated at pH 5.5 in the presence and absence of 37.3 mM sodium oxalate (0.5%) as an inducer of the putative oxalate operon. The *L. gasseri* type strain was pre-adapted to oxalate by two consecutive transfers in MRS broth supplemented with a low concentration of sodium oxalate (3.73 mM / 0.05%), according to the protocol of Azcarate-Peril *et al.* (2006). Thereafter, adapted *L. gasseri* cells were transferred to (i) MRS broth (pH 5.5) and (ii) MRS broth containing 37.3 mM sodium oxalate (pH 5.5), and sampled over time. The growth curves constructed to determine the inhibitory effects of 37.3 mM sodium oxalate on the growth of the pre-adapted *L. gasseri* cells are shown in Figure 3.9.

The 37.3 mM sodium oxalate greatly inhibited growth of the *L. gasseri* DSM 20243 cells, which reached a maximum OD_{600nm} reading of approximately 3.5 after twenty-eight hours (Figure 3.9). This level of growth was much less than that recorded for non-adapted cells grown in 5 mM oxalate, which reached a maximum OD_{600nm} reading of approximately 7.5 in twenty-seven hours (Figure 3.8). Nonetheless, the growth of *L. gasseri* DSM 20243T at the higher oxalate concentration (37.3 mM) suggested that the oxalate-degrading system present

in *L. gasseri* DSM 20243T could be induced by pre-exposure to sub-lethal concentrations of oxalate.

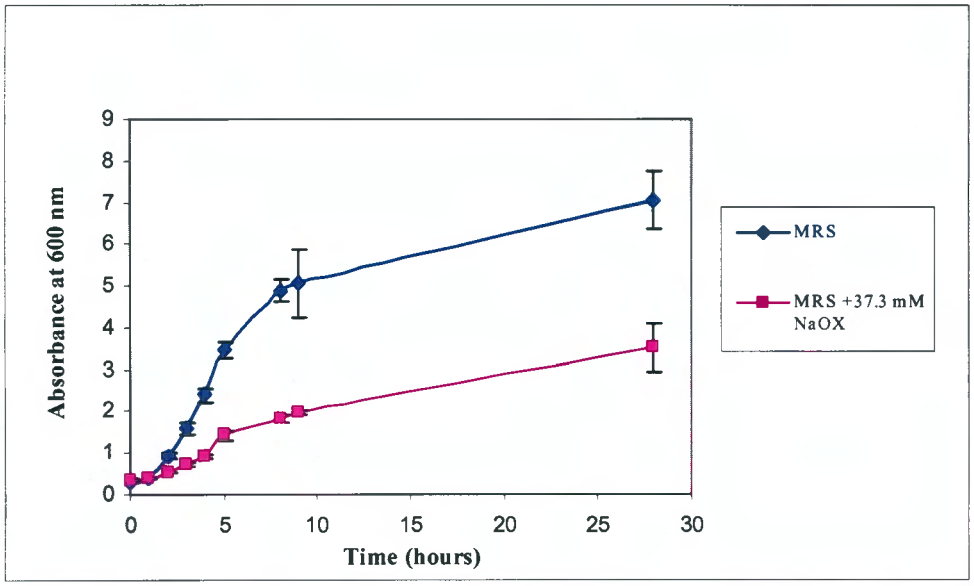

Figure 3.9. Growth curves of pre-adapted *L. gasseri* DSM 20243^T cells in MRS and MRS + 37.3 mM sodium oxalate (NaOX) media. Results reflected are duplicates of two independent experiments and SD values are indicated as error bars.

RNA dot blots were performed to study the conditions under which the *L. gasseri oxc* gene is transcriptionally regulated. A labelled DNA probe, specific for *oxc* mRNA, was hybridized to total RNA extracted from *L. gasseri* cells during mid- and late-log phase growth in MRS and MRS-OX media (Figure 3.9). Samples were collected at time 4 and 6 hours, representing mid- and late-log phase growth, respectively (Figure 3.9). A probe specific for the 16S rRNA gene was used as the internal control.

Exposure of the pre-adapted *L. gasseri* cells to 37.3 mM sodium oxalate (pH 5.5) resulted in a high expression of the putative *oxc* gene during mid-log phase growth (Lane 3, Figure 3.10), and to a lesser extent in late log phase (Lane 4, Figure 3.10).

Figure 3.10. Transcriptional analysis of the *oxc* gene in pre-adapted *L. gasseri* cells at pH 5.5. Autoradiographs of dot blot membranes hybridized with labelled probes specific for *L. gasseri oxc* mRNA and 16S rRNA. Cells were first transferred in MRS broth containing sub-inhibitory concentrations of sodium oxalate (3.73 mM). Total RNA was then extracted from cells during mid- and late log phase growth in MRS (1-2) and MRS-OX (3-4) media, respectively. Blots were performed in duplicate.

In contrast, transcription of the putative *oxc* gene was not detected during mid-log phase growth in the *L. gasseri* cells grown in MRS medium in the absence of oxalate (Lane 1, Figure 3.10). This result indicated that 37.3 mM oxalate induced the transcriptional expression of the putative *L. gasseri oxc* gene under mildly acidic conditions. The low expression of the putative *oxc* gene in late-log phase growth in MRS medium possibly represented basal levels of expression under acidic conditions (Lane 2, Figure 3.10). However, the possibility that the putative *oxc* gene is induced by stress conditions during stationary phase growth cannot be discounted entirely.

3.4.6. Transcriptional analysis of the putative *L. gasseri* DSM 20243^T *oxc-frc* operon by RT-PCR.

The clustering of the *oxc* and *frc* orthologs in *L. gasseri* DSM 20243^T, with an intergenic region of only 6 bp, suggested that these two genes could be co-regulated as an operon. A schematic representation of the putative *L. gasseri frc-oxc* operon is shown in Figure 3.11. The predicted permease located downstream of the putative *frc* and *oxc* genes is transcribed in

the opposite direction to the latter genes (Figure 3.4), and is consequently not expected to be part of the *oxc-frc* operon. RT-PCR was performed to determine if the *frc* and *oxc* orthologs function as an operon.

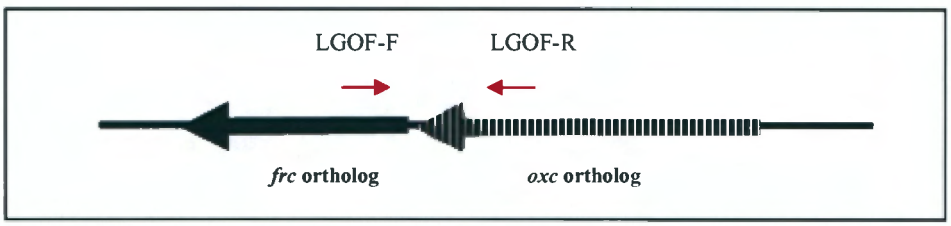

Figure 3.11. Schematic representation of the intergenic region between genes of the putative oxalate degradation pathway in *L. gasseri* DSM 20243. The red arrows represent the LGOF-F and LGOF-R primers that were used to amplify the 6 bp intergenic region between the *frc* and *oxc* orthologs.

The set of primers used to amplify the mRNA of the intergenic region between the *oxc* and *frc* orthologs had been tested on *L. gasseri* DSM 20243^T genomic DNA, to confirm the positions of the putative *frc* and *oxc* genes in relation to each other (section 3.4.3.1). This primer set gave the predicted PCR amplification product size of 586 bp, which was confirmed by sequencing (Lane 2, Figure 3.12).

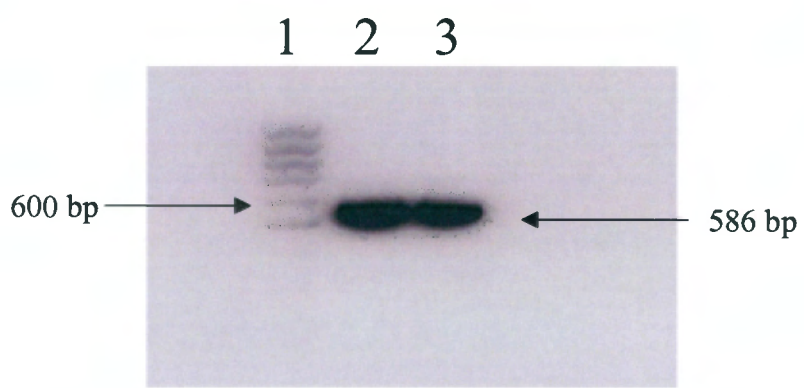

Figure 3.12. RT-PCR analysis of the putative oxalate-degrading genes cluster in *L. gasseri* DSM 20243^T. Agarose- gel electrophoresis of genomic and cDNA PCR amplification products; Lane 1, 100 bp MW marker (Fermentas). Lane 2, genomic DNA PCR amplicon. Lane 3, cDNA PCR amplicon.

RNA extracted from *L. gasseri* cells, grown under the inducing conditions described in section 3.4.5, was used to synthesise the cDNA used in RT-PCR. A PCR amplification product of 586 bp was obtained using the prepared cDNA template (Lane 3, Figure 3.12). These results confirmed that the putative *L. gasseri* DSM 20243^T *frc* and *oxc* genes are expressed on a single mRNA transcript, and presented the first confirmation of an operon of genes in lactobacilli which encoded proteins possibly involved in oxalate degradation. Azcarate-Peril *et al.* (2006) postulated that the *L. acidophilus oxc* and *frc* genes were regulated as an operon, but they did not confirm that these two genes were transcribed on a single mRNA transcript.

3.5. CONCLUSIONS

The *O. formigenes oxc* and *frc* genes encode enzymes involved in this bacterium's catabolism of oxalate, as discussed in Chapter 1. In the present study, bioinformatic screening for orthologs of these genes resulted in the identification of clusters of putative oxalate-degrading genes in *E. coli*, *L. reuteri* and *L. gasseri* genomes. The arrangement of the *frc* and *oxc* orthologs in *E. coli*, *L. acidophilus*, *L. reuteri* and *L. gasseri* genomes was highly conserved across these species; and phylogenetic analysis of the *oxc* orthologs in the lactobacilli revealed a close evolutionary relationship. These results strongly suggested that horizontal gene transfer events could be responsible for the acquisition of oxalate-degrading genes by generalist oxalotrophic gut bacteria. Nucleotide sequencing confirmed the presence and arrangement of the *frc* and *oxc* orthologs in *E. coli*-OX and *L. gasseri* DSM 20243^T. RNA

hybridisation studies and RT-PCR showed that the clustered *frc* and *oxc* orthologs in *L. gasseri* DSM 20243*T* are induced by oxalate and regulated as an operon.

The *in vitro* oxalate-degrading ability of *L. gasseri* DSM 20243^T indicated that bioinformatic screening is a useful approach for the identification of potential probiotics in the management of kidney stone disease. Lactobacilli have been used as probiotics for many decades, and continue to flourish as preferred research candidates in both food and health industries (reviewed in Tannock, 2004). Health benefits associated with consumption of lactobacilli by humans include: improved lactose tolerance (*L. acidophilus* NCFM), pathogen exclusion (*Lactobacillus fermentum* RC-14) and prevention of urinary tract infections in women (*Lactobacillus rhamnosus* GR-1) (as reviewed in Reid, 1999).

Reported health benefits specifically associated with consumption of *L. gasseri* strains include the suppression of *Helicobacter pylori* infections by *L. gasseri* OLL 2716 (Sakamoto *et al.*, 2001), and stimulation of the immune system by *L. gasseri* CECT 5714 (Olivares *et al.*, 2006). In view of this, the oxalate-degrading *L. gasseri* DSM 20243^T is clearly a suitable probiotic candidate in the prophylactic treatment of kidney stone disease. However, a possible limitation of attributing metabolic properties to probiotic candidates based on *in vitro* investigations of pure cultures alone is that, these bacteria may not replicate these properties *in vivo*. Accordingly, the oxalate-degrading ability of the probiotic candidate *L. gasseri* DSM 20243^T was tested in a simulated model of the human colonic microbiota, and is reported in Chapter 4.

CHAPTER 4

Oxalate-Degrading Capacity of the Probiotic Candidate *L. gasseri* DSM 20243^T in a Multi-stage Continuous Culture Simulator of the Human Colonic Microbiota

Contents

4.1. SUMMARY

The ability of *L. gasseri* DSM 20243^T to degrade oxalate in pure culture was confirmed *in vitro*, as reported in Chapter 3. Consequently, its ability to degrade oxalate in simulated gut conditions was tested using a three-stage continuous culture fermentor (CCF) inoculated with faecal bacteria. The CCF modelled environmental conditions in the proximal, transverse and distal colons, and was operated at system retention times analogous to normal human colonic transit rates (30 and 60 hours). The culture medium used comprised dietary substances and other compounds that are typically encountered by bacteria in the human gut, supplemented with 10 mM sodium oxalate. A freeze-dried preparation of *L. gasseri* DSM 20243^T was introduced into the CCF under steady-state growth conditions, and confirmation of the successful establishment of *L. gasseri* in the CCF was obtained through viable cell culture and quantitative Real-Time PCR. Oxalate determination using gas chromatography indicated that *L. gasseri* DSM 20243^T initiated oxalate degradation in the first stage of the CCF. Short chain fatty acid analysis showed that addition of L. *gasseri* to the CCF, at both retention times, did not adversely affect the equilibrium of the microbial ecosystem in the colon simulator. These results suggested that *L. gasseri* DSM 20243^T should colonise the human gut effectively and, therefore, has potential therapeutic use in the management of kidney stone disease.

4.2. INTRODUCTION

4.2.1. The human intestinal microbiota

The human GIT contains a vast, complex and metabolically diverse microbiota, as described in Chapter 1. Many of these microbes have a transient existence; some persist for short periods, whereas others are consistently detected in the gut (Tannock, 1995). It is these microbes that are seemingly permanent colonisers of the GIT that are referred to as the autochthonous or 'normal' microbiota. Much of the microbial colonisation of the GIT is restricted to the colon, where an excess of 400 species, representing more than 190 genera, have been detected (Holzapfel *et al.*, 1998). In this normal microbiota, obligately anaerobic species predominate although facultative anaerobes are also typically encountered.

Humans differ considerably in their extent and variation of microbial composition; nonetheless, 40 bacterial species have been identified as 'commonly detected' (Tannock, 1995). These include *Bacteroides*, *Bifidobacterium*, *Peptostreptococcus*, *Ruminococcus*, *Eubacterium*, *Enterococcus*, *Lactobacillus* and *Clostridium* species. Whilst numerous studies report that predominant species in the adult gut remain relatively stable, various factors, including host, microbiological and environmental factors, affect bacterial colonisation of the gut (Macfarlane & Macfarlane, 2004; Tannock, 1995; Conway, 1995). Some of these factors are summarised in Table 4.1 (adapted from Macfarlane & Macfarlane, 2004).

Table 4.1. Some factors affecting bacterial diversity in the large bowel[a].

Host	Microbiological	Environmental
Diet, host genetics	Competition for limiting nutrients and adhesion sites. Cooperative interactions between microbes.	Amounts and type of substrates available for growth
Colonic transit time	Bacterial genetic, biochemical and physiological traits	pH of gut contents
Drugs, disease, antibiotics	Pathogen inhibition by production of metabolites and/or antagonistic substances by commensal bacteria	Redox potential

[a]Adapted from Macfarlane & Macfarlane (2004)

As mentioned earlier, the importance of microbes and bacteria-host interactions for the maintenance of good health has long been recognised (Metchnikoff, 1908); and GIT bacteria have consequently been the subjects of numerous investigations to understand these interactions. The complications inherent in obtaining samples from different parts of the human GIT have limited most studies on GIT bacteria to faecal bacteria (Kleessen *et al.*, 2000); and much of the knowledge on biodiversity, development and stability of human gut bacteria has come from studies on faecal microbiota using culturing methods (reviewed in Kleessen *et al.*, 2000; Macfarlane & Macfarlane, 2004). More recently, molecular analyses including quantitative Real-Time PCR (qRT-PCR), density gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridisation (FISH) are used in culture-independent analyses of faecal microbiota (reviewed in Macfarlane & Macfarlane, 2004). The advent of these culture-independent methods to study the gut microbiota has brought new knowledge on the groups of bacteria that are known to predominate in faecal samples. Several groups of bacteria that remain undetected by culturing studies have been reported to occur in high numbers using molecular analyses (Macfarlane &

Macfarlane, 2004). For instance, both *Bacteroides* and *Eubacterium* groups are detected in high numbers by culturing and FISH methods. However, while *Atopobium* and *Ruminococcus* groups are detected in higher numbers than bifidobacteria using FISH, the former two bacterial groups remain unreported in most culturing studies. Molecular analyses have their own limitations including hybridisation efficiencies of oligonucleotide probes (FISH) as well as dependency on DNA extraction and PCR amplification efficiencies (qtRT-PCR, DGGE), as noted in Chapter 2. Nevertheless, molecular analyses are increasingly overriding culturing studies as methods of choice in analysing the gut microbiota.

4.2.2. Models for studying the human intestinal microbiota

The colonic microbiota can be studied using *in situ*, *in vivo* or *in vitro* models (Conway, 1995). Most of these models use faecal material for inocula and/or direct analysis due to the earlier-mentioned inaccessibility of the human colon for sampling purposes. Inferences are then made based on studies on faecal samples although faecal samples are admittedly not entirely representative of the colonic microbiota (Conway, 1995).

In situ models

In situ studies involve typically non-invasive investigations using human volounteers (Conway, 1995). In these studies, the volounteers are subjected to the designed experimental conditions, for example particular diets or supplementation, and thereafter-faecal samples are collected and analysed. The primary advantage of this method is that because the investigation is being carried out in humans, the results obtained directly reflect the effect(s) of the parameter(s) under investigation on the normal gut ecosystem. Other advantages include the ease of collecting and processing freshly voided faecal samples, and the potential use of a large number of healthy

individuals (Conway, 1995). Apart from the earlier-stated disadvantage of being unable to sample the human colon directly, ethical approval must be obtained before any studies on human subjects can commence, which may impede investigations.

In vivo models

In vivo investigations usually involve the use of germ-free animals, particularly mice and rats, for studying human microbial interactions (Conway, 1995). Analyses of faecal activities have shown that neither rats nor mice harbour a functional microbiota that is consistent with that found in humans (Rowland *et al.*, 1986 cited in Conway, 1995). A major disadvantage of the use of germ-free animals is that the simple mixes of anaerobes generally employed as inocula, do not constitute a microbiota as complex and metabolically diverse as that encountered in the human gut (Conway, 1995). As a result, it has been proposed that the ideal *in vivo* model would be that of ex-germ-free mice colonised with human colonic microbiota (Fujiwara *et al.*, 1991). One advantage of animal models such as mice and rats is that ethical approval can often be obtained much more easily than with human subjects. In addition, ethical approval is easily granted for investigations which require sampling directly from the mouse/rat gut; as well as bleeding of the animals for concomitant immunological analyses. Valuable information on human gut bacteria has been obtained from *in vivo* models using animals (Sidhu *et al.*, 2001).

In vitro models

A large number of studies on bacterial fermentations involving colonic bacteria have been done using *in vitro* models (reviewed in Conway, 1995). Numerous *in vitro* models have been devised, and validated, that are used to study mono- and mixed cultures of human colonic bacteria under anaerobic conditions (Adams, 1980; Freter *et al.*, 1983; Edwards *et al.*, 1985;

Molly *et al.*, 1994; Macfarlane *et al.*, 1998a). These *in vitro* models range from screw-capped bottles and batch culture fermentors, to single- and multiple-stage continuous culture fermentors (reviewed in Conway, 1995; Macfarlane *et al.*, 1994; Gibson & Fuller, 2000). While screw-capped bottles and other batch fermentations have the advantage of being relatively simple and inexpensive to operate, as well as being amenable to large-scale manipulations, they operate as uncontrolled 'closed' systems. Progression of a batch culture fermentation results in a depletion of substrates, accumulation of bacterial metabolites and thus constantly changing environmental conditions in the fermentor (reviewed in Gibson & Fuller, 2000; Macfarlane *et al.*, 1994). This severely limits the information that can be reliably inferred from batch fermentations to information concerning substrate input and product outputs. Information on actual bacterial fermentation and regulatory processes is often speculative.

Most of the above-mentioned problems inherent in batch culture fermentations are avoided in continuous culture fermentations such as turbidostats and chemostats (Macfarlane *et al.*, 1994). Continuous culture fermentations operate as 'open' systems in which a fixed volume of well-mixed culture is continuously provided with fresh nutrients, at the same rate as culture liquid is removed (Gottschal, 2000). The most common type of continuous culture system used to study bacterial fermentations is the chemostat (Conway, 1995; Gottschal, 2000; Macfarlane *et al.*, 1994). In the chemostat, the growth rate of the culture, which is determined by the rate at which medium is fed to the chemostat, can be strictly controlled by limiting a nutrient in the feed medium (Macfarlane *et al.*, 1994). The dilution rate (D) is defined as "the flow rate of incoming fresh medium divided by the actual volume of the culture in the continuous culture fermentor" (Gottschal, 2000). The reciprocal of (D) is referred to as the system retention time. The continuous culture fermentor (CCF) is an ideal model to study colonic bacteria under carefully

controlled conditions for extended periods. Additionally, it enables the study of bacterial interactions between different species grown as mixed cultures under single- and multiple-nutrient limitations (Macfarlane *et al.*, 1994). However, CCFs are complex and expensive in time and materials to operate.

The analogy of the human colon to a CCF has been criticised as an oversimplification (Conway, 1995; Macfarlane *et al.*, 1994). This is because the CCF is unable to mimic bacteria-host interactions involving host secretions and intestinal mucosal surfaces, both of which reportedly affect the metabolism of the gut microbiota. In addition, the composition of culture medium is often criticised as failing to mimic nutritional environments encountered by bacteria in the large bowel (Macfarlane *et al.*, 1994). Nevertheless, studies using continuous culture fermentors, particularly multi-stage systems, have shown that *in situ* numerical relationships of human colonic bacteria can be duplicated (Molly *et al.*, 1994; Macfarlane *et al.*, 1998a). Moreover, bacterial interactions that have been demonstrated *in vivo* can be mimicked (Edwards *et al.*, 1985; Molly *et al.*, 1994; Macfarlane *et al.*, 1998a).

Although *in situ, in vivo* and *in vitro* models that are used to study the human colonic microbiota have their limitations, each provides valuable information towards understanding the role of colonic microbiota in maintaining human health. The primary aim of the work presented in this chapter was to investigate the oxalate-degrading efficacy of the *L. gasseri* DSM 20243^T probiotic candidate under simulated human gut conditions. This investigation involved the use of a three-stage continuous culture fermentor inoculated with human faeces, which would provide useful information for the further development of *L. gasseri* DSM 20243^T as a probiotic candidate in biotechnological applications as well as in human trials.

4.3. MATERIALS AND METHODS

4.3.1. Type strain and culture maintenance

L. gasseri DSM 20243^T was routinely cultured as described in section 3.3.1. The *L. gasseri* type strain and all anaerobic species were cultured in an anaerobic chamber containing an atmosphere of hydrogen, carbon dioxide, and nitrogen (10:10:80).

4.3.2. Production of freeze-dried *L. gasseri* DSM20243^T cells

After two rounds of sixteen hour growth on MRS agar, a single *L. gasseri* DSM 20243^T colony was inoculated into 30 ml MRS broth supplemented with 5% (w/v) fructo-oligosaccarides (MRS-FOS), and incubated for eighteen hours. This 30 ml eighteen-hour-old culture was then inoculated into 1L of MRS-FOS broth and incubated for 18 hours. The *L. gasseri* cells were harvested by centrifugation at 14,000 g using a Beckman centrifuge, and washed in anaerobic sodium phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH 7.0), supplemented with 10% glycerol. The washed cell pellet was then frozen at -85°C, freeze-dried and packaged into 0.05g aliquots, each of which contained 10^9 viable cells. When steady-state conditions had been established in the continuous culture fermentor (CCF), freeze-dried L. *gasseri* DSM 20243^T cells were fed to V1 of the CCF twice per day, providing 2 x 10^9 viable cells per day for 336 hours at retention time (R) = 60 h, and for 168 hours at R = 30 h.

4.3.3. Fermentation system design, culture media and growth conditions

The CCF used in this study was similar to the model described by Macfarlane *et al.* (1989). The colon simulator comprised three culture vessels V1, V2, and V3 arranged in series, each with a working volume of 300 ml, and was run at total system retention times (R) of either 60 or 30 hours, which are in the range of normal human colonic transit times (Cummings *et al.,* 1992). A schematic representation of the CCF is shown in Figure 4.1.

Temperature within the CCF was maintained at 37°C using a Haake Circulating Water Bath (Macfarlane *et al.,* 1998b). A headspace of O_2-free N_2 was maintained in each fermentor, and culture pH in the three vessels was maintained at 5.5 (V1), 6.2 (V2) and 6.8 (V3) by New Brunswick Modular pH Controllers, to reproduce pH values found in the proximal, transverse and distal colons, respectively (Cummings *et al.,*1987). The sterile culture medium was continuously sparged with O_2 - free N_2 and was fed by peristaltic pump to V1, which sequentially supplied V2 and V3. The growth medium consisted of the following (g L^{-1}) in distilled water: oligofructose (Synergy 1) 4.0; pectin (citrus), 3.0; xylan (oatspelt), 2.0; arabinogalactan (larchwood), 2.0; guar gum, 1.0; mucin (Sigma porcine gastric type III) 4.0; tryptone, 5.0; peptone water, 5.0; yeast extract, 4.5; bile salts no. 3, 0.4; cysteine 0.8; hemin, 0.01; Tween 80, 1.0; NaCl; 4.5; KCl, 4.5; $MgCl_2.6H_2O$, 1.25; $CaCl_2.6H_2O$, 0.05; KH_2PO_4, 0.4 and sodium oxalate, 1.34 (10 mM). Two ml of a trace elements solution was also added, which contained (g l^{-1}); $MgSO_4.7H_2O$, 3.0; $MnCl_2.4H_2O$, 0.32; $FeSO_4.7H_2O$, 0.1; $CoSO_4.7H_2O$, 0.18; $CaCl_2.2H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.18; $CuSO_4.5H_2O$, 0.01; $NiCl_2.6H_2O$, 0.092. The final medium preparation was adjusted to pH 5.5 by the addition of 1 M HCl prior to autoclaving.

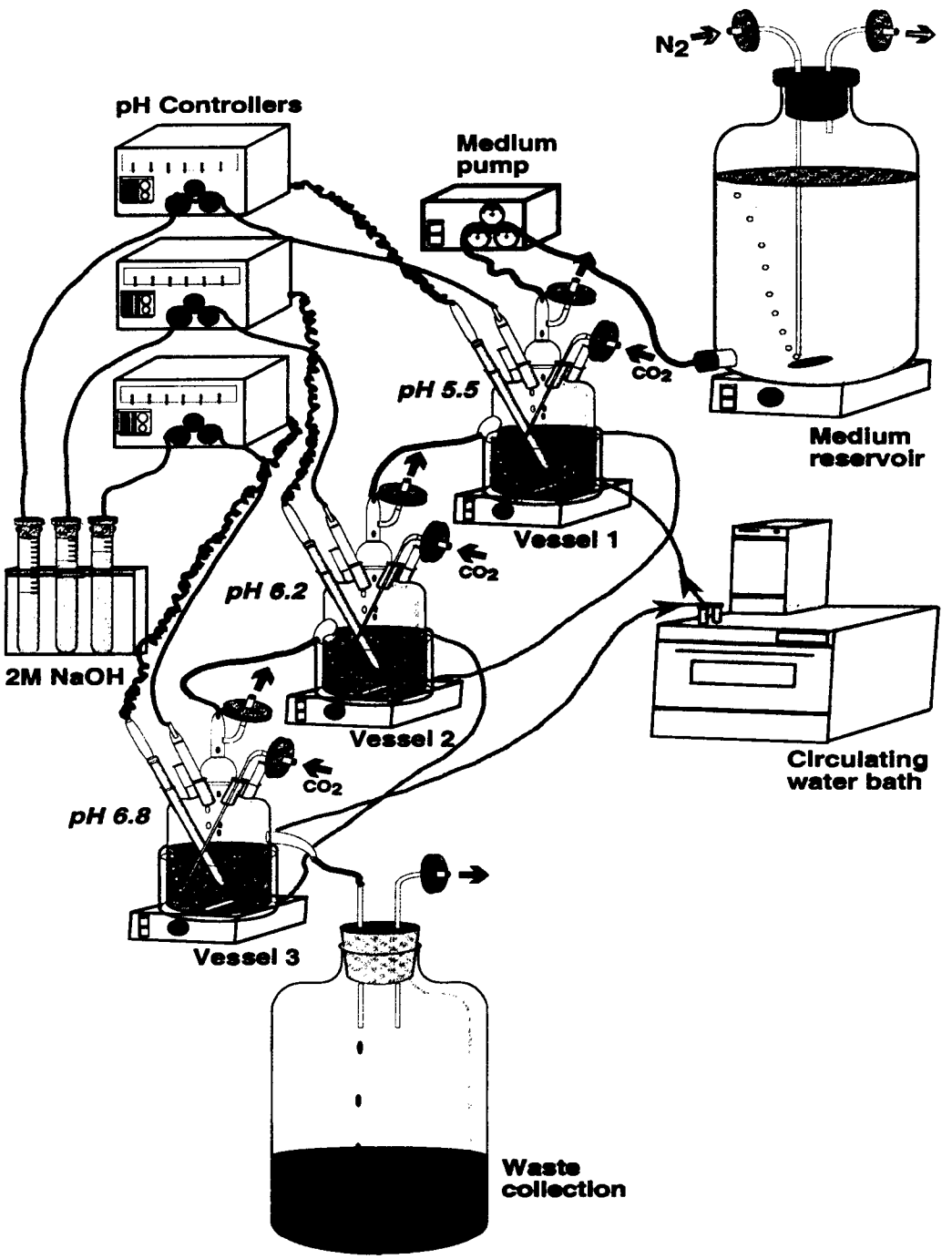

Figure 4.1. Schematic diagram of the three-stage continuous culture system used in this study. Image generously supplied by G. T. Macfarlane (Dundee, UK).

4.3.4. Preparation of faecal material, fermentor inoculation and stabilisation

Faecal samples for inoculating the CCF were provided by a healthy, kidney stone-free 30-year-old white male, who had not taken antibiotics for at least 12 months prior to sampling. Freshly voided whole stools were collected and 20% (w/v) slurries were prepared by macerating the stool in anaerobic sodium phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH 6.2). Large food residues were removed by passing the slurries through two sieves of pore diameter 1 mm and 0.125 mm, respectively. Aliquots (150 ml) of the resulting faecal suspensions were then used to inoculate each of the three fermentation vessels, containing 150 ml double-strength culture medium. After overnight equilibration, medium was supplied to the CCF. Short chain fatty acid (SCFA) analysis was used to confirm that the CCF was operating under steady-state growth conditions.

4.3.5. Short chain fatty acid, oxalate and lactate detection in the CCF

Capillary gas chromatography (GC) was used to measure SCFA, lactate and oxalate concentrations in samples collected from the CCF as previously described (Holdeman *et al.*, 1977). Briefly, for oxalate determinations 1 ml of each sample or standard solution was acidified with 0.4 ml of 50% H_2SO_4, methylated with 1 ml of methanol and extracted into chloroform. For SCFA analysis, 1 ml of each sample or standard solution was acidified with 0.1 ml 50% H_2SO_4 and extracted into ether. Fumarate (10 mM) was used as an internal standard for oxalate and lactate, and 5 mM tert-butyl acetic acid was the internal standard used for SCFA.

All extracted samples were separated by gas chromatography using an Agilent Technologies Model 689N Gas Chromatograph (Agilent Technologies, Stockport, UK), following split

injection (40:1) and using a HP-INNOwax (cross-linked PEG) 30 m x 0.25 mm x 0.25 μm column (No. 19091N-133, Agilent Technologies). Injector and detector temperatures were 250°C and 300°C, respectively. The initial column temperature (120°C) was held for one minute, and then increased in 10°C per minute increments until reaching 260°C, which was maintained for two minutes. Flow rates of the helium carrier gas, H_2, air and N_2 (makeup gas) were set at 1.8 ml per minute, 40 ml per minute, 450 ml per minute and 45 ml per minute, respectively. Samples were quantified by comparison of sample peak heights against those of authentic standards and internal standards using Hewlett Packard Integrated Chemstation Software.

4.3.6. Confirmation of bacterial diversity in the CCF using FISH and culturing studies

Qualitative fluorescent *in situ* hybridisation (FISH) was used to determine that the culture medium supplied to the CCF supported the growth of diverse bacterial species. Samples from all three vessels of the CCF were fixed by mixing them in a 1:3 ratio with 4% (w/v) paraformaldehyde. This mixture (total 1.2 ml) was then incubated for 16 hours at 4°C. After incubation, the samples were centrifuged at 15,000 g for five minutes. The pellet obtained was washed twice in 1 ml of sterile phosphate buffered saline (PBS) at pH 7, and resuspended in a final volume of 300 μl sterile PBS. An equal volume (300 μl) of ice-cold ethanol was added to this mixture, and the fixed samples were stored at –20°C.

FISH was done as previously described (Harmsen *et al.*, 2000). Fixed samples were diluted 10 to 100-fold, depending on the expected numbers of target cells, and the diluted bacterial suspensions (10 μl aliquots) were placed onto teflon-coated slides, with individual 5 mm diameter wells [VWR International, Lutterworth, UK]. To improve cell attachment, the slides

were immersed in a solution containing 0.075% (w/v) gelatin (Fisher G-9391) and 0.01% (w/v) chromium potassium sulfate dodecahydrate at 60°C, and allowed to air dry for sixteen hours at room temperature. Samples were hybridised overnight on glass slides at temperatures of 45, 50, or 54°C depending on the probe used. Where necessary, formamide was added to the hybridisation buffer at levels ranging from 0 - 60%. Where lysozyme treatment was needed to improve efficiency, cells (10 µl aliquots) were incubated before hybridisation with 10 µl of lysozyme (1.0 mg/ml) in 100 mM Tris-HCL (pH 8.5) for 15 minutes, at room temperature. The specificity of the probes used was confirmed by DNA staining using 6-diamino-2-phenylindole (DAPI). The full list of probes used in this study, with their respective hybridization conditions, is shown in Table 4.2.

For enumeration of culturable bacteria in the CCF, samples were collected periodically from the CCF and serially diluted in 10-fold stages in half-strength anaerobic peptone water. Thereafter, 100 µl aliquots of each dilution were plated in duplicate onto selective and nonselective solid culture media. Facultative anaerobes were isolated on nutrient agar (total facultative anaerobes), azide blood agar base (Gram-positive cocci), and MacConkey agar no. 2 (lactose-fermenting bacteria, nonlactose-fermenting bacteria, and enterococci). Anaerobic species were isolated on Wilkins-Chalgren blood agar (total anaerobes); Beerens agar (Bifidobacteria) (Beerens, 1990), azide blood agar base (anaerobic cocci); and rogosa agar (lactobacilli). The *Bacteroides fragilis* group microorganisms were enumerated with bacteroides mineral salts agar (Macfarlane *et al.*, 1990). Aerobic plates were incubated at 37°C for two days and anaerobic plates at 37°C for five days.

Table 4.2. Oligonucleotide probes used for FISH analysis in this study.

Probe	Target bacterial group/species	Sequence (5' – 3')	Hybridisation conditions			Reference
			F[a]	T_H[b]	L[c]	
Eub338	Universal eubacterial probe	GCTGCCTCCCGTAGGAGT	0	50	0	Amman *et al.*, 1990
Bif164	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	0	45	0	Langendik *et al.*, 1995
Bac303	*Bacteroides/Prevotella*	CCAATGTGGGGGACCTT	0	45	0	Manz *et al.*, 1996
Rfla729	*Ruminococcus albus, Ruminococcus flavifaciens*	AAAGCCCAGTAAGCCGCC	20	50	15	Harmsen *et al.*, 2002
Rbro730	*Ruminococus bromii, Clostridium leptum*	TAAAGCCCAGYAGGCCGC	20	50	15	Harmsen *et al.*, 2002
Ecyl387	*Eubacterium cylindroides, Clostridium innocuum, Eubacterium biforme, Eubacterium tortuosum* and related species	CGCGGCATTGCTCGTTCA	20	45	0	Harmsen *et al.*, 2002
Lacb0722	Lactic acid bacteria, lactobacilli, enterococci	YCACCGCTACACATGRAGTTCCACT	20	54	15	Sghir *et al.*, 1998
Ato291	*Atopobium* group	GGTCGGTCTCTCAACCC	0	45	0	Harmsen *et al.*, 2000
Ent	*Enterobacteriaceae* except *Proteus* spp.	CCCCCWCTTTGGTCTTGC	30	50	0	Kempf *et al.*, 2000
Erec482	*Clostridium* cluster XIVa (*Eubacterium rectale/Clostridium coccoides*)	GCTTCTTAGTCAGGTACCG	0	50	0	Franks *et al.*, 1998
Component probes of Erec482						
Lach571	*Lachnospira* group	GCCACCTACACTCCCTTT	60	45	0	Harmsen *et al.*, 2002
Ehal469	*Eubacterium hallii*	CCAGTTACCGGCTCCACC	20	50	0	Harmsen *et al.*, 2002
Rint623	*Roseburia intestinalis*	TTCCAATGCAGTACCGGG	0	54	0	Hold *et al.*,
Ehad	*Eubacterium hadrum*	GACTTGCCATACCACC	0	50	0	2003

[a] Formamide concentration in the hybridisation buffer (%).
[b] Hybridisation temperature (°C).
[c] Lysozyme treatment (min).

4.3.7. Assessment of bacteria in the colon simulator using live/dead staining

The live/dead staining technique using propidium iodide and SYTO 9 was used for quantitative differential counting of the live/dead anaerobic microorganisms in the CCF, as previously described (Fukuda *et al.*, 2004). Briefly, the three components used consisted of A, SYTO 9 dye (3.34 mM, 300 µl solution in DMSO), B, Propidium iodide (20 mM, 300 µl solution in DMSO) and C, Baclight mounting oil. Approximately 1.5 µl each of Components A and B were added to 1 ml of pre-reduced anaerobic water and kept in the dark. Thereafter, 200 µl of the mixture was added to 200 µl of CCF sample, and incubated in the dark for ten minutes in an anaerobic jar at room temperature. After incubation, 10 µl aliquots of each mixture were spotted onto slides and visualised using red and green filters on a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Welwyn Garden City, UK). Five fields were covered on each slide.

4.3.8. Extraction of bacterial chromosomal DNA

Aliquots (1 ml) were collected daily from all culture vessels of the colon simulator. Cells were harvested by centrifugation at 5,000 g for ten minutes, and the pellet obtained was resuspended in 450 µl of sterile water. The cells were treated with lysozyme at 37°C for thirty minutes (50 µl of 50 µg/ml stock) and, thereafter, 25 µl of Proteinase K, 50 µl of 20% (w/v) SDS, 500 µl of H_2O and 350 mg of 0.1 mm glass beads were added to the mixture. Bead beating was done for two minutes and the mixture was then incubated at 60°C for ten minutes. After incubation, the bead beating was repeated for two minutes and the mixture centrifuged at 5, 000 g for three minutes to remove the large cellular debris. DNA was then isolated from the collected supernatant using the QIAmp DNA Mini Kit (Qiagen).

4.3.9. Enumeration of bacteria in the CCF using quantitative Real Time PCR

Real-time PCR was used for culture-independent enumeration of lactobacilli and bifidobacteria in the CCF using previously described 16S rRNA gene primers (Table 4.3). In addition, a set of primers was designed to amplify a 249 bp fragment of the *L. gasseri oxc* gene for enumeration of *L. gasseri* species (Table 4.3). All real-time PCR results were normalized as gene copies per 1 x 10^7 copies of control DNA (eubacterial 16S rRNA gene).

Table 4.3. Primer sets used for real-time PCR in this study

Target gene	Forward (F) and reverse (R) primer sequences (5′- 3′)	Position	Product length (bp)	T (°C)[a]	References
Bifidobacterium 16S rRNA	**F:** AGGGTTCGATTCTGGCTCAG	8	156	62	Kok *et al.*, 1996
	R: CATCCGGCATTACCACCC	164			
L. acidophilus group 16S rRNA**	**F:** GATAGAGGTAGTAACTGGCCTTTA*	478	390	55	Malinen *et al.*, 2003
	R: GCGGAAACCTCCCAA	853			
All eubacteria 16S rRNA	**F:** ACTCCTACGGGAGGCAGCAGT	330	200	56	Nadkarni *et al.*, 2002
	R: GTATTACCGCGGCTGCTGGCA	530			
L. gasseri oxc	**F:** TCGTCATCTGGGAC AAGACCT	964	249	62	This study
	R: TCAATGATGACCTTGGCGAA	1213			

[a]PCR annealing temperature.
* Modified from original published sequence.
** *L. acidophilus* group primers detect *L. acidophilus, L. amylovorous, L. amylolyticus, L. crispatus, L. gasseri* and *L. johnsonii.*

4.3.10. Chemicals

Unless otherwise stated, all chemicals used were obtained from Sigma and biological culture media from Oxoid Ltd. (Basingstoke, Hamps, UK).

4.4. RESULTS AND DISCUSSION

4.4.1. Culture conditions and stabilization of the continuous culture fermentor

The continuous culture fermentor (CCF) used in this study, shown in Figure 4.2, has been previously validated and found to simulate environmental conditions and bacterial activities in gut contents obtained from human sudden death victims (Macfarlane *et al.*, 1998a).

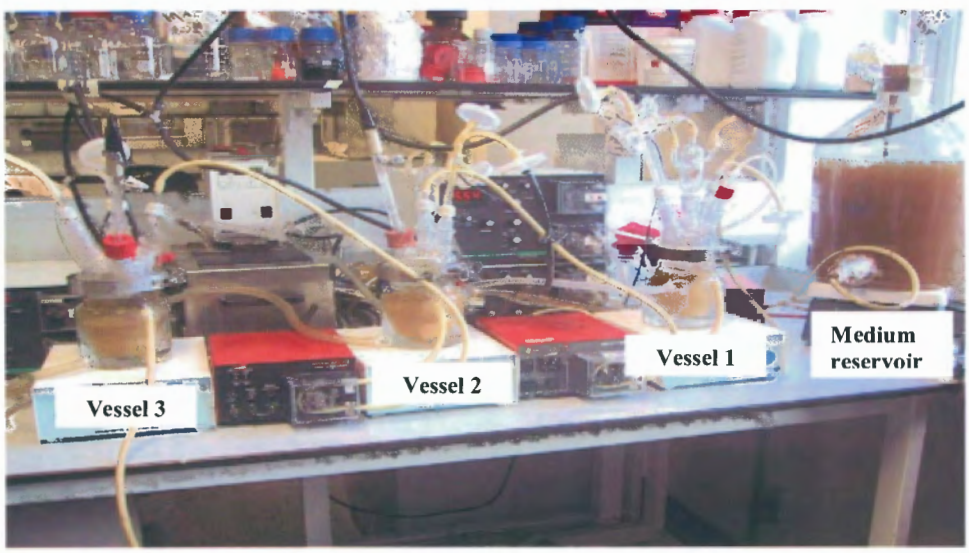

Figure 4.2. The three-stage continuous culture fermentor used to simulate nutritional and environmental conditions in the proximal (vessel 1), transverse (vessel 2) and distal (vessel 3) colon.

The operational system retention time of the CCF was analogous to human colonic transit rates. Normal colonic transit rates have been reported to range between 20 to 120 hours, with a mean value of 60 hours (Cummings *et al.*, 1992). Colonic transit times reportedly affect the metabolic

activity of GIT bacteria (Cummings *et al.*, 1979); therefore, in this investigation, the system retention times of 60 and 30 hours were chosen to represent average and fast colonic transit rates.

Carbohydrates form a large proportion of the human diet, and humans are well equipped to absorb simple sugars, such as glucose, and also to hydrolyse certain disaccharides like lactose (Hooper *et al.*, 2002). On the other hand, humans are limited in their ability to hydrolyse other more complex carbohydrates, for instance, raffinose. Consequently, dietary carbohydrates/substances that are indigestible to humans are the major source of carbon and energy for colonic bacteria. These substances include resistant starch, plant cell wall derived polysaccharides such as cellulose, pectin and xylan, as well as host mucopolysaccharides and mucins (Hooper *et al.*, 2002; Macfarlane *et al.*, 1998b). The culture medium formulated in this study comprised these and other substances that are typically encountered by bacteria in the human gut, supplemented with 10 mM sodium oxalate. The CCF thus modelled nutritional and environmental conditions found in the human proximal, transverse and distal colons; and was operated at system retention times within the range of normal colonic transit rates (30 and 60 hours).

The human colon has been likened to a continuous culture fermentor in which numerous species of bacteria co-exist in a steady state (Conway, 1995). In this steady state, bacterial species actively compete with each other for available carbon and energy sources, whilst also maintaining some syntrophic relationships. The fermentations of these bacteria generate a wide range of low molecular weight products in the human gut (Macfarlane & Macfarlane, 2003). Short chain fatty acids (SCFA) are the major products of bacterial fermentation in the colon, with acetate, propionate and butyrate accounting for 85-95% of total SCFA in all regions of the colon

(Cummings & Macfarlane, 1991). Other SCFA formed include valerate, caproate and the branched chain fatty acids (BCFA) isobutyrate, isovalerate and 2-methylbutyrate.

In this study, quantitative SCFA analysis was used to determine whether the colon simulator, inoculated with faecal bacteria, was operating under steady-state conditions. Steady-state conditions at R = 60 hours were attained after approximately 312 hours and butyrate, acetate and propionate were the predominant fatty acids produced in all CCF vessels (Table 4.4).

Table 4.4. Major SCFA in the CCF under steady-state conditions at R = 60 h[a].

Retention time	Hours[b]	SCFA (mM)	V1	V2	V3
60 h	0	Acetate	10.7 ± 0.5 (15)[c]	21.9 ± 2.4 (24)	29.7 ± 0 (28)
		Propionate	5.3 ± 0.3 (7)	12.3 ± 1.4 (13)	17.1 ± 0.2 (17)
		Butyrate	57.0 ± 3.5 (78)	59.0 ± 6.9 (63)	57.0 ± 1.2 (55)
		Total	73.0 ± 4.3	93.2 ± 11	104 ± 1.4

[a]Results are mean concentrations ± standard deviation (n=2).
[b]Hours of *L. gasseri* administration to V1 of the colon simulator.
[c]Values in parenthesis are molar ratios of individual SCFA.

There is a progressive decrease in the availability of carbon and energy sources from the proximal to the distal colon, resulting in a concomitant decrease in the rates of SCFA formation along the colon (Cummings & Macfarlane, 1991). Accordingly, in this study SCFA formation was maximal in V1 (73 mM). The total SCFA values shown in Table 4.4 are cumulative amounts, therefore, the SCFA produced in V2 and V3 were approximately 20 (93 – 73 mM) and 11 mM (104 – 93 mM), respectively. Butyrate was produced in large amounts in V1, making it the predominant SCFA in all subsequent culture vessels. In contrast, acetate and propionate were produced in relatively equal amounts in all three vessels. Lactate was undetectable in the CCS prior to the addition of *L. gasseri*. Although lactic acid is a fermentation product of numerous

GIT bacteria, including lactobacilli, bifidobacteria, streptococci and enterococci, it is seldom detected as a major fermentation product of mixed anaerobic communities in human faeces or gut contents under normal conditions (Duncan *et al.*, 2004). Lactate-utilising, butyrate-producing bacteria including *Eubacterium* and *Anaerostipes* species have been implicated in this phenomenon (Schwiertz *et al.*, 2002), and these and other related species could have been responsible for the undetectable lactate and high butyrate levels in V1 of the colon simulator.

4.4.2. Assessment of bacteria in the CCF using live/dead staining

As mentioned earlier, the live/dead staining technique was used for quantitative differential counting of the live/dead anaerobic microorganisms in the CCF. The membrane-permeant SYTO 9 (DNA stain) labels live bacteria with green fluorescence, while membrane-impermeable propidium iodide labels membrane-compromised bacteria with red fluorescence (Figure 4.3). Live/dead staining was used to discriminate between live and dead bacteria in the CCF, and thereafter, fluorescent microscopy was used to estimate the proportions of live bacteria in the culture vessels.

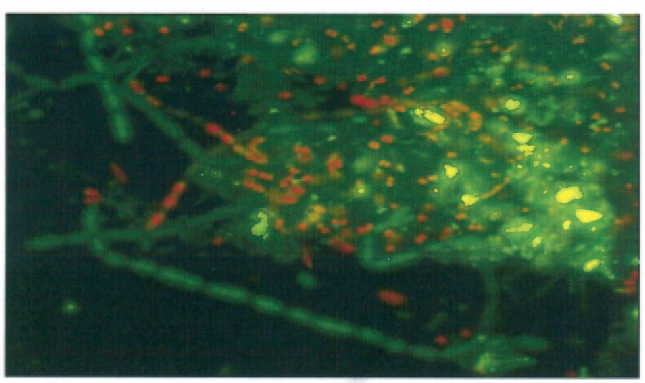

Figure 4.3. Live/dead stained bacterial cells in vessel 2 of CCF at R = 60 h. Live cells are stained green (SYTO 9) and dead bacteria are stained red (propidium iodide). Yellow fluorescence is caused by bacterial aggregates.

Live/dead staining showed that up to 95% of the microbiota in the CCF were viable under the operational culture conditions employed in this study (Table 4.5). Before addition of *L. gasseri* to the colon simulator, the percentage of live bacteria in the CCF was highest in V1, decreasing sequentially in V2 and V3 (Table 4.5). This was the expected result because nutrients depleted sequentially from V1 to V3, as mentioned earlier. After the introduction of *L. gasseri* to the CCF at R = 60 hours, there was a marked reduction in the percentages of live bacteria in all three culture vessels. The overall pattern of V1 having the highest percentage of live organisms, however, remained unchanged. At R = 30 hours, the proportion of live bacteria in all three culture vessels was relatively unaffected by *L. gasseri* administration (Table 4.5). The differences in live/dead counts of the three culture vessels were less pronounced at R = 30 h, as possibly more nutrients were available to the bacteria in V2 and V3 at this shorter system retention time.

Table 4.5. Effect of *L. gasseri* administration on live/dead counts of bacteria in the CCF under steady-state conditions[a]

Retention time	Hours[b]	V1	V2	V3
60 h	0	95 ± 2	90 ± 0	91 ± 1
	168	87 ± 3	78 ± 5	64 ± 2
	336	86 ± 3	80 ± 5	75 ± 2
30 h	0	92 ± 1	86 ± 2	81 ± 2
	96	87 ± 5	85 ± 3	76 ± 6
	168	94 ± 2	85 ± 3	74 ± 3

[a]Results are % live bacteria ± standard deviation (n=5).
[b]Hours of *L. gasseri* administration to V1 of colon simulator.

4.4.3. Bacterial diversity in the simulated colon model

Freshly voided faecal samples for inoculating the CCF were provided by a stone-free healthy white male. Previous studies using FISH have identified the *Bacteroides/Prevotella, Clostridium coccoides/Eubacterium rectale, Ruminoccocus, Eubacterium cylindroides* and *Lactobacillus/Enterococcus* groups, Enterobacteria, as well as *Atopobium* and *Bifidobacterium* species as the major bacterial groups present in faecal DNA (Child *et al.*, 2006; Harmsen *et al.*, 2002). All the oligonucleotide probes used in FISH analysis in this study, which targeted the afore-mentioned range of bacterial groups and species, gave positive fluorescent signals as illustrated in Figure 4.4. The culture-conditions of the CCF were thus able to support growth of the 'normal' microbiota as estimated by FISH. DNA staining using DAPI (blue) was used to distinguish between positive fluorescence and non-specific background staining.

Figure 4.4 (B) shows the bright red positive fluorescent signal of the Bac303 probe, against the dull red haze of background staining. Mixing of the cy3 and FITC labeled probes (red and green, respectively) in single samples, confirmed probe specificities (Figure 4.4 (C) and (D)). Non-specific probe hybridization would have fluoresced yellow (red + green) instead of the clear green and red fluorescence observed. The Clostridium cluster XIVa probe (Erec482) hybridized with a large component of the CCF microbiota in V1 (illustrated in Figure 4.4 (D)), and the earlier-mentioned lactate-utilizing butyrate-producing bacteria, *Eubacterium* and *Anaerostipes* species, are member organisms of this Clostridium cluster XIVa group (Schwiertz *et al.*, 2002). This result further suggested that these species might have contributed to the undetectable lactate and large amount of butyrate produced in V1 of the colon simulator.

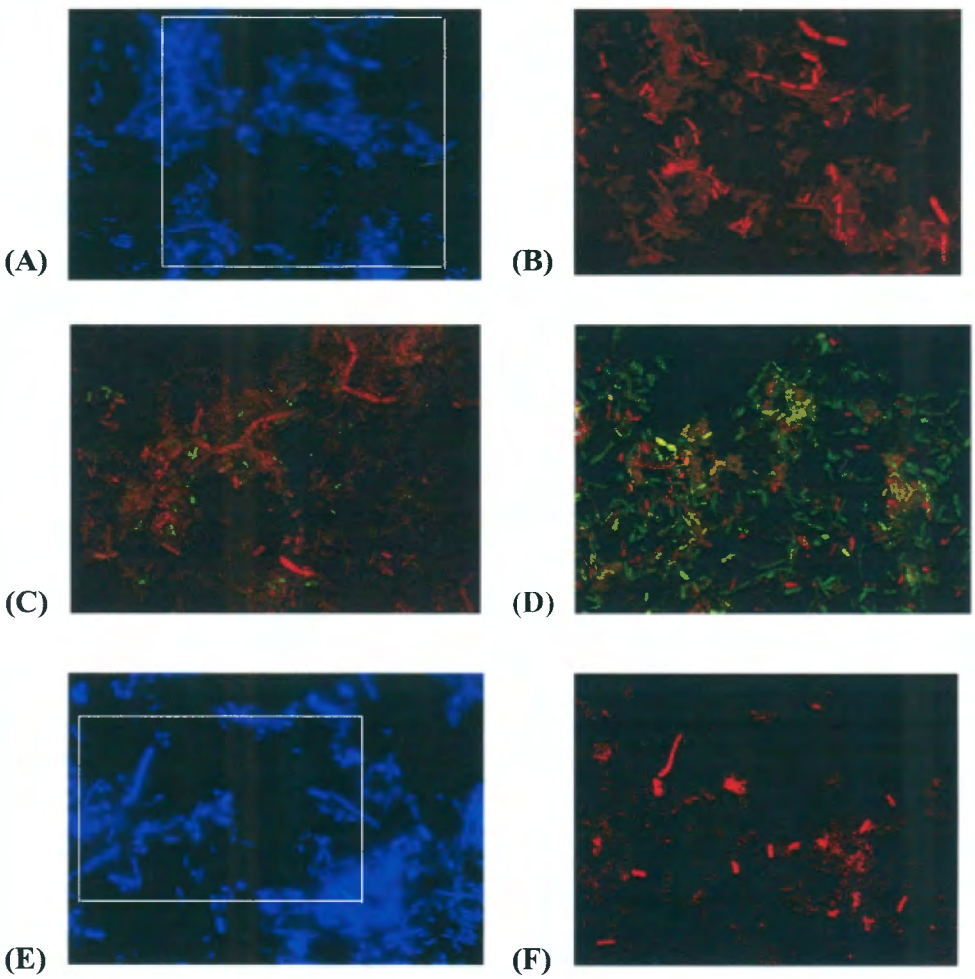

Figure 4.4. FISH images of bacteria from the CCF at retention time 60 h. (A) V3 with Bac303 DAPI stain image. (B) Confocal view (white enclosure from (A)) of V3 with Bac303 showing probe specificity (red). (C) V1 with Ecyl387 (green) and Ato291 (red). (D) V1 with Erec482 (green) and Bac303 (red). (E) V2 with Ato291 DAPI stain image. (F) Confocal view (white enclosure from (E)) of V2 with Ato291 showing probe specificity (red).

Synthetic culture media have been extensively used to quantitate faecal bacterial populations for many years (Finegold *et al.*, 1983; Borriello, 1986; Macfarlane *et al.*, 1998). However, numerous arguments have been advanced on the limitations of the information inferred from studies on culturable faecal microbiota (Conway, 1995; Macfarlane & Macfarlane, 2004). Not all bacteria are culturable thus synthetic culture media have been postulated to overestimate the numbers of important groups of bacteria including bifidobacteria and *Bacteroides* species (Macfarlane & Macfarlane, 2004). In addition, synthetic media often lack the complex nutritional requirements of certain bacteria and are unable to duplicate the highly evolved nutritional syntrophic relationships between some bacteria. Nonetheless, culture-based studies have identified *Bacteroides* species as the most prevalent faecal flora (Moore & Holdeman, 1974; Finegold *et al.*, 1983; Duerden, 1980). Other major bacterial populations have been identified as anaerobic non-sporeforming Gram-positive cocci, eubacteria, bifidobacteria, clostridia and lactobacilli (Finegold *et al.*, 1983).

In this study, culturing studies showed that Gram-positive cocci, lactose-fermenting bacteria, nonlactose-fermenting bacteria, enterococci, bifidobacteria, anaerobic cocci, lactobacilli, and *Bacteroides* species were all viable in the CCF (Table 4.6.). Anaerobic bacteria have been reported to outnumber aerobes and facultative anaerobes considerably in the colon (Simon & Gorbach, 1984). In this investigation, total anaerobes outnumbered total facultative anaerobes approximately 100-fold in the CCF (Table 4.6).

Table 4.6. Enumeration of culturable bacteria in the CCF using selective media[a].

Retention time	Hours[b]	Culture medium	V1 (CFU/ml)	V2 (CFU/ml)	V3 (CFU/ml)
60 h	0	Aerobic blood azide	$1.1 \pm 0.1 \times 10^7$	$7.1 \pm 0.6 \times 10^6$	$7.3 \pm 1.1 \times 10^6$
		Maconkey	$1.6 \pm 0.0 \times 10^6$	$1.1 \pm 0.0 \times 10^5$	$5.5 \pm 2.1 \times 10^5$
		Nutrient agar	$2.0 \pm 0.0 \times 10^7$	$1.7 \pm 0.0 \times 10^7$	$1.4 \pm 0.6 \times 10^7$
		Anaerobic blood azide	$9.5 \pm 0.2 \times 10^7$	$2.0 \pm 0.4 \times 10^8$	$4.6 \pm 0.1 \times 10^8$
		Bacteroides mineral salts	NO GROWTH[c]	$3.2 \pm 1.2 \times 10^7$	$4.9 \pm 0.6 \times 10^8$
		Beerens	$2.5 \pm 0.7 \times 10^7$	$2.4 \pm 0.2 \times 10^7$	$4.5 \pm 0.7 \times 10^6$
		Rogosa	$4.0 \pm 2.8 \times 10^6$	$6.5 \pm 0.7 \times 10^6$	$4.0 \pm 0.0 \times 10^6$
		Wilkins-Chalgren blood agar	$6.0 \pm 1.4 \times 10^8$	$2.5 \pm 0.7 \times 10^8$	$8.0 \pm 2.0 \times 10^8$
	336	Aerobic blood azide	$2.8 \pm 1.3 \times 10^6$	$2.1 \pm 0.1 \times 10^6$	$2.0 \pm 0.0 \times 10^6$
		Maconkey	$2.5 \pm 0.7 \times 10^5$	0	$9.0 \pm 1.4 \times 10^5$
		Nutrient agar	$2.2 \pm 2.6 \times 10^7$	$3.0 \pm 0.0 \times 10^6$	$3.0 \pm 1.0 \times 10^6$
		Anaerobic blood azide	$5.0 \pm 1.4 \times 10^7$	$7.5 \pm 0.2 \times 10^7$	CONTAM[d]
		Bacteroides mineral salts	$3.0 \pm 1.4 \times 10^7$	$2.9 \pm 0.2 \times 10^7$	$7.9 \pm 0.7 \times 10^8$
		Beerens	$2.8 \pm 1.1 \times 10^7$	$8.2 \pm 0.9 \times 10^7$	$2.1 \pm 0.9 \times 10^7$
		Rogosa	$3.6 \pm 0.6 \times 10^6$	$2.5 \pm 2.1 \times 10^6$	$3.0 \pm 0.0 \times 10^6$
		Wilkins-Chalgren blood agar	$3.5 \pm 0.7 \times 10^8$	$4.0 \pm 0.0 \times 10^8$	$1.5 \pm 0.3 \times 10^9$
30 h	0	Aerobic blood azide	$1.5 \pm 0.1 \times 10^6$	$2.1 \pm 0.1 \times 10^6$	$3.2 \pm 0.6 \times 10^6$
		Maconkey	0	0	$5.5 \pm 0.7 \times 10^5$
		Nutrient agar	$2.0 \pm 0.0 \times 10^6$	$2.0 \pm 0.0 \times 10^6$	$3.2 \pm 1.2 \times 10^6$
		Anaerobic blood azide	$8.0 \pm 4.2 \times 10^7$	$4.9 \pm 0.6 \times 10^8$	CONTAM
		Bacteroides mineral salts	NO GROWTH	NO GROWTH	$2.5 \pm 2.1 \times 10^7$
		Beerens	$6.4 \pm 0.2 \times 10^7$	$6.5 \pm 2.1 \times 10^7$	$5.5 \pm 0.2 \times 10^7$
		Rogosa	$3.5 \pm 0.7 \times 10^6$	$2.7 \pm 0.7 \times 10^6$	$3.0 \pm 0.4 \times 10^6$
		Wilkins-Chalgren blood agar	$1.5 \pm 0.7 \times 10^8$	$9.5 \pm 0.7 \times 10^8$	$3.4 \pm 0.4 \times 10^8$
	168	Aerobic blood azide	$2.2 \pm 0.4 \times 10^6$	$1.5 \pm 0.1 \times 10^6$	$7.5 \pm 0.7 \times 10^5$
		Maconkey	$1.0 \pm 0.0 \times 10^5$	0	0
		Nutrient agar	$3.0 \pm 0.0 \times 10^6$	$2.6 \pm 0.0 \times 10^6$	$1.0 \pm 0.4 \times 10^6$
		Anaerobic blood azide	$1.1 \pm 0.0 \times 10^8$	$7.2 \pm 0.5 \times 10^8$	$5.0 \pm 0.3 \times 10^8$
		Bacteroides mineral salts	$3.2 \pm 4.0 \times 10^8$	$1.6 \pm 0.1 \times 10^8$	$1.2 \pm 0.2 \times 10^8$
		Beerens	$1.4 \pm 0.6 \times 10^8$	$2.3 \pm 0.7 \times 10^7$	$1.7 \pm 0.6 \times 10^7$
		Rogosa	$8.0 \pm 1.4 \times 10^5$	$1.8 \pm 0.3 \times 10^6$	$1.3 \pm 0.1 \times 10^6$
		Wilkins-Chalgren blood agar	$3.5 \pm 0.7 \times 10^8$	$1.1 \pm 0.6 \times 10^9$	$2.5 \pm 0.7 \times 10^8$

[a]Results are mean values ± standard deviation (n=2)
[b]Hours of *L. gasseri* administration to V1 of the colon simulator
[c]No growth recorded but presence of *Bacteroides/Prevotella* sp. confirmed using semi-quantitative FISH
[d]Fungal contamination present on plates following incubation

Anaerobic cocci, *Bacteroides* sp. and bifidobacteria were the predominant populations. Enumeration of *Bacteroides* was difficult because no growth was detected on 10^{-4} dilution plates on two sampling days (V1, 0 hours at R = 60 h; V1 and V2, 0 hours at R = 30 h). It remains unclear if *Bacteroides* numbers were undetectable at this dilution, or if the problem lay with the culturing techniques. *Bacteroides* are reportedly fastidious when grown on BMS agar (G.T. Macfarlane, personal communication), and semi-quantitative FISH using the Bac303 probe detected high numbers of *Bacteroides/Prevotella* sp. throughout the feeding study at both retention times (results not shown). Additionally, there was contamination of anaerobic azide plates on two occasions (V3, 336 hours at R = 60 h and V3, 0 hours at R = 30 h). Nonetheless, results obtained from both culture-dependent and culture-independent analyses confirmed that the culture conditions of the colon simulator used in this investigation supported the growth of bacterial groups known to predominate in faecal samples.

4.4.4. Establishment of *L. gasseri* DSM 20243^T in the CCF

At R = 60 hours, V1 of the steady-state CCF was inoculated with a freeze-dried preparation of *L. gasseri* DSM 20243^T, providing 2 x 10^9 viable cells per day. This did not affect SCFA molar ratios of butyrate, acetate and propionate, and lactate was still not detected up to 336 hours at R = 60 hours, and up to 168 hours at R = 30 hours (Table 4.7). Viable counts before and at the end of the feeding study indicated that administration of *L. gasseri* to the CCF did not affect the ratios of the major bacterial populations in the CCF (Table 4.6).

Table 4.7. Effect of *L. gasseri* administration on production of the major SCFA in the CCF under steady-state conditions[a].

Retention time	Hours[b]	SCFA (mM)	V1	V2	V3
60 h	0	Acetate	10.7 ± 0.5 (15)[c]	21.9 ± 2.4 (24)	29.7 ± 0 (28)
		Propionate	5.3 ± 0.3 (7)	12.3 ± 1.4 (13)	17.1 ± 0.2 (17)
		Butyrate	57.0 ± 3.5 (78)	59.0 ± 6.9 (63)	57.0 ± 1.2 (55)
		Total	73.0 ± 4.3	93.2 ± 11	104 ± 1.4
	168	Acetate	14.5 ± 1.7 (18)	22.6 ± 0.1 (24)	35.5 ± 2.2 (30)
		Propionate	6.2 ± 0.6 (8)	11.7 ± 0.1 (12)	18.5 ± 0.1 (16)
		Butyrate	61.0 ± 1.8 (74)	61.0 ± 0.8 (64)	63.0 ± 2.0 (54)
		Total	81.7 ± 4.1	95.3 ± 1.0	117 ± 4.3
	336	Acetate	14.3 ± 1.2 (18)	23.4 ± 0.4 (24)	33.5 ± 3.2 (29)
		Propionate	5.7 ± 0.5 (7)	12.3 ± 0.4 (13)	19.4 ± 1.5 (17)
		Butyrate	60.0 ± 4.0 (75)	61.0 ± 2.8 (63)	62.0 ± 3.1 (54)
		Total	80.0 ± 5.7	96.7 ± 3.6	115 ± 7.8
30 h	0	Acetate	11.0 ± 0.1 (16)	18.0 ± 0.2 (21)	35.4 ± 5.6 (29)
		Propionate	4.7 ± 0.2 (7)	7.5 ± 0.3 (9)	19.4 ± 1.8 (16)
		Butyrate	54.0 ± 2.5 (77)	59.0 ± 2.8 (70)	67.0 ± 2.9 (55)
		Total	69.7 ± 2.8	84.5 ± 3.3	122 ± 10.3
	96	Acetate	12.0 ± 0.2 (16)	23.3 ± 0.8 (26)	34.6 ± 2.5 (32)
		Propionate	4.0 ± 0.1 (5)	6.6 ± 0 (8)	15.4 ± 0.4 (14)
		Butyrate	58.0 ± 2.2 (79)	59.0 ± 0.7 (66)	59.0 ± 0.5 (54)
		Total	74.0 ± 2.5	88.9 ± 1.5	109 ± 3.4
	168	Acetate	13.0 ± 1.7 (18)	33.1 ± 2.9 (33)	34.3 ± 1.7 (34)
		Propionate	3.5 ± 0.2 (5)	8.5 ± 0.4 (8)	12.1 ±0.4 (12)
		Butyrate	54.0 ± 0.1 (77)	59.0 ± 0.7 (59)	54.0 ± 1.1 (54)
		Total	70.5 ± 2.0	101 ± 4.0	100 ± 3.2

[a]Results are mean concentrations ± standard deviation (n=2).
[b]Hours of *L. gasseri* administration to V1 of the colon simulator.
[c]Values in parenthesis are molar ratios of individual SCFA.

Confirmation of the successful establishment of *L. gasseri* in the CCF was obtained through viable cell culture and quantitative Real-Time PCR (qRT-PCR). Prior to *L. gasseri* inoculation, only one colony morphology of a *Lactobacillus* species was identified on Rogosa agar when

samples were plated from all three culture vessels of the CCF. After addition of *L. gasseri*, a second distinctive colony morphology was identified on the *Lactobaciilus*-selective Rogosa agar. The second colony morphology was that of a spreading colony with erose edges, distinctive of *L. gasseri* DSM 20243^T growing on this culture medium. Partial sequencing of the 16S rRNA and *oxc* genes verified that these colonies were *L. gasseri* DSM 20243^T. The freeze-dried probiotic candidate preparation had therefore remained viable during production, storage and use; all of which are desirable characteristics for its biotechnological manipulation.

Before addition of *L. gasseri* to the CCF, the number of culturable lactobacilli in all three culture vessels was approximately 10^6 cfu/ml (Table 4.6). During the course of *L. gasseri* administration, lactobacilli counts ranged between 10^6-10^7 cfu/ml in all three vessels, but the increases were not statistically significant at either retention time. Culturable bifidobacteria numbers remained steady at 10^7-10^8 cfu/ml throughout the study (Table 4.6). Quantitative Real-time PCR (qtRT-PCR) results confirmed that lactobacilli and bifidobacteria numbers remained relatively stable throughout the feeding experiment (Table 4.8).

The primer set used to amplify lactobacilli was the *L. acidophilus* group primer set that amplifies the 16S rRNA gene of *L. acidophilus, L. amylovorous, L. amylolyticus, L. crispatus, L. gasseri* and *L. johnsonii.* The *L. acidophilus* group is abundant in the human gut and is routinely used to quantify lactobacilli in qtRT-PCR (Nadkarni *et al.*, 2002). At R = 60 hours, total lactobacilli numbers in V1 and V2 increased by two orders of magnitude after 168 hours, but they remained approximately the same in all the culture vessels when R was decreased to 30 h. At R = 60 hours, numbers of *L. gasseri* in the CCF increased by at least three orders of magnitude after 168 hours (Table 4.8). When retention time was reduced to 30 hours, *L. gasseri* numbers increased

only in V1 after 96 hours. This was not unexpected as bacterial cell washout is reportedly higher at shorter system retention times (Conway, 1995).

Table 4.8. Real-time PCR enumeration of lactobacilli and bifidobacteria in the CCF under steady-state growth conditions[a]

Target species	Retention time	Hours[b]	V1	V2	V3
L. gasseri	60 h	0	1.0×10^2	1.3×10^2	2.2×10^3
		168	2.3×10^5	2.5×10^5	5.9×10^5
		336	1.6×10^5	2.5×10^5	1.4×10^5
	30 h	0	1.8×10^2	2.2×10^4	3.1×10^4
		96	2.8×10^4	3.5×10^4	4.6×10^5
		168	4.8×10^3	6.2×10^4	7.5×10^4
L. acidophilus group	60 h	0	2.9×10^2	3.7×10^2	1.6×10^4
		168	2.6×10^4	1.8×10^4	1.6×10^5
		336	2.7×10^4	4.1×10^4	3.1×10^4
	30 h	0	3.5×10^2	2.3×10^3	1.1×10^4
		96	8.4×10^3	4.3×10^3	1.5×10^5
		168	1.5×10^3	7.6×10^3	2.0×10^4
Bifidobacterium	60 h	0	0.91×10^1	0.49×10^1	2.08×10^2
		168	1.64×10^2	0.51×10^2	4.01×10^2
		336	4.79×10^2	7.79×10^1	3.38×10^2
	30 h	0	0.82×10^2	1.16×10^2	2.47×10^2
		96	0.93×10^2	0.42×10^2	8.31×10^2
		168	0.30×10^1	0.18×10^2	0.17×10^2

[a]Results are expressed as gene copies per 1×10^7 copies of eubacterial control DNA.
[b]Hours of *L. gasseri* administration to V1 of the colon simulator.

There were some discrepancies noted in the *L. acidophilus* group vs. *L. gasseri* numbers obtained with qtRT-PCR (Table 4.8). In some instances, *L. gasseri* numbers exceeded total *L. acidophilus* group numbers, although the former is a member organism of the latter group. Since the *L.acidophilus* group primer set is based on the 16S rRNA gene, while the *L. gasseri* primer set used was based on the *oxc* gene, the discrepancies could be attributed to differences in primer

efficiencies for the respective PCR amplifications. The *oxc* gene primer set designed in this study was specific for the *L. gasseri oxc* sequence, as illustrated by the melting curve analysis of the *L. gasseri oxc* gene qtRT-PCR amplification products from CCF samples (Figure 4.5).

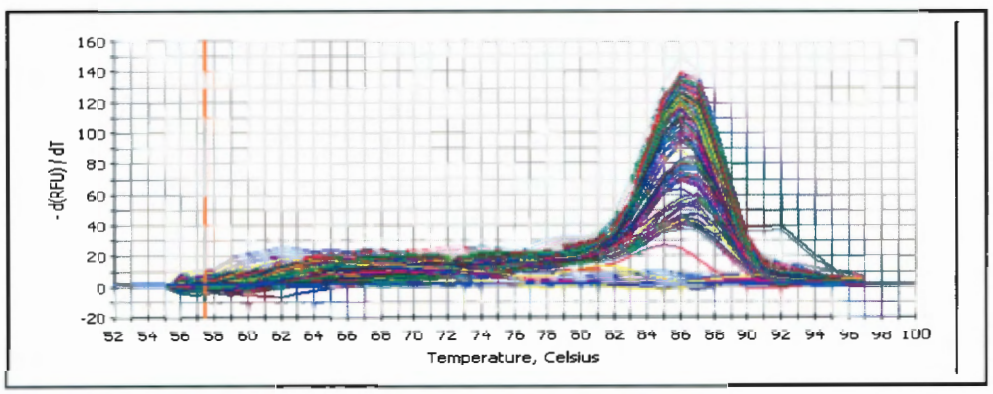

Figure 4.5. Melt curve graph for qtRT-PCR amplification products of CCF samples using *L. gasseri oxc* gene-specific primers.

4.4.5. Oxalate-degrading activities of *L. gasseri* DSM 20243^T in the CCF

During the establishment of steady-state conditions at R = 60 h, baseline oxalate degradation in V1 of the CCF was 4% (0 hours, Table 4.9.). After 168 hours of feeding *L. gasseri* to the CCF, oxalate-degrading activity had increased to 41%. This increase in oxalate-degrading activity is concomitant with the 1000-fold increase in *L. gasseri* numbers in V1 (Table 4.8). The increased rates of oxalate breakdown remained at approximately 40% when measured after 336 hours of feeding at this retention time. When R was reduced to 30 hours, the CCF was operated for 144 hours before feeding of *L. gasseri* recommenced. *L. gasseri*, nonetheless, was not flushed out of the CCF and was still detected via culture and qRT-PCR (Day 0, R = 30 hours, Table 4.8).

Table 4.9. Oxalate-degrading activity in V1 of the CCF under steady-state conditions.

Retention time	Hours[b]	Oxalate (mM)	% Oxalate-degrading activity
60 h	0[c]	9.0 ± 0.12	4.0 ± 1.3
	168	5.3 ± 0.30	41 ± 3.3
	336	6.1 ± 0.75	32 ± 8.3
30 h	0	7.5 ± 0.29	20 ± 3.1
	96	7.5 ± 0.27	20 ± 2.9
	168	7.7 ± 0.47	18 ± 5.0

[a]Results are means ± SD (n=2).
[b]Hours of *L. gasseri* administration to V1 of the colon simulator.
[c]Growth medium oxalate concentration after autoclaving was 9.4 ± 0.19 mM.

At R = 30 hours, oxalate-degrading activity did not increase above 20%, the baseline value recorded at this retention time until 168 hours (Table 4.9). This was possibly due to reduced numbers of *L. gasseri* at R = 30 hours (Table 4.8). Alternatively, greater substrate availability and higher imposed specific growth rates at R = 30 hours may have affected *L. gasseri* 20243^T physiology, affecting its abilities to metabolise oxalate. This observation warrants further investigation, because decreased oxalate degradation in continuous cultures when the retention time is reduced has also been reported for *O. formigenes* strain Va3 (Duncan *et al.*, 2002).

Oxalate degradation by *L. gasseri* specifically could not be measured in V2 and V3 because these two vessels had endogenous oxalate-degrading activity approximating 95% under steady-state culture conditions prior to addition of *L. gasseri* 20243^T to the CCF (results not shown). All three culture vessels (V1, V2 and V3) were inoculated with the same faecal inoculum, and only differed in their operational pH values. The stability of sodium oxalate in the culture medium at these pH values was tested to investigate if pH effects were responsible for the high level of intrinsic oxalate-degrading activities observed in V2 and V3. Three preparations of culture

media were made as described in the Materials and methods section, at final pH values of 5.5 (V1), 6.2 (V2) and 6.8 (V3), respectively. The results obtained showed that pH effects alone did not account for the observed phenomenon (Table 4.10).

Table 4.10. Effect of pH on stability of sodium oxalate in culture medium[a].

pH of Culture Medium	Oxalate concentration (mM)
5.5 (V1)	10.8 ± 0.99
6.2 (V2)	10.9 ± 0.21
6.8 (V3)	10.1 ± 1.27

[a]Results expressed are mean ± SD (n=2).

Further investigations using cell-free extracts of culture supernatant from V2 and V3, revealed the presence of a substance in these vessels, which immediately removed any oxalate introduced to the cell-free supernatants obtained from these culture vessels. When 1 ml of 50 mM sodium oxalate was added to 4 ml of cell-free supernatants from V2 and V3 (final concentration 10 mM sodium oxalate), only 0.8 mM sodium oxalate was immediately detectable by GC. This oxalate-removing substance in V2 and V3, was absent from V1, and is yet to be identified. The immediate degradation of oxalate measured in V2 and V3 is of interest since it may indicate the presence of intrinsic oxalate degrading capacity in the microbiota established in these regions of the CCF.

4.5. CONCLUSIONS

The results of this investigation show that *L. gasseri* DSM 20243^T has oxalate-degrading capacity under colon-simulated conditions, and could play a significant role in the metabolism of this compound in humans. As discussed in Chapter 1, the colon is the major site of oxalate absorption in the body, therefore any probiotic used to prevent kidney stone disease would have to survive passage to the large bowel and establish itself there, at least temporarily. A candidate probiotic preparation of *Lactobacillus gasseri* DSM 20243^T was shown to have good survival properties in the colon-simulated conditions employed in this study. In addition, *in situ* studies have reported excellent survival properties of *L. gasseri* in humans (Conway *et al.*, 1987). Most of the probiotic benefits attributed to lactobacilli have largely resulted from work on *L. acidophilus,* however, recent advances in molecular taxonomy have identified six distinct species of *Lactobacillus* that comprise what is now referred to as the 'acidophilus group' (Kullen *et al.*, 2000). Amongst these species is *L. gasseri,* reportedly the most dominant strain of the acidophilus group found in the human GIT (Benno *et al.*, 1989 cited in Saito, 2004).

As mentioned in Chapter 1, reducing oxalate levels in the diet is one of the measures taken to reduce hyperoxaluria (Siener *et al.*, 2005). Unlike the specialist oxalotroph *O. formigenes,* which is most likely to require co-administration of oxalate to facilitate colonization of the gut when administered as a probiotic, *L. gasseri* could become established in the colon despite oxalate-reduced diets, due to its greater metabolic flexibility. Moreover, this organism is known to be resistant to a number of antibiotics (Cho *et al.*, 2000), making it a robust alternative to the reportedly antibiotic-sensitive *O. formigenes* (Duncan *et al.*, 2002; Sidhu *et al.*, 1998).

In summary, while the results of all investigations using *in vitro* models of the human colon should be interpreted with caution given the limitations of these models, they undoubtedly provide valuable information on the metabolism of the gut microbiota. *L. gasseri* DSM 20243^T fulfils many of the requirements of a probiotic candidate. It was originally isolated from the human gut, it is non-pathogenic, non-toxic, viable during use and has good colonisation potential in the GIT. The current study has demonstrated its survival under simulated colonic conditions with the ability to exert a potentially beneficial effect through the degradation of oxalate. Accordingly, *L. gasseri* DSM 20243^T may be of utility in the prophylactic treatment of kidney stone disease.

CHAPTER 5

General Conclusions

The first major aim of the work reported in this thesis was to study the faecal microbiota of a sample population of black and white South Africans, with respect to oxalate-degrading bacteria. It was postulated that qualitative differences in oxalate-degrading gut bacteria could explain the lower incidence of kidney stone disease in the black South African population, relative to the white population. Most investigations on oxalate-degrading bacteria playing a role in the prevalence or reduction of kidney stone disease have focussed on the specialist oxalotroph *O. formigenes*, as described earlier. The present study gives the first report on investigations into the possible role of generalist oxalate-degrading bacteria in explaining South African kidney stone statistics.

The results of the preliminary investigations of this study indicated that *O. formigenes* could play a role in the lower incidence of kidney stone disease in black South Africans, relative to white South Africans. The 70% incidence of *O. formigenes* in the black South African population was within the 60 – 80% reported for other stone-free populations, as discussed earlier. However, it must be recognized that the sample size of twenty individuals (ten each) used in this pilot study is statistically small, and these results may not be entirely reflective of the entire South African male population. Accordingly, the incidence of *O. formigenes* in South African males is being investigated in an ongoing study involving ninety South African males, and the present investigator is involved in this research. The ninety test subjects in the afore-mentioned study comprise thirty stone-free black subjects, thirty stone-free white subjects and thirty stone-forming

white subjects. Furthermore, quantitative Real-Time PCR will be used to determine the numerical predominance of *O. formigenes* in the black and white South African males. The results from these analyses will provide useful information based on a more statistically representative sample size, and additional knowledge on the oxalate-degrading bacteria of stone-free vs. stone-forming white South Africans will be obtained.

Lewandowski (2003) suggested that a quantitative difference in lactobacilli contributed to the differences in kidney stone disease prevalence in black and white South Africans. In the present study, qualitative rather than quantitative differences in oxalate-degrading lactic acid bacteria and bifidobacteria were evaluated to explain the seemingly anomalous low incidence of kidney stones in the black South African population, relative to the white population. Qualitative differences were noted in the predominant culturable lactic acid bacteria in the two population groups, as determined by oxalate degradation assays and DGGE analyses. Furthermore, the hypothesis that generalist oxalate-degrading species play a significant role in human oxalate homeostasis was also supported by the finding that some individuals, in whom *O. formigenes* was undetectable, had culturable lactic acid bacteria and bifidobacteria with very high oxalate-degrading capacities. These results, hitherto unreported for South African and other populations, could provide better understanding of the importance of generalist oxalate-degrading gut bacteria in human oxalate homeostasis. The experiments of the present study will have to be repeated on a larger sample population before more conclusive inferences are made regarding the potential role of lactic acid bacteria and bifidobacteria in addressing South African kidney stone statistics. In addition, these investigations would also have to consider the biodiversity of bifidobacteria, in addition to lactic acid bacteria, in contributing to differences in oxalate-degrading abilities.

The second major aim of the work presented in this study was the identification and characterisation of novel oxalate-degrading bacteria for potential use as probiotics in the prophylaxis of kidney stone disease. The identification of novel oxalate-degrading bacteria was attempted using two major strategies. In the first approach, the rarity of kidney stone disease amongst black South Africans made the faecal microbiota of this population a potential reservoir of novel oxalate-degrading species. The faecal microbiota from a black test subject were grown on an oxalate-enriched medium, and random colony selection resulted in the isolation of two novel oxalate-degrading bacteria, an *E. coli* strain and a *C. innocuum* isolate. These results showed that the black South African population harboured novel oxalate-degrading species, and this warrants further investigations into the possible role of uncharacterised bacterial species in explaining South African kidney stone disease statistics. Parallel studies would need to be undertaken on the faecal microbiota of black and white South African populations, for a complete comparative analysis.

The *E. coli* strain isolated in this study lost oxalate-degrading ability upon repeated sub-culture *in vitro*. This phenomenon, also seen in the *E. faecalis* strain isolated by Hokama *et al.* (2000) and the *P. rettgeri* strain isolated by Hokama *et al.* (2005), presents an intriguing question on the possible regulatory mechanisms involved in the catabolism of oxalate by these bacteria *in vivo*. The putative *E. coli frc* gene encodes a functional acyl-CoA transferase that utilises oxalate as a substrate (Gruez *et al.*, 2003). It can, therefore, be assumed that the putative *oxc* gene encodes a functional oxalyl-CoA decarboxylase; but functionality studies still have to be done on the *E. coli oxc* ortholog cloned in this study to prove this. Thereafter, studies focussing on the promoter(s) of the putative oxalate-degrading genes of the *E. coli* strain isolated in this study could help in resolving the regulatory mechanisms involved in this bacterium's metabolism of oxalate.

Very little is known about the *in vivo* regulation of seemingly 'silent' genes that have been identified in the genomes of *E. coli* and other bacteria. However, a few mechanisms have been described that could possibly explain the *in vivo* activation of the putative *frc-oxc* 'operon' identified in *E. coli*-OX. Promoter regulation by invertible regions of DNA, for example, could possibly explain the observed loss of the oxalate-degrading phenotype in the *E. coli* strain isolated in the current study. Invertible regions of DNA located within or near promoter regions have been shown to act as "on-off" switches in the "flip-flop" transcriptional regulation of genes. In *Bacteroides fragilis*, for instance, invertible DNA regions (*fin*) are reportedly responsible for the "flip-flop" regulation of numerous sets of genes, including the genes involved in polysaccharide antigenic variation (Patrick *et al.*, 2003; Cerdeño-Tárraga *et al.*, 2005). Polysaccharide antigenic variation is seemingly a mechanism this bacterium has evolved to evade detection by its host immune system. Similar invertible regions of DNA have been identified in phase variation of type 1 fimbriae in *E. coli* (Abraham *et al.*, 1985), and flagellar phase variation in *Salmonella typhimurium* LT2 (Kutsukake *et al.*, 2006). The *B. fragilis fin* regions were identified in the whole-genome sequencing project of this bacterium (Cerdeño-Tárraga *et al.*, 2005; Patrick et al., 2003). Accordingly, sequencing the promoter region of the putative *frc-oxc* operon of oxalate degrading vs. non-oxalate-degrading strains of *E. coli*-OX, could be used to investigate the possible role of invertible DNA sequences in this bacterium's regulation of oxalate metabolism.

Promoter activation by insertional sequence (IS) elements is an alternative explanation for the oxalate-degrading phenotype observed only in freshly isolated *E. coli*-OX in the present study. The *E. coli bgl* operon is an example of an otherwise 'silent' operon that requires activation by

insertional mutations of the promoter regions in wild-type strains (Schnetz & Rak, 1988). The genes of the *bgl* operon encode enzymes involved in the uptake and metabolism of aryl β-glucosides. The *E. coli bgl* operon is activated by integration of IS5 elements into its promoter region. Thereafter, the activated operon is subject to induction by substrate. A similar transient activation may have occurred when the wild-type *E. coli*-OX isolated in this study was grown on oxalate-enriched medium. This activation could perhaps have been followed by excision of the 'activating' IS element from the promoter when the bacterium was sub-cultured on non-oxalate-containing medium. Alternatively, oxalate metabolism could be regulated by the action of more specific protein transcriptional factor, which was inactivated by *in vitro* culture conditions.

Although oxalate-degrading metabolic properties have not been described for *E. coli* and *C. innocuum* prior to this study, these two species were undesirable candidates for further characterisation as probiotic candidates in managing kidney stone disease. Consequently, the second approach taken to isolate novel probiotic candidates focussed on GRAS lactobacilli as a reservoir. The *O. formigenes oxc* gene was used to screen lactobacilli genomes *in silico*, which led to the identification of a novel oxalate-degrading strain of *L. gasseri*. Bioinformatic screening proved to be the more effective approach because it drew from a pool of organisms that are already administered as probiotics to treat a variety of disorders, and are also amenable to biotechnological manipulations.

The high oxalate-degrading capacities reported for the culturable lactic acid bacteria and bifidobacteria isolated from the black population in the present study, make these bacteria an excellent pool of potentially novel oxalate-degrading *Lactobacillus* and *Bifidobacterium* species.

Some oxalate-degrading lactobacilli (*L. acidophilus* and *L. gasseri*) and bifidobacteria (*B. lactis*) have recently been shown to contain oxc *genes*, which appear to be involved in these bacteria's catabolism of oxalate. For this reason, colony hybridisation studies using bifidobacteria- and lactobacilli-specific *oxc* DNA probes could be used to identify *oxc* gene-containing colonies from the MRS pools of lactobacilli and bifidobacteria generated from the test groups in the present study. This strategy could prove to be quicker and less expensive than a physiological random screening of colonies.

The oxalate-degrading ability of *L. gasseri* DSM 20243^T under colon-simulated conditions reported in this study, presented the first such study of a generalist oxalotroph. Most investigations on generalist oxalate-degrading probiotic candidates have involved human consumption of bacterial preparation containing at least four species of lactic acid bacteria and bifidobacteria. While these studies have shown that urinary oxalate levels are reduced in the test subjects, the contributions of the individual bacteria remain poorly characterised. Consequently, the combination of species used in these preparations does not necessarily represent the most efficacious probiotic product. The use of *in vitro* human simulations with single probiotic candidates, on the other hand, provides valuable information on the oxalate-degrading capacity of specific generalist oxalatrophs in the presence of normal gut microbiota, as well as other preferential substrates. Such studies would allow preparation of probiotics with higher oxalate-degrading efficiencies, which could then be tested *in vivo*.

The results obtained in this study showed that *L. gasseri* DSM 20243^T had significant oxalate-degrading ability under simulated gut conditions. These results provided a viable probiotic

candidate for use in managing kidney stone disease. However, further characterisation of this bacterium is needed to address some of the limitations encountered in the present study. The *L. gasseri* probiotic candidate had approximately 40% oxalate degrading capacity at retention time (R) 60 hours, which dropped to 20% when R was reduced to 30 hours. It was hypothesised that the reduction in retention time affected the oxalate-degrading capacity of *L. gasseri*, as reported in *O. formigenes* oxalate degradation fermentor studies. In the present study, however, *L. gasseri* could not be washed out of the continuous culture fermentor (CCF) after the experiments at R = 60 hours, and the results of the shorter retention time may have consequently been compromised. To address this problem, and ascertain if retention time affects *L. gasseri*'s ability to metabolise oxalate, the experiments reported in this study will have to be repeated using separate fermentors for each retention time.

Furthermore, the operational parameters of the CCF could also be varied, including oxalate concentration in the culture medium, retention time and faecal inocula to allow for greater comparative analyses. A more extensive quantitative analysis on the effects of *L. gasseri* administration on the gut microbiota could also be undertaken using an extensive set of FISH oligonucleotide probes and real time PCR primers. The results obtained from such investigations would provide invaluable information for the further development of *L. gasseri* DSM 20243^T as a probiotic candidate. Clinical trials with well-controlled human subjects would then be the ultimate test of the use of *L. gasseri* DSM 20243^T, as an alternative to *O. formigenes*, in the management of kidney stone disease.

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