Evaluation of five *Bifidobacterium* isolates as potential probiotics and genetic analysis of their ability to withstand oxidative stress

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LIST OF ABBREVIATIONS

A	adenosine
BaT	*Bifidobacterium adolescentis* ATCC 15703^{T}
BSA	bovine serum albumin
bp	base pair
C	cytosine
CFE	cell free extract
cfu	colony forming units
Cm^{R}	Chloramphenicol resistance
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
Em^{R}	erythromycin
G	guanosine
g	gram
Hrs	hours
LB	Luria-Bertani medium
Log	logarithmic
Min	minutes
M	molar
NADH	nicotinamide adenine dinucleotide (reduced form)
NCBI	National Centre for Biotechnology Information
Nm	nanometers
OD_{600nm}	optical density measured at wavelength of 600 nm
P	plasmids
PCR	polymerase chain reaction
G+C	guanine + cytosine
GIT	gastrointestinal tract
GRAS	generally regarded as safe
Hsp60	heat shock protein
IPTG	isopropyl-µ-D-thiogalactopyranoside
kb	kilo base
LAB	lactic acid bacteria
PCR	polymerase chain reaction
rRNA	ribosomal RNA
T	thymidine
µ	micro
â	beta
á	alpha

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Abstract

The diverse microbiota of the human gastrointestinal tract plays a major role in the general health of humans. A number of bacterial strains with distinctive properties have been isolated and used commercially as probiotics in order to harness these health benefits and offer them to unhealthy hosts. There are set criteria that have to be followed before new probiotics can be introduced into the market. These criteria help regulate this rapidly growing industry of probiotics and help to prove the efficacy of the probiotic products.

A faecal sample from a healthy donor was found to have numerous *Bifidobacterium* isolates with extremely high sucrase activity. Five *Bifidobacterium* isolates were randomly selected from this sample for further characterisation as potential probiotics with the ability to utilize fructo-oligosaccharide substrates in the gut. Phylogenic identification of the isolates to the species level was carried out using sequences of the 16S rRNA gene, 16S-23S rRNA gene spacer region, the heat shock protein (*hsp60)* and Elongation factor Tu (*tuf*). The sequences of all these genes showed the highest percentage identity to *Bifidobacterium adolescentis* ATCC 15703^T which implied that all of the isolates were *Bifidobacterium adolescentis* strains. Sequence alignments of some of these genes and DNA fingerprinting profiles from Randomly Amplified Polymorphic DNA provided evidence suggesting these isolates were possibly novel strains of *B. adolescentis* as differences were detected between the isolates themselves and *B. adolescentis* ATCC 15703^T.

The ability of the isolates to utilise oligosaccharides with the sucrose moiety at the end of the molecule was investigated. The 5 isolates were found to grow differently

in different carbohydrates and also found to be extremely efficient at hydrolysing these carbohydrates. The presence of genes encoding β-fructo-furanosidase and sucrose phosphatase, enzymes suspected of being responsible for the high sucrose activity, was confirmed by PCR.

When evaluating the potential of the 5 *B. adolescentis* isolates for use as probiotics, all 5 isolates and *B. adolescentis* ATCC 15703^T were unable to grow at low pH, but isolate *B. adolescentis* B95 and *B. adolescentis* ATCC 15703^T remained viable after 5 hours at pH 2. Isolates B92, B94 and *B. adolescentis* ATCC 15703^T showed minimal growth in media containing 0.5% and 1% bile salts. All of the isolates, with the exception of isolate B91 and B93, were found to remain viable after 5 hours of exposure to 0.5% bile salts. Although the isolates showed low levels of adhesion potential, isolate B91 and B93 showed the highest adhesion potential. All the isolates were found to be resistant to kanamycin and streptomycin and were also found to inhibit the growth of more than half of the pathogen bacterial strains used.

Only *B. adolescentis* ATCC 15703^T was able to grow to a small extent in aerobic conditions but isolates B91, B93 and *B. adolescentis* ATCC 15703^T were viable after 2 hours of aerobic exposure. The presence of an oxidative stress response system in *B. adolescentis* was, therefore, investigated. The presence of *trxB (*encoding thioredoxin reductase) and *baiC* (encoding NADH oxidoreductase) genes in all the isolates and *B. adolescentis* ATCC 15703^T was confirmed by bioinformatics and PCR. The *trxB* genes from *B. adolescentis* isolate B93 and *B. adolescentis* ATCC 15703^T were cloned and expressed in *Escherichia coli* JM109 to determine the role of thioredoxin reductase in oxidative stress response. Several attempts were also made to clone the same *trxB* gene into a *Lactococcus lactis trxB* mutant strain to prove functionality by complementation of the mutant.

Chapter 1: Introduction to study

Literature Review

Contents

1.1 Concept of probiotics

The human gastrointestinal tract (GIT) is sterile at birth but acquires microorganisms throughout the human life (Collins *et al.,* 1998). The Nobel Prize winner, Elie Metchnikoff, was one of the first people to hypothesize in the 1900's, that these natural gut colonizing microbes offer health benefits to the host (Parvez *et al.,* 2006). The concept of probiotics came from the attempts to exploit these potential health benefits of natural microorganisms. Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts (Borriello *et al.,* 2003; Perez-Conesa and Lopez, 2005; Sullivan and Nord, 2002).

Some of the health benefits attributed to the presence of these microorganisms include stimulation of the immune response, reduction of the risk of cancer and serum cholesterol, alleviation of symptoms of lactose-intolerance and the prevention of the colonisation and growth of pathogens in the gastrointestinal tract (GIT) (Perez-Conesa and Lopez, 2005; Ward and Roy, 2005). Probiotics are said to improve digestion and increase the natural resistance to infectious disease (Collins and Gibson, 1999). Extensive research is being conducted to confirm these proposed health benefits.

There are several requirements for strains to qualify as probiotics. One of the requirements is that the strains should be isolated from the same species as its intended use. The probiotics must remain viable when passing though the human stomach and its harsh conditions. They then have to colonise the lower GIT in large numbers and still be able to metabolize (Jayamanne and Adams, 2006). In addition to exerting beneficial effects on the host, the probiotics should be non-pathogenic and should not produce any toxins (Collins and Gibson, 1999). It is also important for

probiotics to have antimicrobial activity (Yazid *et al*., 2000). During manufacturing, storage and usage, the probiotics strains should remain viable (Collins and Gibson, 1999).

Once bacterial strains have been proven not to cause any harm to humans, they are classified as Generally Recognised as Safe (GRAS). Lactic acid bacteria (LAB) have the GRAS status and a history of safe consumption in fermented products and dietary supplements (Leahy *et al.,* 2005). LAB strains such as *Lactobacillus acidophilus* have been safely used for more than 70 years (Salminen *et al.,* 1998). Microorganisms that are commonly used as probiotics include various species of the genera *Lactobacillus, Bifidobacterium, Streptococcus and Saccharomyces* (Collins *et al.,* 1998). Some of the strains used as probiotics are shown in Table 1.

Table 1 Most commonly used LAB used in probiotic preparations (adapted from Parvez *et al.,* 2006)

Lactobacillus *sp.*	**Bifidobacterium *sp.***	**Enterococccus *sp.***	***Streptococcus* sp.**
L. acidophilus	*B. bifidum*	*E. faecalis*	*S. cremoris*
L. casei	*B. adolescentis*	*E. faecium*	*S. salivaris*
L. delbrueckii spp. (bulgaricus)	*B. animalis*		*S. diacetylactis*
L. celliobiosus	*B. thermophilum*		*S. intermedius*
L. curvatus	*B. longum*		
L. fermentum			
L. lactis			
L. plantarum			
L. reuteri			
L. brevis			

1.2 Bifidobacteria

Bifidobacteria are anaerobic, Gram positive bacteria with a high G + C content and rod-like shape with a characteristic Y-shaped (bifid) end (Scardovi, 1986). They are commonly found in the GIT of humans and animals and the human oral cavity and vagina. Bifidobacteria are members of the lactic acid bacteria (LAB) group, which constitute a significant portion of the probiotic human intestinal microbiota and produce lactic acid as the major end product during fermentation of carbohydrates. The *Bifidobacterium* genus, a member of the family *Bifidobacteriaceae* and order *Bifidobacteriales,* currently consists of 32 species (Collins *et al*., 1998; Yu *et al.* 2009, Zhi *et al*., 2009).

Bifidobacteria are among the first colonizers of the neonatal intestines and dominate the microbiota of the full-term neonate especially in breast-fed babies (He *et al.,* 2001; Edwards and Parrett, 2002). Bifidobacteria approximately make up 3-6% of the adult microbiota (Schell *et al.,* 2002) which is in excess of 10^{10} cells per gram of adult intestinal contents but these numbers are believed to decrease with age. *Bifidobacterium adolescentis a*nd *Bifidobacterium longum* are the predominating strains in adults (Hopkins *et al.,* 1998).

1.2.1. Bifidobacteria as probiotics

A number of characteristics have resulted in the use of bifidobacteria as probiotics. These probiotic activities include the reduction of cholesterol, the lowering lactose intolerance, and reduction of health risks such as dental caries, allergies and cancer. Other activities include reduction of bacterial and viral diarrhoea, and the alleviation

of chronic inflammatory diseases such as pouchitis and ulcerative colitis (Marco *et al*., 2006).

Bifidobacterium strains have been shown to secrete antimicrobial compounds. For instance, a protein factor produced by *Bifidobacterium* probiotic strain has been suggested to prevent infection by out-competing pathogenic viruses or bacteria for binding sites on epithelial cells (Picard *et al.,* 2005). Bifidobacteria contribute to the acidity of the large intestinal tract by hydrolyzing indigestible complex carbohydrates into acetic and lactic acid. These acids influence the microbial balance in the GIT and inhibit pathogen growth (Yazid *et al*., 2000).

1.3 Prebiotics and Synbiotics

The supply of fermentable carbohydrates is an important growth-limiting factor as the probiotics strains have to remain viable and metabolically active upon reaching the GIT. Simple sugars are absorbed efficiently in the small intestines but complex carbohydrates are not digested by humans and, therefore, pass through to the lower part of the GIT (Collins *et al.,* 1998). These complex carbohydrates serve as substrates for the growth of GIT microbiota and can be termed prebiotics.

Prebiotics are described as non-digestible food ingredients that selectively stimulate the growth and/or activity of one or a limited number of bacteria in the colon, thereby beneficially affecting the host by improving health. Natural or synthesised complex carbohydrates are used commercially as prebiotics to enrich the growth of desired probiotic strains (Dunne *et al.,* 2001; Perez-Conesa and Lopez, 2005).

Synbiotics is the combined use of probiotics and prebiotics. This is believed to beneficially affect the host by improving the survival and implantation of live microbial

dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (Gibson and Roberfroid, 1995).

1.4 Carbohydrate preference and utilisation

Carbohydrates play an important role in the GIT of humans and besides their physiological effect; they also affect the gut ecosystem. This significantly contributes to the well-being of humans. The ability of bifidobacteria to digest an oligosaccharide is determined by the type, number and sequence of the component monosaccharide (Roberfroid *et al.,* 1998). For instance, carbohydrates with a degree of polymerization of less than ten monomers are fermented twice as fast as those consisting of more than ten monomers (Hopkins *et al.,* 1998). This is an indication of the specificity and substrate preference in *Bifidobacterium* spp. Once the oligosaccharides and polysaccharides are depolymerised down to their monomeric constituents, they are incorporated into the fructose-6-phosphate shunt for energy production (Amaretti *et al.,* 2007).

A number of oligosaccharides cannot be digested by mammalian enzymes and these carbohydrates are available in the colon for bacterial growth. The sucrose subunit is common in most of these carbohydrates and those used commercially as prebiotics (Kullin *et al*, 2005). Sucrose is a disaccharide made up of fructose and glucose. Sucrose is the most abundant disaccharide in the environment because it is widespread in higher plant tissues (Reid and Abratt, 2005). For instance, raffinose is a soybean oligosaccharide that contains a galactose unit linked to a sucrose unit. The other example is Raftilose P95, which is a fructo-oligosaccharide derived from partially hydrolyzed chicory root inulin with *n*-repeating fructose units ranging

between 2 and 8 and joined by β (2, 1) bonds with a terminal glucose unit (Kullin *et al*, 2005). Probiotics that can utilize the sucrose unit can, therefore, utilize a wide range of carbohydrates as prebiotics although they may not be able to completely degrade them.

The catabolism of sucrose involves the breaking of the glycoside linkages between the glucose and the fructose by the catabolic enzymes sucrose-6-phosphate hydrolase, encoded by the *cscA* gene, and sucrose phosphorylase, encoded by the *spl* gene. Sucrose-6-phosphate hydrolase converts sucrose-6-P into glucose-6-P and fructose. Sucrose phosphorylase converts sucrose into glucose-1-P and fructose. Glucose-6-P is directed into the hexose pathway for energy production and then the glucose-1-P is converted to glucose-6-P and utilized likewise (Kitaoka and Hayashi*,* 2002; Reid and Abratt, 2005).

It is important that in *vitro* results of substrate preferences of isolated bacteria be complemented by *in vivo* results. Probiotics are usually administered as combinations of strains to enhance cross-feeding. Metabolic products produced from dietary prebiotics by one bacterial species may be used to provide substrates to support the growth of other populations though extensive metabolic interactions (Belenguer *et al.,* 2006). There are common practices and protocols used to for evaluation of probiotic potential aimed at standardising the approaches used. The most important of these are reviewed below.

1.5 Criteria for selecting probiotics

The Food and Agriculture and World Health Organisation Guidelines (2002) list the criteria for the pre-selection of microorganisms as a probiotics. The core basis of the criteria is the establishment of the safety of the final probiotics for use by humans. These are some of the general approaches used when isolating and characterizing potential probiotics:

1.5.1 Identification

It is common practice that strains administered as probiotics to humans should be isolated from humans. This minimizes the risk of unpredictable detrimental effects that may come with the introduction of foreign microbes from foreign origins (Collins *et al.,* 1998). It is also crucial that reliable methods are used for identifying new strains since probiotic capacities are often strain-dependent (Mohania *et al*., 2008). The trend for identification methods has moved from phenotypic to genotypic methods as the latter generate more sensitive and accurate results (Mohania *et al*., 2008).

The most commonly used genotypic method is the method that targets 16S rRNA gene because it is present in all bacteria, is functionally constant, and is composed of highly conserved domains (Vandamme *et al.*, 1996). Miyake *et al*. (1998) demonstrated the use of *Bifidobacterium* 16S rRNA gene identification and phylogenetic analysis. The main limitation of using 16S rRNA genes is that very closely related strains of the same species cannot be distinguished due to the conserved nature of the gene. In such cases, additional DNA sequences are used to

support the 16S rRNA gene phylogenic analysis and distinguish closely related strains (Leblond-Bourget *et al.*, 1996).

One of these additional DNA sequences is the 16S-23S rRNA gene intergenic spacer region, also described as the internally transcribed spacer (ITS) region. In eubacterial DNA, the rRNA genetic loci include 16S, 23S, and 5S rRNA genes, which are separated by the ITS regions (Barry *et al.* 1991). These ITS regions are more variable both in length and sequences among species of the same genus due to frequent insertion-deletion events. The ITS regions are a useful tool for differentiating prokaryotic strains (Leblond-Bourget *et al.*, 1996).

Housekeeping genes such as *hsp60*, the heat shock protein, and *tuf*, encoding the elongation factor Tu have been used for phylogenic analysis because of their highly conserved primary structures and ubiquity. *Hsp60* gene sequences have been shown to be useful in differentiating species and in confirming 16S rRNA gene phylogenic analysis of *Bifidobacterium* spp. (Jian *et al*., 2001). The elongation factor Tu gene (*tuf)* has also been in the phylogenic analysis of bifidobacteria and lactobacilli (Chavagnat *et al*., 2002; Ventura *et al.*, 2003). Other genes used for the genus, species and strain identification and differentiation include the sequence analysis of *recA* (Eisen*,* 1995)*, atpD, ldh* and pyruvate kinase encoding genes (Ventura and Zink, 2003*;* Ward and Roy *et al.,* 2005).

There are several other methods used for identification and differentiation of strains that do not rely on gene sequencing but rather use DNA fingerprinting. One such method is the Randomly Amplified polymorphic DNA (RAPD) technique used for

differentiation between strains within the same species. The technique uses short random sequence primers about 9 to 10 bases in length. These primers anneal randomly to chromosomal DNA sequences at low annealing temperatures. The resulting amplification products of regions of the bacterial genome are separated by agarose gel electrophoresis and a characteristic pattern of bands particular to a bacterial strain is observed (Ventura *et al*., 2004b). RAPDs have been used in the identification and differentiation of *Bifidobacterium* and *Lactobacillus* strains (Vincent *et* al., 1998; Oh-Sik *et al.,* 2002).

Other DNA fingerprinting methods include denaturing gradient gel electrophoresis (DGGE) which is based on the discontinuous phenomenon of strand dissociation that allows resolution of fragments differing by as little as a single nucleotide substitution. Restriction enzyme profiles from pulsed-field gel electrophoresis (PFGE) (Ward and Roy *et al.,* 2005) and amplified ribosomal DNA restriction analysis (ARDRA) (Ventura *et al*., 2004b) can also be used.

1.5.2 Ability of the strain to persist within the GIT

Each day the human stomach secretes about 3 litres of gastric juice which has a fluctuating pH of approximately pH 2 (Dunne *et al.*, 2001; Vernazza *et al*., 2006) and contains bile salts which are synthesized in the liver from the catabolism of cholesterol and secreted from the gall bladder into the small intestines (Chung *et al*., 1999; Dunne *et al.*, 2001). The acid and bile together create harsh conditions in the upper part of the GIT. Acid and bile resistance of ingested probiotic strains is, therefore, a survival requirement that enables the passage of viable probiotic strains

through the upper part of the GIT allowing them to successfully colonise the lower GIT and function as expected (Dunne *et al.,* 2001; Delgado *et al.,* 2008). As part of the screening process fro suitable probiotic strains, it is therefore, important that probiotics are tested for sensitivity to conditions of low pH and high bile salts concentration.

1.5.3 Adhesion to gut epithelial tissue

Probiotic strains need to colonise the GIT so as to exert beneficial effects on the host. To achieve this, the strains must compete with the resident microbiota in order to be persistent and maintain their beneficial activity *in situ.* The ability to colonize the GIT should be assessed during pre-selection of probiotic bacterial strains (Crociani *et al.,* 1995; Cepelejnik *et al.,* 2007). The adhesion tests differ according to the materials used as an adherence surface to simulate the human gut. Some assessments use intestinal mucus while others use HT-29 and Caco-2 cells. HT-29 and Caco-2 cells are cultured human intestinal cell lines that are used because they express morphologic and physiological characteristics of normal human enterocytes and have been exploited to reveal the mechanisms mediating enteropathogen adhesion (Crociani *et al.,* 1995). Yeast cells are used to test the adherence as they have mannose-containing sugar moieties on their cell wall that resemble those found on the epithelial lining of the gut (Alderich *et al.,* 1996; Pretzer *et al.,* 2005). Different capacities for human mucus adherence have been reported for some probiotic, dairy and clinical LAB strains (Kirjavainen *et al.,* 1998; op den Camp *et al.,* 1985; He *et al.,* 2001).

1.5.4 Antimicrobial activity against potentially pathogenic bacteria

One of the benefits associated with probiotics use, is the protection of the host by inhibition of pathogens. The pathogen inhibition is proposed to occur by probiotics exerting competitive exclusion due to blockade of adhesion sites, stimulation of the host immune system and production of inhibitory substances (Picard *et al.*, 2005). LAB strains produce a number of pathogen inhibitory substances including organic acids, hydrogen peroxide, bacteriocins, nisin and bacteriocin-like substances (Dunne *et al.,* 2001). These substances exhibit inhibitory activity against potentially pathogenic bacteria such as strains of *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, Escherichia coli* and *Streptococcus* (Oh-Sik *et al.,* 2000; Dunne *et al.,* 2001). *In vitro* tests that evaluate antimicrobial activity against potentially pathogenic bacteria are required for probiotic potential evaluation (Salminen *et al*., 1998).

1.5.5 Antibiotic profiles

Antibiotic therapy can significantly affect the microbial balance in the intestine and especially can reduce the viability of indigenous bifidobacteria. This may result in an increase in the numbers of pathogens. Bifidobacteria can be used to restore and re-establish the microbial balance, thereby preventing diseases (Kheadr *et al*., 2007). For example, administering *B. longum* was found to reduce the incidence of ampicillin associated diarrhoea and the time required for recolonization of the intestine (Black *et al*., 1991).

For this reason, it is useful to know the antibiotic sensitivity and resistance profiles of the potential probiotics in order to decide which of probiotics to administer when using particular medication. This is to avoid the antibiotics nullifying the effects of the administered probiotics (Moubareck *et al.,* 2005).

1.5.6 Oxidative Stress and tolerance

Probiotic products in dairy product or tablets are, to some extent, exposed to air either during manufacturing, distribution or consumption (Talwalkar and Kailasapathy, 2003). Bifidobacteria are described as strictly anaerobic microorganisms (Scardovi, 1986) but some strains have shown the capacity to tolerate certain levels of oxygen exposure (Kawasaki *et al.,* 2006) while some bifidobacteria are unable to form colonies on plates without the aid of chemicals such as cysteine which lower the redox potential (Nebra *et al.,* 2002). Other Bifidobacteria display weak growth under the conditions of partial aeration and differences in oxygen tolerance between *Bifidobacterium* species have been reported (de Vries, W. and Stouthamer, 1969, Meile *et al.,* 1997, Shimamura *et al.,* 1992, Ahn *et al.,* 2001, Talwalkar and Kailasapathy, 2003). Differences in the degree of their sensitivity to oxygen exposure have been shown to be species and strain specific (Meile *et al*., (1997).

Molecular oxygen on its own is not toxic, but reactive oxidative species (ROS) are toxic to cells. ROS are formed when molecular oxygen accepts one, two or three electrons to form superoxide radicals, hydrogen peroxide and hydroxyl radicals, respectively (Xianqin and Kesen, 2007). Oxidative stress occurs when abnormally high levels of ROS are generated, resulting in DNA, protein and lipid damage (Kullisaar *et al*., 2002).

Several methods have been used to minimize the effects of oxidative stress in probiotics. For example, in dairy products, oxygen sensitive probiotic strains are protected from oxygen damage by microencapsulation, oxygen impermeable packaging material, adding special high oxygen consuming strain together with the

sensitive strains, and use of acorbate and L-cysteine as oxygen scavengers (Talwalkar and Kailasapathy, 2004). These methods have been shown to reduce the effects of oxidative stress, but it is still more advantageous to use aerotolerant probiotic strains.

Anaerobes that can tolerate ROS have enzymes such as NADH oxidase, NADH peroxidise and superoxide dismutase (SOD) as enzymes for antioxidative defence (Shimamura *et al.,* 1992; Talwalkar and Kailasapathy, 2003). Manganese has also been shown to act as a scavenger of oxygen and hydrogen peroxide (Imlay and Linn 1988, Shin and Park, 1997; Horsburgh *et al.,* 2002).

The inability of lactic acid bacteria to synthesize heme proteins means *Bifidobacterium* spp. lack catalase, a key enzyme involved in oxygen detoxification. ROS are usually eliminated by SOD and hydrogen peroxide by catalases. The absence of an effective oxygen scavenging mechanism means that most *Bifidobacterium* spp. are highly susceptible to the accumulation of ROS in the cell, eventually leading to cell death (Talwalkar and Kailasapathy, 2004). The few that have tolerance to ROS accumulation possibly have alternative protective mechanisms.

NADH oxidase and NADH peroxidase activities have been considered significant in the oxygen tolerance of a few members of *Bifidobacterium* spp. as maximum activities of these enzymes were found in most aerotolerant strains (Shimamura *et al.,* 1992). The interrelatedness between these two enzymes, however, makes it difficult to determine the activities of these enzymes individually as the various

assays for the estimation of NADH oxidase and NADH peroxidise activities have been shown to contradict each other (Talwalkar and Kailasapathy, 2003; Shin and Park, 1997). In other bacteria, thiol-specific redox reactions have been linked with the elimination of ROS. Thioredoxins are maintained in a reduced form by a thioredoxin reductase (Baker *et al.,* 2001). These genes have been shown to be induced under conditions of oxidative stress in *Bacillus subtilis* and in *Lactobacillus plantarum* (Serrano *et al.* 2007). There is overall, limited molecular information on oxidative stress response systems, especially in bifidobacteria

This ability of GIT microbiota to help out with ROS scavenging is a useful probiotic benefit. The antioxidant defence systems of humans and GIT microbiota are tightly linked since components of the human antioxidant defence system are derived from foodstuffs and/or provided by GI microbiota. The use of probiotic strains such as *L. fermentum ME-3* with physiologically effective antioxidative properties, have been proposed for the management of the oxidative stress levels in the human gut lumen, inside mucosa cells and in the blood (Mikelsaar and Zilmer, 2009).

Understanding the effects of oxygen on the probiotics, their ability to withstand that exposure, and the mechanisms involved in oxidative stress response will aid the design of protection methods and also useful for selection of ROS scavenging probiotics that are more robust probiotic strains with longer shelf lives.

1.6 Background to Study

In a small independent study it was demonstrated that black South Africans have a higher incidence of carbohydrate mal-absorption compared to white South Africans.

It was also found that white South Africans are more susceptible to GIT infections compared to black South Africans (Segal, 2002). The hypothesis put forward to explain these differences, was that the carbohydrate malabsorption may result in accumulation of prebiotic substrates that could stimulate the growth of some of the probiotic strains which lower the risk of GIT infections.

In a study on kidney stone disease, differences in microbial diversity and efficiency in carbohydrate utilisation in the GITs of people from the different racial groups were investigated. It was in this study, that faecal samples from one of the donors from the black population were shown to possess very high sucrase activity. It was then decided to analyse 5 of the microorganisms that were responsible for this high activity with a view to their possible use as probiotics to enhance gut health.

1.7 Aims and objectives

This study was aimed at identifying the 5 *Bifidobacterium* isolates mentioned above identified using different molecular tools. The probiotic potential of these isolates would also be evaluated using some of the methods in the probiotics screening criteria. The probiotic potential assessment will focus on the high ability of the isolates to utilise sucrose and other oligosaccharides that contain the sucrose moiety. The carbohydrate preference will give an indication of the type of carbohydrates that could be used as prebiotics for these isolates. The presence of an oxidative stress response mechanism in these isolates will also be investigated as the presence of such a system is advantageous to probiotics strains.

Chapter 2: Identification of five novel bacterial isolates

2.1 Introduction

The isolation of microbes from human and animal intestines for use as probiotics is common and so most of the bacteria that are currently administered as probiotics include species of the GIT genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Mohania *et al.,* 2008). The best way to avoid health risks and misleading claims, and to ensure efficient quality control of approved strains, is to identify strains thoroughly after the isolation. Probiotic strain identification is also important as the probiotic capacities and features of microorganisms have been shown to be strain-dependent (Lick, 2003; Callon *et al.* 2004*;* Mohania *et al.,* 2008).

Advances in molecular taxonomy have resulted in a number of effective techniques and methods being used for identifying organisms. These molecular methods and the information derived from their usage, provide means of complete description of the taxonomy of a strain (Prasad *et al.,* 1999). Genotypic methods are currently preferable for identification as compared with phenotypic methods as they generate more accurate results. Phenotypic methods are limited in that they lack reproducibility, have poor discriminatory power, are laborious, and the results may vary depending on the bacterial growth conditions (Mohania *et al.,* 2008).

Different taxonomical methods have been used for identifying *Bifidobacterium* spp., and one such method is the sequence analysis of the 16S rRNA gene. The 16S rRNA gene is a conserved universal marker whose product has constrained functions and is therefore relatively unaffected by evolutionary environmental pressures. The main reason that the 16S rRNA gene is widely and commonly used as an identification tool is because of its size and conserved nature. (Woese, 1987; Kimura *et al*., 1997)

Many genes other than the 16S rRNA gene have also been used for bacterial identification. One of these is the internally transcribed 16S-23S rRNA gene spacer (ITS) region which is used in addition to the 16S rDNA sequence (Ludwig and Schleifer, 1994; Rudi *et al.,* 2007; Albuquerquea *et al.,* 2009). The ITS region is under 10 times more evolutionary pressure than the 16S rDNA and thus provides greater genetic variation. These variations can, therefore, be used for differentiating closely related bacteria (Ventura and Zink, 2002; Leblond-Bourget *et al.* 1996).

The other gene sequence used for identification is that of the heat shock protein gene *(hsp60*). The heat shock response is designed to increase bacterial survival and results in the activation of numerous proteins that are involved in the maturation of newly synthesised proteins and in the refolding or denaturing of the denatured proteins (Ventura *et al.*, 2004a). The sequence conservation of the heat shock protein genes is due to their vital role in cell survival and this qualifies them as tools for phylogenetic analysis (Ventura *et al.*, 2004). *Hsp60* sequences have been shown to reproduce the same phylogeny trends as the 16S rRNA gene sequences (von Ah *et al.,* 2007).

Tuf genes offer an alternative or additional method for identification and discrimination of closely related taxa (Hsin-Chin and Wen-Zhe, 2008). The *tuf* gene codes for the elongation factor Tu (EF-Tu) which is a guanosine-5'-triphosphate (GTP) binding protein that plays a role in protein synthesis (Ventura *et al.,* 2003; Ventura and Zink*,* 2003; Chavagnat *et al.,* 2002). During translation, the EF-Tu facilitates the elongation of polypeptides from the ribosome and aminoacyl-tRNA during translation (Chavagnat *et al.,* 2002). Properties of *tuf* that make it suitable for

taxonomic studies include the presence a single copy of the gene in Gram positive bacteria. The gene sequence length is short and it is, therefore, easy to sequence and analyse (Chavagnat *et al.,* 2002; Hsin-Chin and Wen-Zhe, 2008; Ventura and Zink*,* 2003).

In all the above mentioned cases, the genes are isolated and amplified by PCR and then sequenced. The sequence homology comparisons to existing sequences in NCBI database are done by BLAST (Basic Local Alignment Search Tool). This algorithm provides a powerful and rapid specificity test against the largest available nucleotide database (NCBI) (Altschul *et al*., 1990).

There are other useful and rapid methods for classification of isolates and that do not require sequencing. One such method is the Randomly Amplified Polymorphic DNA (RAPD) technique. RAPD is a PCR-based discrimination method in which non-specific short primers anneal randomly to DNA. The annealing is random because the PCR is set to annealing conditions of low-stringency. Different sizes and numbers of DNA fragments are then produced by the RAPD-PCR and these form different patterns or profiles after electrophoresis (Vincent *et al.,* 1998; Ward and Roy *et al.* 2005; Sakata *et al*. 2002).

The different molecular methods described above were used to find the identity of the 5 isolates mentioned in Chapter 1.

2.2 Materials and methods

2.2.1 Strains and growth conditions

The isolates were anaerobically cultured in MRS broth (de Man *et al*., 1960). The anaerobic chamber (Forma Scientific, Model 1024) used was maintained at an

atmosphere consisting of 5% H_2, 10% CO_2 and 85% N_2. The *Bifidobacterium adolescentis* ATCC 15703 type strain (BaT) was obtained from American Type Culture Collection (ATCC), Manassas, VA, USA and was also cultured anaerobically in MRS broth (de Man *et al*., 1960). *Escherichia coli* JM109 (Yanisch-Perron *et al.,* 1985) was used for cloning purposes and cultured aerobically at 37ºC in Luria Bertani (LB) broth (Sambrook and Russel, 2001). *E. coli* JM109 competent cells were prepared by the $CaCl_2$ method and recombinants were selected by plating on LB agar containing 100 µg/ml ampicillin (Sambrook *et al*., 1989).

2.2.2 DNA extraction

Bacterial cells from 5 ml broth culture grown for 18 hours were harvested by centrifugation at 10 000 rpm (Eppendorf centrifuge 5415 C). The cells were re-suspended at room temperature for 30 minutes in 200 µl of lysis buffer: 1% Triton X-100, 20 mM Tris-HCl at pH 8.5, 2 mM EDTA at pH 8.0 and 20 mg/ml lysozyme. Proteinase K was added to the re-suspension mixture to a final concentration of 100 µg/ml and incubated at 37ºC for an additional 30 minutes. Genomic DNA from these cells was extracted using the Fermentas Genomic DNA extraction kit, following the manufacturer's instructions with the addition of 0.5 mg of RNase A and incubation at 37 ºC for 10 minutes after the manufacturers second centrifugation step. The plasmid DNA was extracted using the Bioflux kit, according to the manufacturer's instructions. DNA quantification was performed using the Nanodrop spectrophotometer (Nanodrop Technologies).

PCR products were purified before sequencing using the QIAquick PCR purification kit (QIAGEN) as per the manufacturer's instructions. For cloning purposes, the

required band was excised from the gel after electrophoresis and purified using the Gel extraction kit (PEFLAB Biotechnologie GmbH). The purified PCR product was cloned into the pTZ57R/T vector (Fermentas InsTAclone Kit) and transformed into *E. coli* JM109 $CaCl_2$-competent cells using standard protocols (Draper *et al.,* 1988).

2.2.3 DNA amplification

DNA PCR amplifications were carried out using a GeneAmp 9700 machine (Applied Biosystems). The PCR reactions were performed in 50 µl volumes: 25 µl of Fermentas 2X master mix (0.05 units/ µl Taq DNA polymerase in reaction buffer, 1.5 mM $MgCl_2$ and dNTPs), 2.5 µl of each primer and 100 ng of genomic DNA template was added to the reaction mix and then the volume was made up to 50 µl using nuclease free water. When the PCR had been completed, 1 µl of loading buffer was added to 10 µl of PCR reaction mixture. The PCR conditions of the different experiments are shown in Table 2.1. The presence of PCR products was determined by 0.8 % agarose gel electrophoresis and visualised using ethidium bromide stained gels. The RAPD-PCR products were electrophoresed using 2% agarose gels. Gels were visualised by UV transillumination using the GEL/CHEMI Doc machine (Biopad). As a molecular weight maker, λ phage DNA digested with *Pst*I was used.

Table 2.1 Primers and conditions used for PCR reactions

Primer	Primer sequence	PCR conditions	Target	Reference
F27 R5	AGAGTTTGATCCTGGCTCAG ACGGITACCTTGTTACGACTT	96°C 2'; 25 cycles of 96°C of 30 s; 55°C for 30 s; 72°C 1' 30 s; final one cycle 72°C 3'	Universal 16S rRNA gene	Wheeler *et al*., 1996
Im26-f Im3-r	GATTCTGGCTCAGGATGAACG CGGGTGCTICCCACTTTCATG	94°C 5'; 30 cycles of 94°C 30 s; 57°C for 30 s; 68°C 1' 30 s; one cycle 57°C 30 s and one final cycle 68°C 7'	*Bifidobacterium* 16S rRNA gene	Kaufman *et al.,* 1997
M13-f M13-r	GGTGTAAAACGACGGCCAGT GGCAGGAAACAGCTATGACC	94°C 2'; 30 cycles of 95°C 30 s; 55°C for 30 s; 72°C 30 s; final cycle 72°C 5'	pTZ57R/T	Yanisch-Perron *et al.,1985*
16-1A 23-1B	GAATCGCTAGTAATCG GGGTTCCCCCATTCGGA	94°C 2'; 30 cycles of 95°C 30 s; 55°C for 30 s; 72°C 30 s; final cycle 72°C 5'	ITS	Tannock *et al*., 1999
OPA-02 OPA-13 OPA-15 OPA-18	TGCCGAGCTG CAGCACCCAC AGGTTGCAGG AGGTGACCGT	94°C 2'; 45 cycles of 94°C 1'; 30°C 1'; 72°C 1' 30 s; final one cycle 72°C 2'	Random for RAPDs	Vincent *et al.,* 1998
H60F H60R	GG (ATGC) GA (CT) GG (ATGC) AC (ATGC) AC (ATGC) AC (ATGC) GC (ATGC) AC (ATGC) GT TC (ATGC) CC (AG) AA (ATGC) CC (ATGC) GG (ATGC) GC (CT) TT (ATGC) AC (ATGC) GC	94°C 5'; 30 cycles of 94°C 30 s; 55°C 30 s; 72°C 1' ; final one cycle 72°C 10'	HSp60 gene	Jian *et al.,* 2001
Tuf-1 Tuf-2	GAGTACGACTTCAACCAG CAGGCGAGGATCTTGGT	95°C 3'; 30 cycles of 95°C 30 s; 52°C 30 s; 72°C 2' ; final one cycle 72°C 10'	Tuf gene	Ventura and Zink, 2003

2.2.4 Colony PCR for checking Transformants

A single colony on a plate was picked with a sterile toothpick and resuspended in 10 µl of sterile water. The cell suspension (4 µl) was used as a template in the PCR using the M13F and M13R primers shown in Table 2.1.

2.2.5 Bioinformatics analysis

The sequences obtained were identified by comparing them to sequences available in Genbank using the BLAST algorithm (Altschul *et al*., 1990). The multiple sequence

alignments were performed using the DNAMAN program (version 4.13; Lynnon biosoft, Quebec, Canada). The Neighbour-joining phylogeny trees were analysed by clustal X and drawn using MEGA 4.0 (Kumar *et al*., 2007).

2.3 Results

2.3.1 16S rRNA gene sequencing

The initial identification of the five isolates was by PCR of the 16S rRNA gene using Im26-f and Im3-r, bifidobacteria-specific primers (Table 2.1). The nucleotide sequences of the 16S rDNA PCR amplicons (1.35 kb) were analysed by screening them against the NCBI GenBank by using the BLAST algorithm. The analysis showed that all the isolates had the highest nucleotide similarity to *Bifidobacterium adolescentis* ATCC 15703 with 99% identity. A phylogenetic tree was constructed using the neighbour- joining method (Fig. 2.1), using the 16S rDNA sequences of the isolates and those of *Bifidobacterium spp.* available in the GenBank database; all of the isolates grouped with *Bifidobacterium adolescentis* ATCC 15703 with isolate B92 being less related to the other 4 isolates.

In previous studies, some of the species that the 5 isolates cluster with in this study, have been grouped and termed the *B. adolescentis* group. The group includes *Bifidobacterium adolescentis* ATCC 15703^{T}, *Bifidobacterium dentium, B. ruminantium, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium merycicum and Bifidobacterium angulatum* (Ventura *et al*., 2006 and Ventura *et al*., 2007).

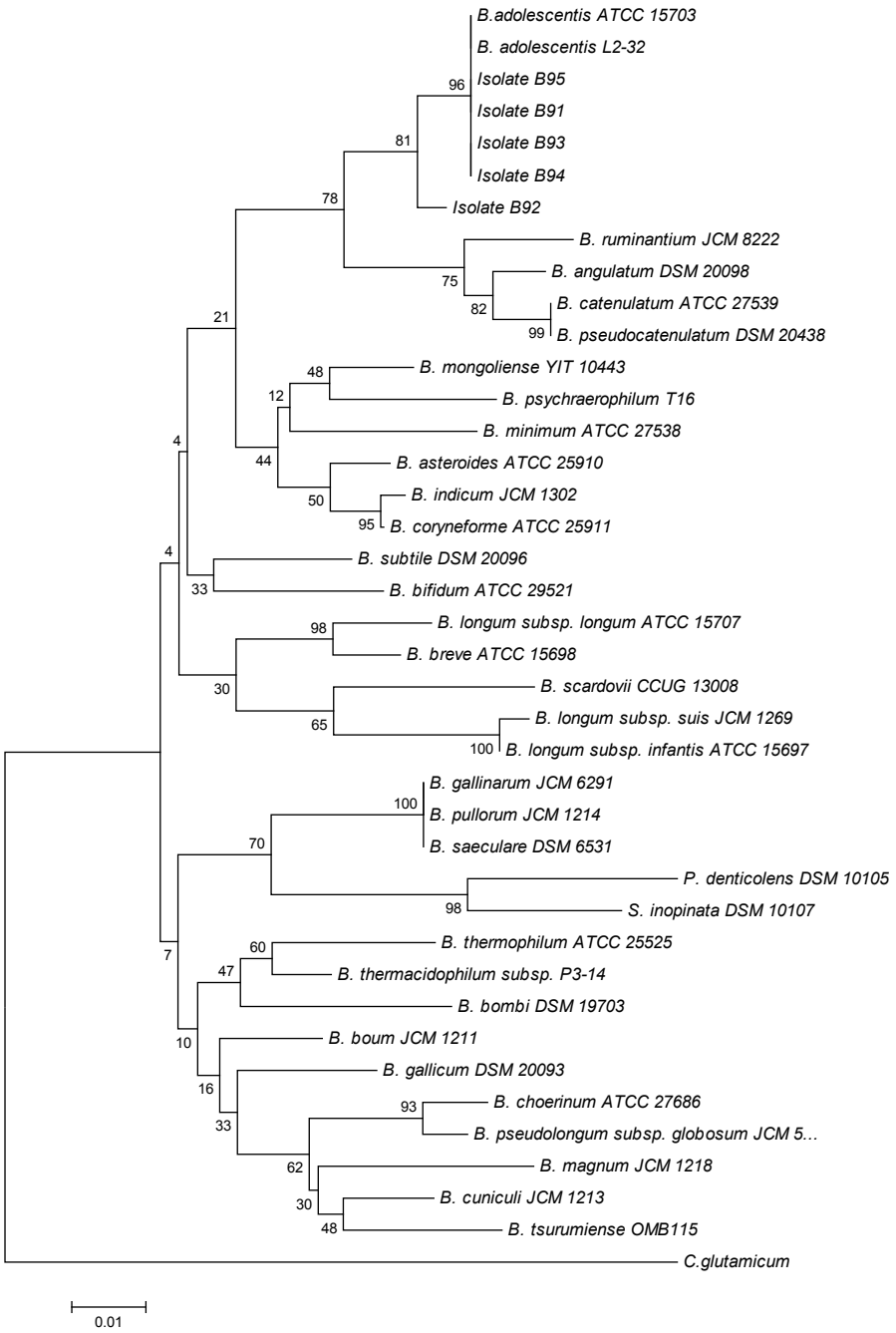


Figure 2.1 Phylogenetic tree obtained by using the 16S rRNA genes of different *Bifidobacterium* spp. and the 5 novel isolates. The trees were calculated by the neighbour-joining method and rooted using *Corynebacterium glutamicum*. Bootstrap values are indicated for a total of 1,000 replicates.

2.3.2 RAPD profiles

To determine if there are any overall genomic differences between the isolates and *Bifidobacterium adolescentis* ATCC 15703, RAPD-PCR was conducted. The RAPD

analysis using OPA 2, OPA 13, OPA 15 and OPA 18 primers revealed 3 different RAPD profiles (Fig. 2.2). The first profile was displayed by isolates B91, B93, B94 and B95. The second profile was from isolate B92 while the last profile was from BaT, indicating clear differences in the genome sequences between these 3 groups.

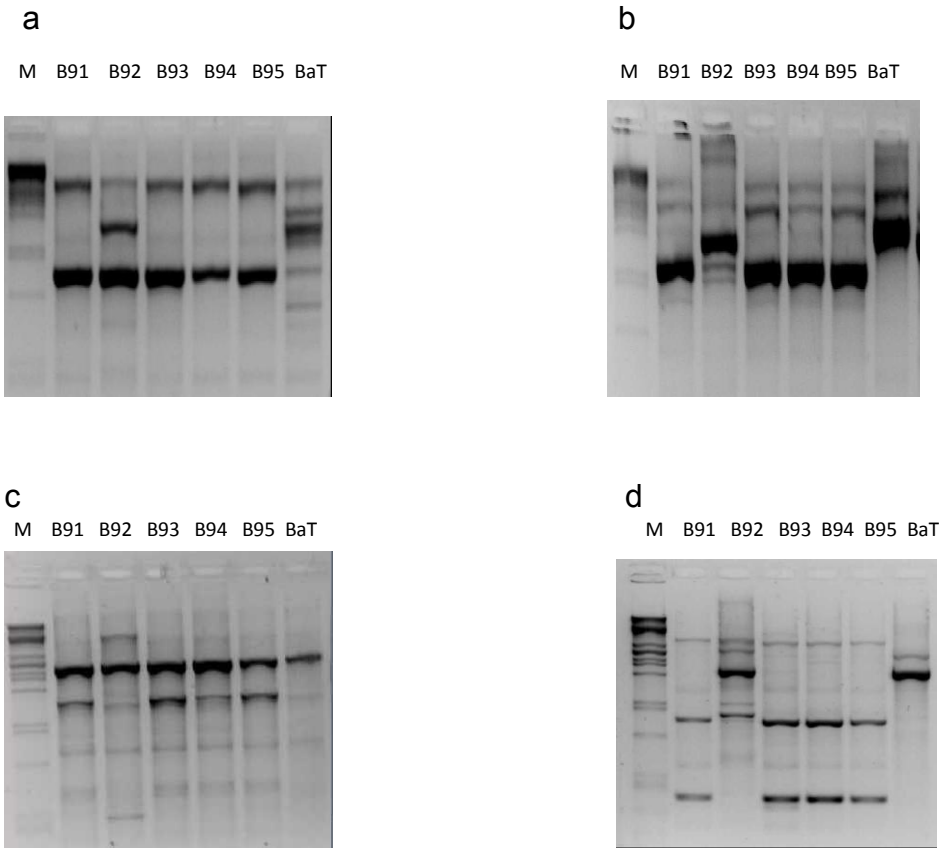


Figure 2.2 RAPD analysis of the 5 isolates obtained using primers a) OPA 15; b) OPA 13; c) OPA 02; d) OPA 18. (M) molecular weight marker of λ-phage DNA digested with *Pst*I, Isolates B91, B92, B93, B94, B95 and *Bifidobacterium adolescentis* ATCC 15703^{T} (BaT) were loaded in the lanes indicated.

2.3.3 16-23S Internal Transcribed Space (ITS) region

Using 16-1A and 23-1B primers (Table 2.1), the intergenic region between the 16S rRNA and 23S rRNA of each of the 5 novel isolates was successfully isolate by PCR and cloned into *E. coli* JM109 using the PTZ57/T vector. The inserts were then sequenced using the M13 primers. DNA sequence analysis of the region using the

BLAST algorithm showed that the ITS regions had the highest levels of nucleotide identity to *Bifidobacterium adolescentis* ATCC 15703^T for all the isolates. When analysed with other bifidobacteria, all of the isolates clustered with other *B. adolescentis* strains (Fig. 2.3). Isolates B93, B94 and B95 formed a cluster with 97% nucleotide identity to *Bifidobacterium adolescentis* ATCC 15703^T while isolate B92 and isolate B91 were different from each other and the group with 93% and 98% sequence identity, respectively.

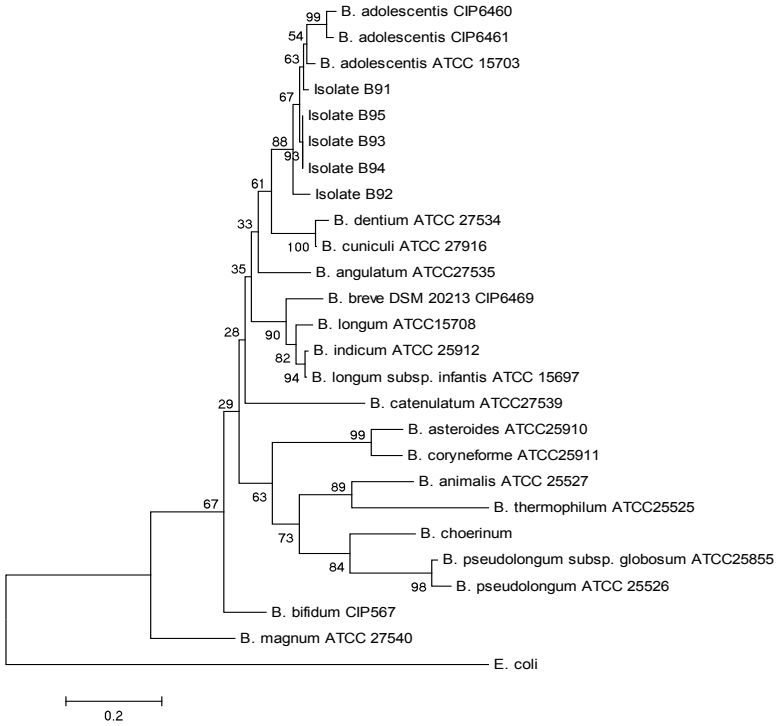


Figure 2.3 Phylogenetic tree of the 16-23S rRNA intergenic spacer region from the 5 *Bifidobacterium* isolates. The trees were calculated by the neighbour-joining method and rooted using *E. coli.* Bootstrap values are indicated for a total of 1,000 replicates.

2.3.4 Heat Shock Protein (Hsp60)

The PCR amplicons of the gene encoding the heat shock protein (*Hsp60*) from all the isolates were obtained using primers H60F and H60R (Table 2.1) and were successfully sequenced. The BLAST sequence identity analysis of the heat shock

protein genes was again highest for *Bifidobacterium adolescentis* ATCC 15703^T for all the isolates. Isolates B91, B94 and B95 had 98% identity to *Bifidobacterium adolescentis* ATCC 15703^T while isolate B92 and isolate B93 had 93% and 97% sequence identity, respectively. Isolate B92 had the same nucleotide identity of 93% to *Bifidobacterium adolescentis* ATCC 15703^T and *Bifidobacterium ruminantim,* and showed 92% and 90% identity *B. dentium* and *B. pseudocatenulatum*, respectively (Table 2.2). The isolates cluster together and with the members of *B. adolescentis* group (Fig. 2.4).

Table 2.2 *Hsp60* sequence % identity of the 5 *Bifidobacterium* isolates with partial Hsp60 of 4 closely related *Bifidobacterium* species.

	B. adolescentis ATCC 15703^T (AP009256)	**B. ruminantium (AF240571)**	**B. dentium (AF240572)**	**B. pseudocatenulatum (AY166555)**
B91	98	92	91	91
B92	93	93	92	90
B93	97	90	91	90
B94	98	91	92	90
B95	98	92	92	91

2.3.5 Elongation factor Tu (*tuf*)

The elongation factor Tu gene sequences were obtained from the 5 novel isolates by PCR using primers, Tuf-1 and Tuf-2. The BLAST analysis of these elongation factor Tu sequences showed that all the isolates had the highest identity to *B. adolescentis* ATCC 15703^T. Isolates B94 and B95 showed 99% nucleotide identity to *B. adolescentis* while isolate B92 and isolate B93 showed 98% identity while isolate B91 showed 97% identity. The 5 isolates clustered together with *B. adolescentis* ATCC 15703^T and *B. ruminantium* which have been classified to be in the *B. adolescentis* group (Fig. 2.5).

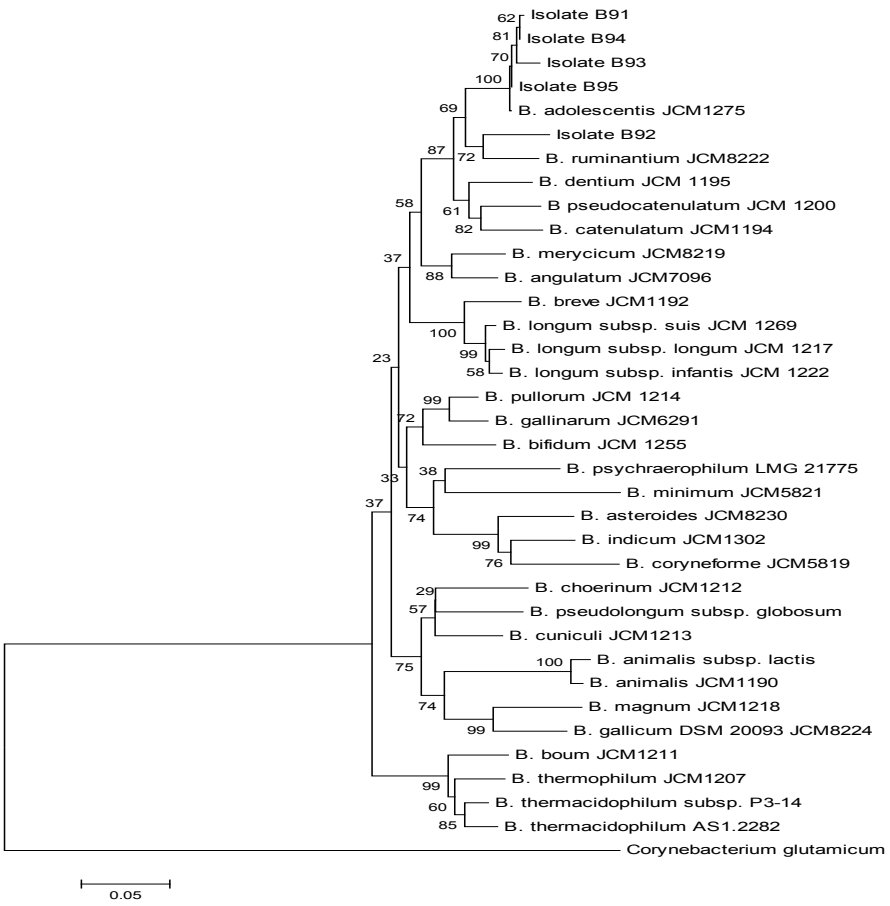


Figure 2.4 Phylogenetic trees obtained using the *Hsp60* genes. The trees were calculated by the neighbour-joining method and rooted using *Corynebacterium glutamicum*. Bootstrap values are indicated for a total of 1,000 replicates.

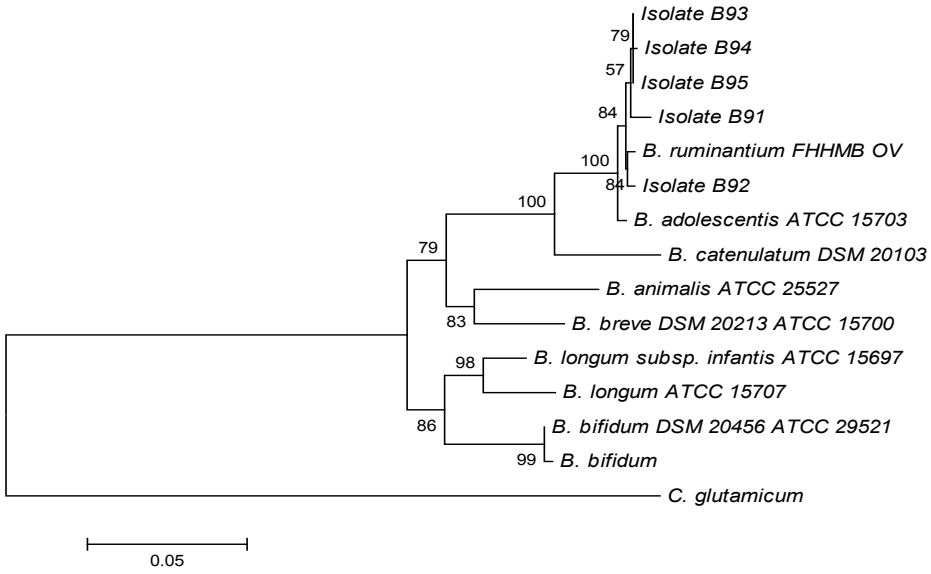


Figure 2.5 Phylogenetic tree obtained by using the *tuf* genes. The trees were calculated by the neighbour-joining method. Bootstrap values are indicated for a total of 1,000 replicates.

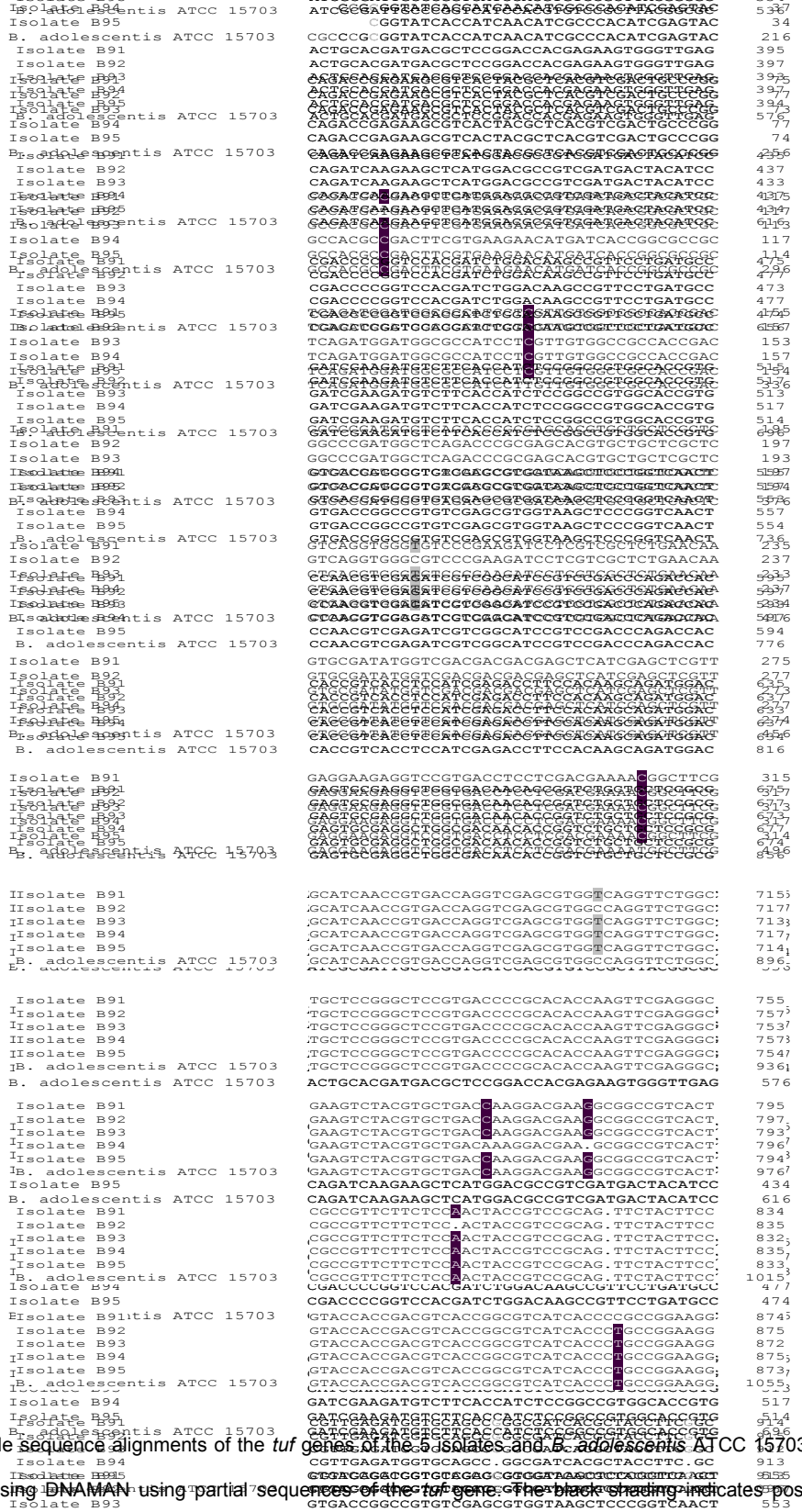


Figure 2.6 Multiple sequence alignments of the *tuf* genes of the 5 isolates and *B. adolescentis* ATCC 15703^{T}. The alignment was generated using DNAMAN using partial sequences of the *tuf* gene. The black shading indicates positions where one isolate differs from the others by one base while the grey indicates where 2 strains have a different base from the rest.

The *tuf* genes from *B. adolescentis* ATCC 15703^{T} and the isolates showed some single base differences (Fig. 2.6). A single amino acid difference in the protein

sequence of isolate B92 was also found further suggesting that isolate B92 is different from the other isolates and *B. adolescentis* ATCC 15703^T.

Table 2.3 Summary of the % nucleotide identity of the isolates to *B. adolescentis* ATCC 15703 using different sequences.

	16S rDNA	**16-23S ITS**	*Hsp60*	*Tuf*
Isolate B91	99	98	98	97
Isolate B92	99	93	93	98
Isolate B93	99	97	97	98
Isolate B94	99	97	98	99
Isolate B95	99	97	98	99

2.4 Discussion

Analysis of the 16S rDNA confirmed that the isolates belonged to the genus, *Bifidobacterium*. It could not be determined whether all the isolates were the same strain as the 16S rRNA gene sequences of all 5 isolates showed 99% nucleotide identity to *B. adolescentis* ATCC 15703^T. The difficulty in distinguishing between certain species of bifidobacteria using 16S rDNA has been attributed to the high levels of similarity that can be as high as 93 to 99%. These high similarities have been found within five different groups of *Bifidobacterium* species (Zhu *et al.,* 2003*;* Miyake *et al.,* 1998).

The main limitation of the use of 16S rDNA sequence is the poor resolving power and inability to discriminate between closely related bacterial species or subspecies. This is because the conserved nature of 16S rRNA genes has resulted in very low variability between genes. The 16S rRNA gene is a universal marker but different bacterial species have different copy numbers of the gene. This has lead to an over-

and under-representation of some bacterial species when using 16S rRNA genes as targets and this has also resulted in the difficulty of identifying a collective conserved region for the design of universal primers (Baker *et al*., 2003; Mohania *et al.,* 2008; Kimura *et al.,* 1997).

The RAPD profiles however, showed distinctive differences between the isolates. The 3 RAPD profiles suggest that isolates B91, B93 B94 and B95 are very closely related and are possibly the same strain while B92 and *Bifidobacterium adolescentis* ATCC 15703^{T} are different strains. The variation of RAPD profiles has been used to show differences between species and strains (Vincent *et al.,* 1998; Ward and Roy, 2005; Matto *et al.,* 2004). This methodology gives good levels of discrimination and does not require prior knowledge of the genome (Vincent *et al.,* 1998; Ward and Roy, 2005; Sakata *et al*. 2002).

The 16S rRNA nucleotide sequences do not show differences between closely related species or the strains of the same species, because of the conserved nature of the 16S rRNA molecule due to its low evolutionary rate (Leblond-Bourget *et al.,*1996). This means that in order to separate strains within the *Bifidobacterium spp.*, information obtained from the 16S rDNA sequences must be complemented with information from alternative identification tools.

The other tools were, therefore, used to further reveal these strain differences and showed that all of the isolates were still highly similar to *B. adolescentis* ATCC 15703^{T}. Compared to the 16S rDNA, the 16S-23S ITS region is under less evolutionary pressure and thus has greater genetic variation (Ventura and Zink, 2002), hence the decision to use it for further classification of the isolates. The 16S-23S ITS regions and the sequences of *Hsp60* and *tuf* all revealed differences

between the isolates (Table 2.3). Combined, these methods show that all the isolates are strain of *B. adolescentis*, and that B91, B93, B94 and B95 are probably the same strains with very minor sequence differences. Information from the RAPD analysis and the sequence analysis from the four different DNA regions suggests that B92 is the most different from all these other isolates and from the type strain.

There are other molecular methods that can be used to distinguish between closely related strains. Ventura *et al.* (2006) suggested the use of sequences from four or five housekeeping genes besides the 16S rRNA gene for the discrimination of species and subspecies within the genus *Bifidobacterium.* This suggested concatenation of genes sequences for phylogenetic purposes allows a significant increase in the discriminatory power between taxa (Ventura *et al.,* 2006).

2.5 Conclusions

All of the taxonomic methods used in order to identify the isolates, lead to the conclusion that all of the isolates are *Bifidobacterium adolescentis* strains. The consistent result is that isolate B92 is different from all the other 4 isolates and from the *B. adolescentis* type strain. The other consensus is that isolate B94 and B95 are possibly the same strain as all the identification tools used gave identical results consistently. The combined data from isolates B91 and B93 was not very conclusive but both seem to be closely related to isolate B94 and B95. However, despite some of the strains being identical at the genome level, strains have been shown to be different physiologically so substantially that they maybe considered separate strains (Mohania *et al.,* 2008). For this reason, all 5 isolates were characterised with respect to their ability to utilize different prebiotic substrates as discussed in the next chapter.

Chapter 3: Carbohydrate preference and utilization by the five *B. adolescentis* isolates

Contents

3.1 Introduction

Complex polysaccharides cannot be hydrolysed by the enzymes in the GIT's of most mammals. These non-digestible polysaccharides reach the lower part of the GIT intact and can be hydrolysed by β-fructo-furanosidase from the GIT microbial inhabitants such as bifidobacteria (Warchol *et al*., 2002). The ability of probiotic bacteria to utilize carbohydrates that are indigestible by their host enables the use of such carbohydrates as prebiotics for the enrichment of specific strains in the GIT.

Most of the mono-saccharides and disaccharides are hydrolyzed in the upper part of the mammalian GIT (Roberfroid *et al.,* 1998). The lower GIT inhabitants, therefore, exist in an environment rich in complex oligosaccharides and only through the hydrolysis of these complex carbohydrates are mono-saccharides and disaccharides accessible. Some of these oligosaccharides contain the fructose chain joined to a glucose end units in the same configuration as in sucrose molecules. This bond is broken to release glucose which is then used for energy production in the bifid shunt (van der Broek *et al*., 2008). This sucrose unit is found in a number of readily available carbohydrates such as raffinose, which is widespread in plants and is not completely digested by humans and reaches the colon intact (Kaditzky, 2008). Genes involved in the hydrolysis of oligosaccahrides containing this sucrose unit are, therefore, important to lower GIT inhabitants and in selecting ideal prebiotics.

The isolation of the 5 *B. adolescentis* isolates in this study was based on their high sucrase activity. This meant that the isolates have the potential to utilize oligosaccharides that contains fructose unit that is attached to a glucose unit. In this study, the carbohydrate utilization preferences of the 5 *B. adolescentis*, their ability to degrade sucrose, and the presence of genes on their chromosome linked to sucrose

utilisation, were investigated. *B. adolescentis* is an ideal candidate for the study of carbohydrate utilization as it is one of the most abundant bifidobacteria in the human colon and thus has potential to play significant role in the diet and colonic health (Belenguer *et al*., 2006).

3.2 Materials Methods

3.2.1 Bacterial strains and culture conditions

The isolates were anaerobically cultured at 37°C (as described in chapter 2) in Basal Yeast (BY) medium containing 1% (w/v) added carbohydrate. The carbohydrates used were glucose, sucrose, raffinose (Merck), Raftiline GR (Savannah fine chemicals, RSA) or Raftilose P95 (Orafti, Belgium) depending on experimental media requirements.

3.2.2 Carbohydrate preference

Single colonies were isolated from BY agar plates with 1% (w/v) sucrose inoculated into BY broth with 1% added sucrose, and incubated anaerobically at 37ºC for 18 hours. The cultures were transferred into fresh BY media supplemented with 1% (w/v) of the different carbohydrates to an initial OD_{600nm} of 0.1. The absorbance values were measured every 24 hours for 96 hours during bacterial growth.

3.2.3 Sucrase activity Assay

The isolates were anaerobically cultured for 18 hours at 37°C in 100 ml Basal Yeast (BY) medium containing 1% (w/v) sucrose. Cell free extracts (CFE) were obtained from these cultures as described by Trindade *et al.* (2003). The CFE were tested for

activity against Raftiline, raffinose, and sucrose using the dinitrosalicylic acid (DNS) assay as previously described (Trindade *et al.,* 2003).

3.2.4 Protein assay

To determine the total protein in the CFE, protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (BSA) being used as the protein standard at concentrations of 0.1 to 0.8 mg/ml.

3.2.5 Sucrose utilization genes

The presence of bifidobacterial genes possibly involved in sucrose utilization, were identified on the genome of *B. adolescentis* ATCC 15703^T using bioinformatic tools. The method of Kullin *et al.* (2005) was used to isolate the β-fructo-furanosidase gene (*cscA*) and the sucrose phosphorylase (*spl*) genes. The PCR primers used to isolate *cscA* were: Forward 5'-CCCAAGCTTGTTCTACACCGACAAGCAGC-3' and the Reverse 5'-GAAGATCTCCGTCCATCAGTATAGGCG-3'. The primers used to isolate the *spl* were: forward 5'-CCCAAGGTTTCACTGCGCGACTGGAC-3' and the reverse 5'-CGGGATCCATGAACCCAGCTTACGC-3'. The PCR constituents were as described in Chapter 2. For both gene targets, the PCR protocol consisted of one initial cycle of 94°C for 5 minutes and then 25 cycles of denaturation at 96°C for 30 seconds; annealing was at for 59°C for 30 seconds; extension was at 72°C for 1 minute and 30 seconds followed by one final cycle of extension at 72°C for 5 minutes.

3.3 Results

3.3.1 Carbohydrate preference

Isolate B91 grew best in BY media supplemented with 1% sucrose or glucose reaching OD values of 1.0 and 0.8, respectively. Low OD values were observed in media supplemented with 1% raffinose, Raftilose or Raftiline with the lowest growth being in media supplemented with Raftiline (Fig. 3.1).

Within the first 24 hours, isolate B92 grew well in BY media with 1% sucrose but at the end of the incubation period, the best overall growth was in media with raffinose reaching OD values above 0.9. Isolates B92 also grew well in media supplemented with 1% glucose or Raftiline. Isolate B92 stopped growing in media supplemented with 1% Raftilose after 24 hours of incubation and at the end of the 96 hours, growth in this substrate was the lowest as compared to the other four substrates

Isolate B93 grew best in media supplemented with 1% sucrose and also grew quite well in media supplemented with 1% Raftiline or raffinose with the cell density reaching OD values 0.8 and above. Isolate B93 was in lag phase for 48 hours when glucose was the carbohydrate substrate but grew to an OD of about 0.8 following that. Throughout the 96 hours of incubation, isolate B93 did not show any growth in media with Raftilose.

Isolate B94 was the only isolate that did not grow in BY media supplemented with 1% glucose. In BY media supplemented with 1% sucrose, isolate B94 grew so well it reached the high OD value of about 1.2 and to an OD of about 0.9 in BY media supplemented with 1% Raftiline. There was a 48 hours lag phase when isolate B94 was growing in BY media supplemented with 1% raffinose but growth initiated

reaching an OD value of about 0.7 after the 96 hours of incubation. Minimal growth of isolate B94 was observed in Raftilose.

Isolate B95 grew best in BY media supplemented with 1% raffinose reaching an OD value of 0.7. Growth in the rest of the substrates was in the OD range of 0.3-0.5 which was low when compared to growth of the other isolates under similar conditions.

Growth of *B. adolescentis* ATCC 15703^T was generally high in all the tested substrates. *B. adolescentis* ATCC 15703^T grew best in glucose, sucrose and Raftilose with the cell density reaching OD values above 0.9 in all these substrates. There was lower growth of *B. adolescentis* ATCC 15703^T in raffinose and Raftiline but this was still quite high when compared to growth of the other isolates in these substrates as the OD values were above 0.7.

Overall, *B. adolescentis* ATCC 15703^T showed the widest range of carbohydrate preference managing to grow relatively well in all the tested substrates. Sucrose was the most preferred carbohydrate substrate by 3 of the 5 *B. adolescentis* isolates, namely isolate B91, B93 and B94. Isolate B95 preferred raffinose but seemed to grow poorly in all 5 substrates. Isolate B91, B92, B93 also grew well in glucose while isolate B94 and B92 could also grow well in Raftiline. Raftilose was the least preferred carbohydrate as all 5 *B. adolescentis* isolates showed low levels of growth when it was used as a substrate (Fig. 3.1).

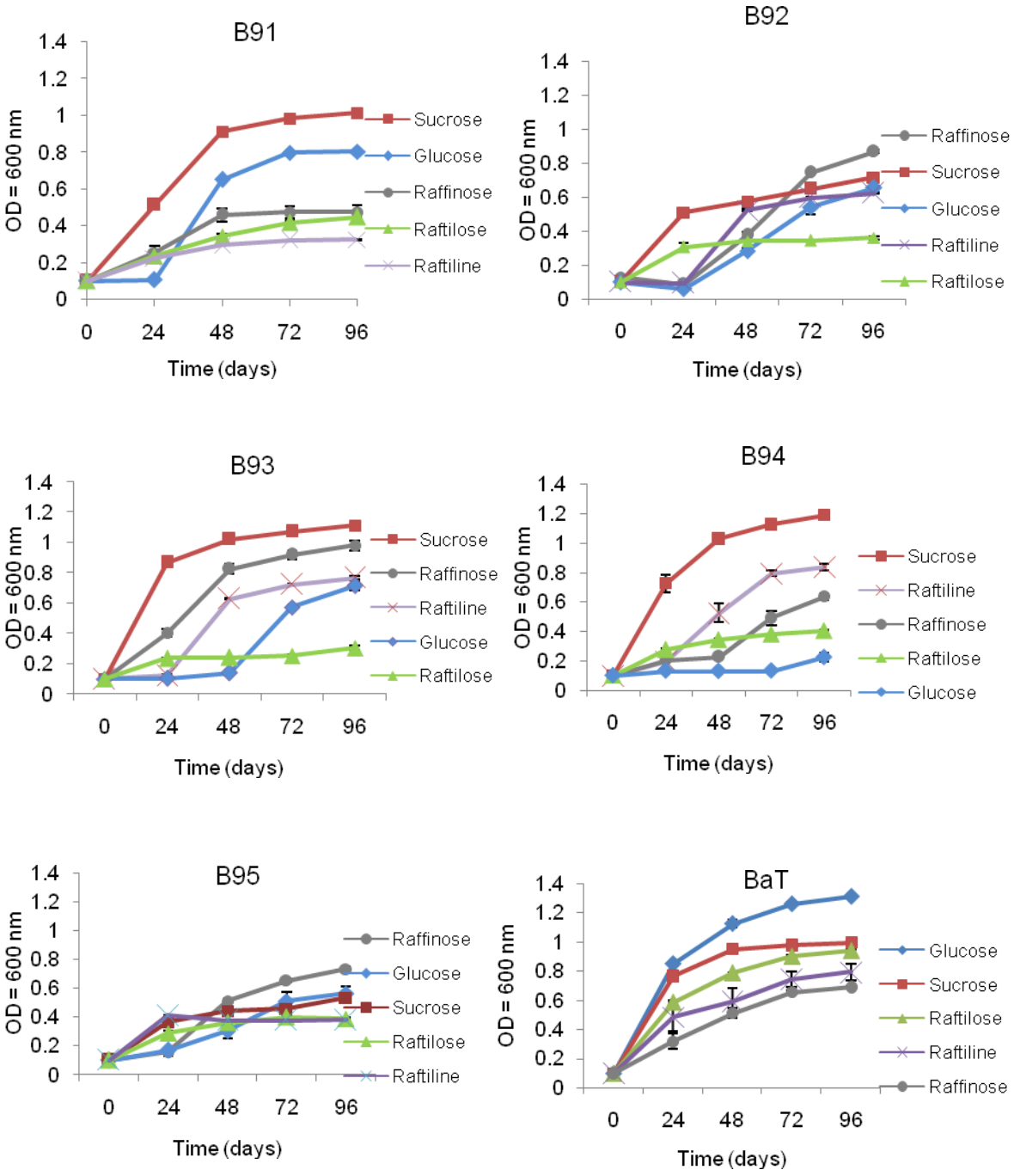


Figure 3.1 Carbohydrate preferences of the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^{T}. Changes OD=600$_{nm}$ were measured over a period of 96 hours with the bacteria growing in BY media supplemented with 1% glucose, sucrose, raffinose, Raftilose or Raftiline.

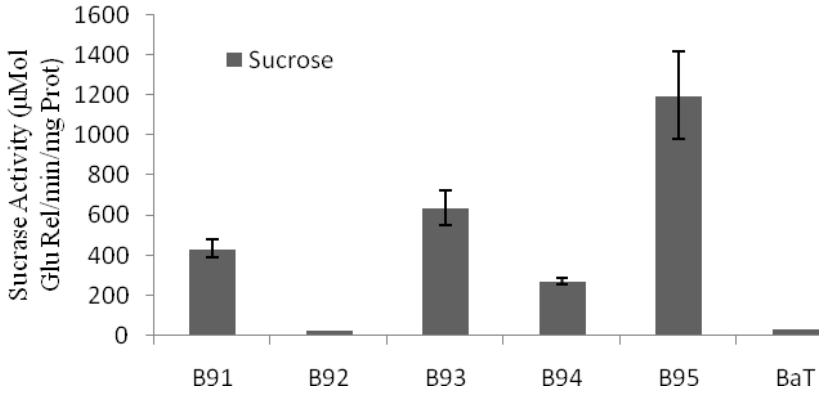
3.3.2 Sucrase activity Assay

Following growth in media containing sucrose, the CFE from the 5 *B.* adolescentis and *B. adolescentis* ATCC 15703^T were tested for ability to hydrolyze raffinose, sucrose and Raftiline using the DNS assay. The highest sucrase activity was observed when sucrose was used as a substrate for the DNS assay with isolate B95 showing the highest activity of all the isolates followed by isolates B93, B91 and B94. Isolate B92 and *B. adolescentis* ATCC 15703^T showed the least activity of all the tested strains (Fig 3.2A).

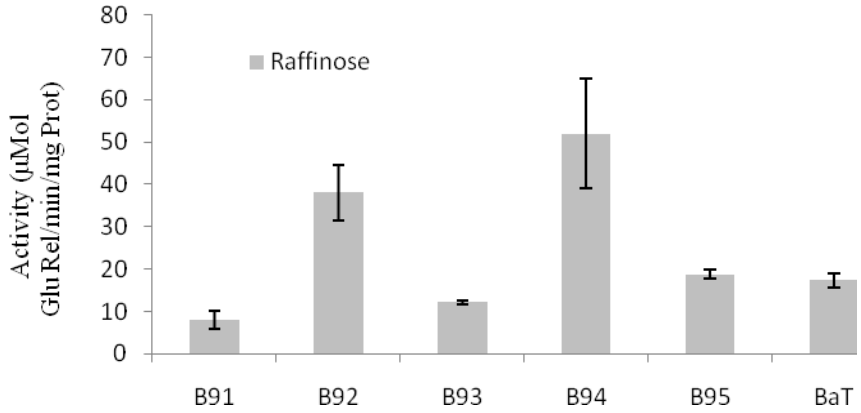
The sucrase activity of all the strains was lower when either raffinose or Raftiline was used as a substrate instead of sucrose. The magnitude of the activity observed when sucrose was a substrate was almost 30 fold the activity when either raffinose or Raftiline were used (Fig. 3.2). When 1% raffinose was used as a substrate, isolate B94 showed the highest activity of all the isolates followed by isolate B92 (Fig. 3.2B). *B. adolescentis* ATCC 15703^T and isolate B95 had almost the same activity and, isolates B91 and B93 had the least activity.

When Raftiline was used as a substrate, isolate B94 again had the best activity followed by *B. adolescentis* ATCC 15703^T (Fig 3.2C). Isolates B92 and B93 had the average level of activity of the isolates with isolate B95 having slightly lower activity compared them. Isolate B91 had the least activity of all of all the isolate. Generally, the patterns of carbohydrate preference seem to be different for each isolate but all the isolates of showed the highest sucrase activity against sucrose.

A



B



C

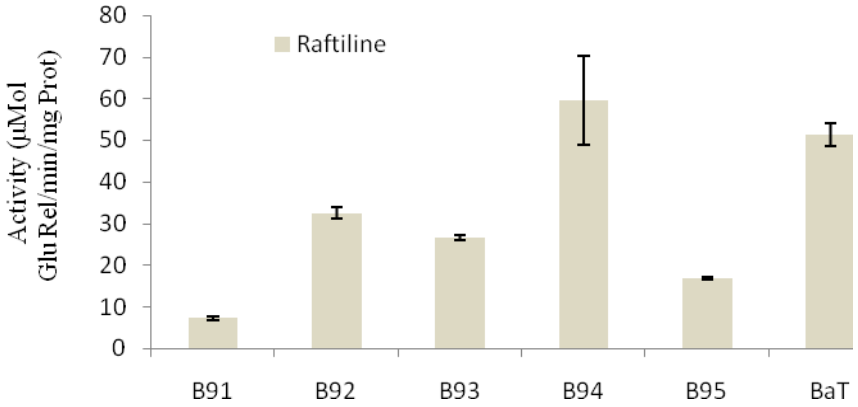


Figure 3.2 Sucrase activity of the 5 isolates and BaT after growth in media supplemented with 1% sucrose. The CFEs were then tested for sucrase activity when A) sucrose B) raffinose or C) Raftiline were the substrates.

3.3.3 Sucrose Utilization genes

The ability of the isolates to utilize sucrose so efficiently led to a bioinformatic analysis of genes that are possibly involved in sucrose catabolism. Two clusters of genes were found on the genome of *B. adolescentis* ATCC 15703^T. The first cluster consisted of 3 genes encoding the β-fructo-furanosidase (*cscA*) [BAD 1150]; galactoside permease (*lacY*) [BAD 1149] and a LacI-type transcriptional regulator [BAD 1148] (Fig. 3.3). The second cluster consisted of genes encoding sucrose phosphorylase (*spl*) [BAD 0078]; a hypothetical permease [BAD 0079] and a transmembrane transport protein (*shiA*) [BAD 0080] (Fig. 3.3).

The presence of the β-fructo-furanosidase gene (*cscA*) and sucrose phosphorylase gene (*spl*) was also confirmed in the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T by PCR (Fig 3.4).

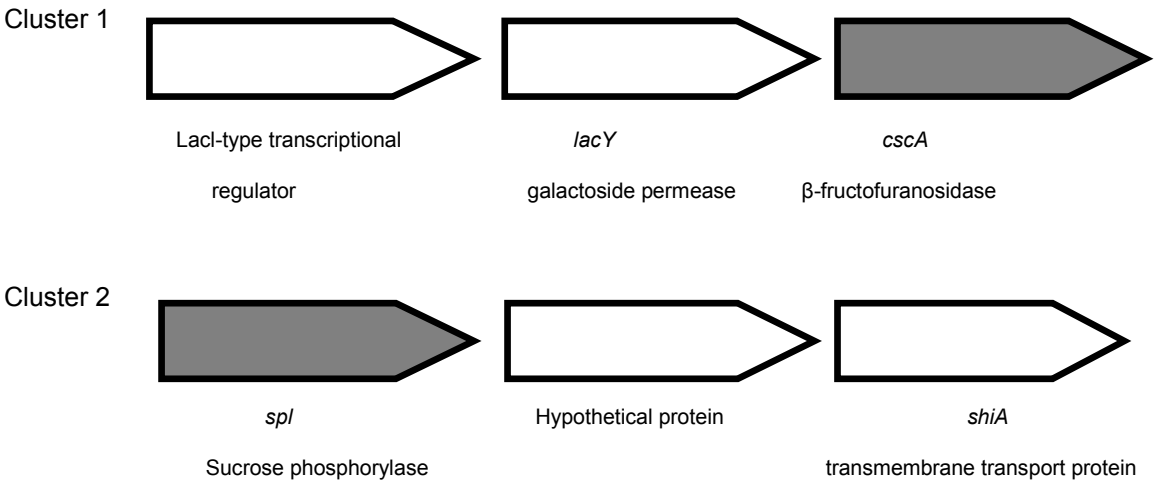


Figure 3.3 Arrangement of genes on the genome of *B. adolescentis* ATCC 15703^T that have been linked with sucrose utilization in other bifidobacteria.

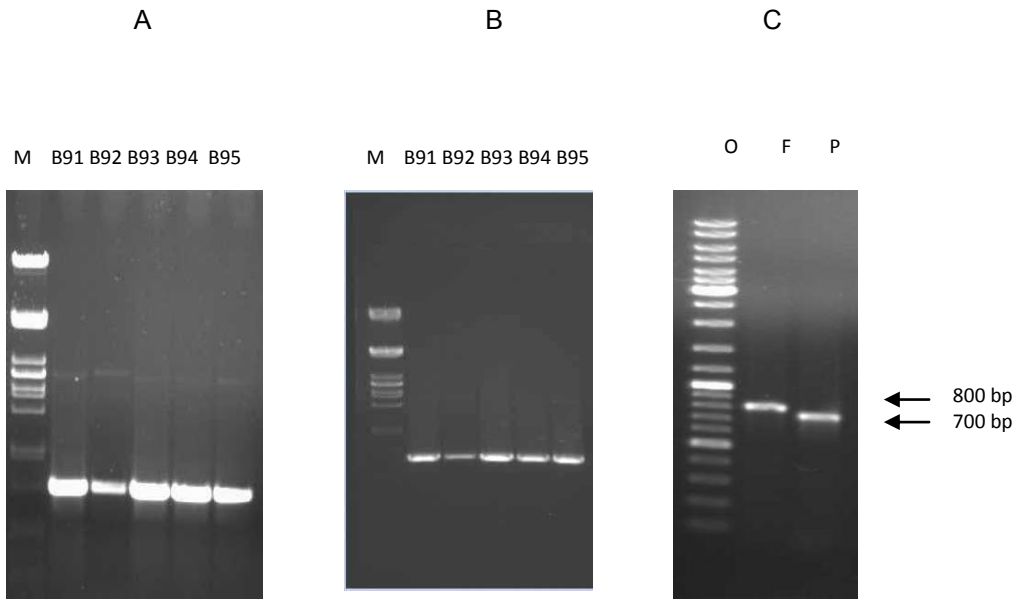


Figure 3.4 Confirmation of the presence of sucrose catabolism genes in *B. adolescentis* strains A) β-fructo-furanosidase genes (*cscA*) in 5 *B. adolescentis* isolates B) sucrose phosphorylase (*spl*) genes in 5 *B.adolescentis* strains C) β-fructo-furanosidase (F) and sucrose phosphorylase (P) genes in *B. adolescentis* ATCC 15703^{T}. (M) Lambda DNA digested with *Pst*I and (O) O'GeneRuler^{TM} DNA ladder mix.

3.4 Discussion

It has previously been shown that sugar metabolism by lactic acid bacteria is both species and strain dependent (Van der Meulen *et al*., 2004). In this study, it was, therefore, of interest to determine which carbohydrate catabolism characteristics were shown by the *B. adolescentis* strains. It was evident that all 5 isolates and *B. adolescentis* ATCC 15703^{T} were all able to grow differently in media supplemented with the different carbohydrate substrates used in this study. The high sucrase activity means that the *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^{T} were have the ability to hydrolyse carbohydrates that carry the sucrose unit such as raffinose and Raftiline.

The β (2-1)-glycosidic bonds that cannot be digested in the upper part of the human GIT are hydrolyzed by bifidobacteria that produce β-fructofuranosidases (Van der

Meulen *et al*., 2004). The hydrolysis of sucrose by a combination of β-fructofuranosidase and sucrose phosphorylase, results in phosphorylated glucose, which is directed into the hexose pathway for energy production (Kitaoka and Hayashi, 2002; Reid and Abratt, 2005). The presence of these genes was confirmed by PCR in all the 5 isolates and BaT and this could explain the ability of these isolates to utilize these carbohydrates.

The ability of mammalian GIT bacteria to utilize these complex carbohydrates has been accredited to the large number of their genes that are dedicated to sugar metabolism. This may be possibly due to the high presence of complex carbohydrates and the high competition from the microflora (Ventura *et al*., 2006). Most bifidobacteria are genetically well equipped for sugar utilization. For example, *B. adolescentis* has 4.8% of annotated genes allocated to the modification of carbohydrates (Den Broek and Voragen, 2008). In *B. longum* and *B. breve* UCC2003, more than 8.5% predicted total proteins are involved in metabolism of carbohydrates which is 50% more than other bacteria (Ventura *et al*., 2007).

There are numerous strains and species of other anaerobes in the human gut that in interact with bifidobacteria and some of these have been shown to influence the ability of *Bifidobacterium spp.* to metabolize particular carbohydrate. For example, *Bifidobacterium* spp. have been shown to ferment inulin inefficiently but thrive on fructo-oligosaccharides. It is therefore, likely that there is cross-feeding from the other microorganisms that can hydrolyze complex substrates to fructo-oligosaccharides that are internalized and utilized by *Bifidobacterium spp.* (Den Broek and Voragen, 2008). *B. adolescentis*, for example was shown to have a cross-feeding link to butyrate-producing anaerobes in the human gut (Belenguer *et al*., 2006).

The high preference of the strains for certain carbohydrates has been credited to a more efficient transport system for those particular carbohydrates (Hopkins *et al*., 1998). The other reason for this preference has also been linked to the amount of ATP produced due to the stoichiometry of the fructose-6-phosphate shunt. The carbon distribution among the substrates results in the different distributions of carbon fluxes through the fermentative pathway (Amaretti *et al*., 2007).

Different studies have linked a number of genes in other bifidobacteria to the hydrolysis of sucrose and fructo-oligosaccharides. Sucrose in *B. animalis* subsp. *lactis* and *B. longum* biotype *longum* NCC2705 is hydrolysed by sucrose–phosporylase which is coded for by *scrP* (Kullin *et al*., 2006, Trindade *et al*., 2003). In *B. breve* UCC2003, β-galactosidase coded for by *fos*, hydrolyses fructo-oligosaccharides and lactose (Ryan *et al*., 2005) and the *bfrA* operon in *B. animalis* subsp. *lactis* DSM10140 codes for β-fructofuranosidase which is linked with the hydrolysis of fructo-oligosaccharides, sucrose, inulin and raffinose.

It has been shown that glucose is not always ideal for culturing bifidobacteria (Rad and Petr, 2000). *B. adolescentis* MB 239 was shown to exhibit low levels of growth in glucose (Amaretti *et al*., 2007) and *B. animalis* DN-173 010 was found to be unable to metabolize glucose (Van der Meulen et al., 2004). This could explain the inadequate growth of isolate B94 in glucose as compared to the other carbohydrates. Glucose would give a higher ATP output in the fructose-6-phosphate shunt so this low glucose preference is probably due to poor substrate transport systems (Amaretti *et al*., 2007).

These carbohydrate utilization results also further support the conclusions from Chapter 2 that *B. adolescentis* ATCC 15703^T is possibly a different strain from these

5 isolates. The *B. adolescentis* ATCC 15703^T grew very well in all the carbohydrates and had very low sucrase activity when tested on sucrose (Fig. 3.1 and 3.2 A). The same argument could be made for isolate B92. In general the strains have different physiological features and this further suggests that they are different strains of *B. adolescentis*.

The 5 *B. adolescentis* isolates and BaT have diverse range of carbohydrates preferences as they grew at differently in the different carbohydrate substrates. Depending on the carbohydrate preference of each isolate, the results mean either raffinose, Raftiline or Raftilose have the prebiotic potential to enrich the growth of the *B. adolescentis* isolates.

The high concentration of substrates available for fermentation in the colon encourages the growth of bacteria adapted using these substrates. These adapted microbes have been linked to the reduction of bowel disease occurrences in the black South African population (Segal, 2002). The *B. adolescentis* isolates used in this study were from a black South African volunteer and they have an excellent ability to optimally utilize sucrose. It was then decided to further investigate the probiotic potential of the *B. adolescentis* isolates as a platform for future plans to investigate possible health benefits to the GIT. These studies are reported in Chapter 4.

Chapter 4 *In vitro* evaluation of the five *B. adolescentis* isolates as potential probiotic cultures

4.1 Introduction

The current view of probiotics is that they are an effective alternative treatment to traditional prophylactic and therapeutic regimes (Sleator and Hill, 2008). This has resulted in a continuous search for new probiotic strains that offer different health benefits. Most of the focus has been on lactobacilli and bifidobacteria as they are commonly used as probiotics due to the numerous potential health benefits they posses (Collins and Gibson, 1999; Yazid *et al.*, 2000). Different criteria for selecting potential probiotic strains have been outlined. For example, to minimize the health risks from potential strains intended for human use, the criteria demands that the strains should be isolated from humans and be classified as non-pathogenic (Collins *et al.,* 1998). The main focus of these selection criteria is to demonstrate the safety, metabolic activity and viability of these isolates while in the gut (Collins *et al.,* 1998).

The GIT is a hostile environment as it contains digestive enzymes, bile salts, low surface tension and an immune response (Oh-Sik *et al.*, 2002). For isolates to be used as probiotics, they should have enough transit tolerance to pass though this harsh GIT environment (Prasad *et al.,* 1999; Vernazza *et al.*, 2006). Isolates that can de-conjugate the bile acids can therefore grow in bile as well as in low pH conditions (Oh-Sik *et al.*, 2002). The transit tolerance of potential probiotic strains are tested *in vitro* under conditions that simulate those of the GIT.

Once the probiotics have survived the harsh transit conditions, they should have the ability to adhere and colonise the GIT thereby becoming part of the intestinal microbiota (Oh-Sik *et al.*, 2000). Probiotic strains that have adhesive properties stand a better chance of colonisation. Production of special inhibitory compounds by the probiotic strains also provides an added advantage for colonisation over

pathogens (Hütt *et al.*, 2006; Oh-Sik *et al.*, 2002). The screening of antimicrobial activities and isolation of such inhibitory compounds is also part of the process of selecting the most suitable strain for probiotic use (Ross *et al.*, 2005). Once isolated and identified, only strains that carry the GRAS status will be used as probiotics (Leahy *et al.*, 2005).

In this study, the 5 *B. adolescentis* strains were evaluated for suitability as probiotics using steps from the above mentioned criteria. The isolates were cultured on media that contains bile salts and has a pH and that closely resemble those of the GIT. The ability of these isolates to adhere to the GIT walls and to each other was evaluated by using yeast cells and auto-aggregation/sedimentation times. Other characteristics of the isolates that were investigated include their antibiotic profiles as well as their ability to inhibit and out-compete pathogens in terms of growth. The ability of the isolates to tolerate oxygen was also evaluated in order to give an indication of the ability of the isolates to remain viable during production and storage if selected for probiotic use. Oxygen tolerance also implies the presence of mechanisms responsible to scavenging toxic oxygen metabolites in the GIT and this could be beneficial to the host.

4.2 Materials and methods

4.2.1 Growth conditions

Unless otherwise stated, the 5 *B. adolescentis* isolates described in chapter 2 and *B. adolescentis* ATCC 15703^T (BaT) were cultured and maintained as in Chapter 2. Yeast strains were grown in YPD media (Sherman *et al.*, 1986) at 30°C for 20 hours. *Clostridium botulinum, Clostridium perfringens, Enterococcus faecalis* and *Enterococcus faecium* strains were grown on Brain Heat Infusion agar and incubated

anaerobically at 37°C. *Escherichia coli* ATCC 25922, *Salmonella typhimurium* and *Staphylococcus aureus* were grown aerobically at 37°C on LB agar.

4.2.2 Ability to tolerate low pH

The ability of the cells to withstand acidic conditions was measured using the method of Gusils *et al.* (2002) modified as follows: The pH of MRS-cys broth was adjusted by adding 50% HCl to 100 ml MRS-cys broth to give pH values of pH 1, 2 and 3. All of the media including unadjusted MRS-cys broth (pH ≈6.8), were reduced for 24 hours in an anaerobic environment before being used. Using broth cultures of the isolates and *B. adolescentis* ATCC 15703^T that had been grown in MRS-cys (pH≈6.8) at 37°C for 18 hours, the different media were inoculated to a starting OD_{620nm}= 0.1. The experiment was performed in triplicate with absorbance values at OD_{620nm} recorded after 0, 2, 4, 7, 10 and 12 hours using the S1000 Diode Array Spectrophotometer (Labotec). The viability of the strains was tested after 0 and 5 hours of acid exposure by diluting and plating 100 µl from the pH 2 samples and MRS-cys (pH ≈6.8) control on MRS-cys agar. Colonies were counted and the surviving fraction determined.

4.2.3 Ability to tolerate bile salt

The modified method of Gusils *et al.* (2002) was used for the examination of the tolerance of the isolates to bile salts. The MRS-cys broth with 0.5 and 1% (w/v) Ox bile salt (LP0055, Oxoid – sodium glycocholate and sodium taurocholate) and MRS-cys broth without any added bile salts were reduced for 24 hours in an anaerobic environment before being used. The broth cultures of the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T that had been incubated at 37°C for 18 hours were inoculated into the fresh media to a starting OD_{560nm}= 0.1. Absorbance values

were taken after 0, 2, 4, 7, 10 and 12 hours using the S1000 Diode Array Spectrophotometer (Labotec). The experiment was performed in triplicate. To test the viability of the strains after exposure to bile salts, 100 µl of the samples were plated on MRS-cys agar after 0 and 5 hours.

4.2.4 Ability to inhibit growth of pathogens

The ability of the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T to inhibit the growth of pathogens was measured. This was done by inoculating 10 µl of the culture onto a MRS-cys plate and incubating aerobically for 48 hours. The bacteria were killed by inverting the plate for 20 minutes on a sterile round filter paper, soaked in chloroform. The plate was then overlaid with 0.7% sloppy agar containing the different representative pathogen indicator strains. In order to ensure formation of a lawn on the plate the formula below was used to calculate the volume to inoculate in the sloppy agar:

$$OD_{600nm} \times \mu l = 4 \qquad (1)$$

was used to calculate the volume of the starting inocula for *Escherichia coli* ATCC 25922 and *Salmonella typhimurium.* The formula:

$$OD_{600nm} \times \mu l = 160 \qquad (2)$$

was used calculate the volume of *Clostridium botulinum, Clostridium perfringens, Enterococcus faecalis, Enterococcus faecium* and *Staphylococcus aureus* to add into the sloppy agars. The diameters of the zones of inhibition were measured after 24 hours of aerobic or anaerobic incubation depending on the bacterial strain.

4.2.5 Antibiotic resistance profiles

MRS broth cultures of the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T that had been incubated at 37°C for 18 hours were diluted to OD_{600nm}= 0.257 and 100 µl were plated on MRS-cys plates. The plates were left to dry for 15 minutes and disks of various antibiotics were placed on the plates and incubated anaerobically for 48 hours. The antibiotics (µg per disc) used were: Vancomycin (30), Penicilin G (10), chloramphenicol (30), tetracycline (30), Kanamycin (30), erythromycin (15), ampicillin (10), rifampicin (5) and streptomycin (10). The diameters of the zones of clearance around the disks were measured and used to calculate the area of growth inhibition. The experiment was performed in triplicate.

4.2.6 Adhesion assays

4.2.6.1 Auto-aggregation

Cultures of the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T were incubated in MRS at 37°C for 18 hours. Separate tubes, each containing 4 ml of each culture, was centrifuged at 10,000 g for 2 minutes and washed twice with 0.85% saline solution. The supernatant was filter sterilized with a 0.4 micron filter and stored in a separate tube. The pellet in the one tube was re-suspended in 2ml of 0.85% saline solution while the other pellet was re-suspended in 1 ml 0.85% saline solution and 1 ml of the supernatant. The tubes were vortexed and the time taken for the top half of the tube to begin clearing was recorded as the start of the auto-aggregation. The overall degree of auto-aggregation was evaluated after two hours by the comparison of the clarity in the top half of the tube as well as the cells that had already settled for all the different isolates. The highest score was graded

(+++++) while lowest score was awarded (+) and (0) where there was no aggregation.

4.2.6.2 Yeast Agglutination

The ability of probiotic strains to agglutinate yeast has been used as an indicator for colonisation ability *(*Alderberth *et al.,* 1996*)*. The 5 B. adolescentis isolates and *B. adolescentis* ATCC 15703^{T} were incubated at 37°C for 18 hours and 2 ml of each of the cultures was centrifuged at 10,000 g for 10 minutes in sterile 2 ml tubes. The supernatant from each tube was filter sterilized using a 0.4 micron filter and stored in separate 2 ml tubes. The pellets were re-suspended in 2 ml of 1X Phosphate Buffer Saline (PBS) solution at pH 7.4. Yeast cells were incubated in YPD media (Sherman *et al.*, 1986) at 30°C for 20 hours. The yeast cells were stained with safranin and washed with distilled water. The staining and washing were repeated 4 times, and then the yeast cells were re-suspended in 1 ml PBS buffer. For each of the isolate samples, 20 µl of the yeast suspension was mixed on a glass slide with 20 µl of the bacterial cells and examined for agglutination under an Olympus CX40 bright field microscope. To test the effect of the supernatant on aggregation, the bacterial pellet was re-suspended in equal volumes of the filter sterilized supernatant, and 20 µl was mixed with 20 µl of safranin stained yeast cells.

4.2.7. Oxygen Tolerance

4.2.7.1 The Relative Bacterial Growth Ratio

The Relative Bacterial Growth Ratio (RBGR) method of Talwalkar *et al.* (2001) was used to evaluate the ability of the isolates to grow aerobically. The RBGR of each culture was determined by dividing its absorbency during aerobic growth in MRS with

shaking, by its absorbency during anaerobic growth in MRS-cys. The experiment was performed in triplicate.

4.2.7.2 Ability to survive aerobic exposure

The ability of the isolates to survive exposure to aerobic conditions was investigated. The 5 isolates and B*. adolescentis* ATCC 15703^T were cultured aerobically at 37°C for 18 hours. These were diluted to a starting OD_{600nm} of 0.1 and cultured aerobically for 24 hours and OD_{600nm} measured. The same cultures were then transferred into the anaerobic chamber and incubated for 24 hours at which point OD_{600nm} was measured again. Cultures incubated anaerobically for the duration of the experiment were used as controls.

To determine the viability of the isolates during the aerobic exposure isolates B91, B92 and B93 and *B. adolescentis* ATCC 15703^T were cultured aerobically for 48 hours using the same starting OD_{600nm} as above. Samples of 100 µl, were taken after 2, 24 and 48 hours. These were diluted and plated on MRS-cys agar and the plates were incubated anaerobically for 48 hours.

4.3 Result

4.3.1 Ability to tolerate low pH

Probiotics are generally administered in the form of a food substance or a tablet. The probiotics then have to survive the transit through the harsh conditions in the upper GIT such as the acidic conditions in the stomach. In order to determine whether the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T could tolerate acidic conditions, they were incubated at low pH for 12 hours. After the incubation, all the

isolates showed significant growth in the control MRS-cys media pH 6.8 (Fig. 4.1). There was however, no sign of bacterial growth in the MRS-cys with pH 1, 2 or 3.

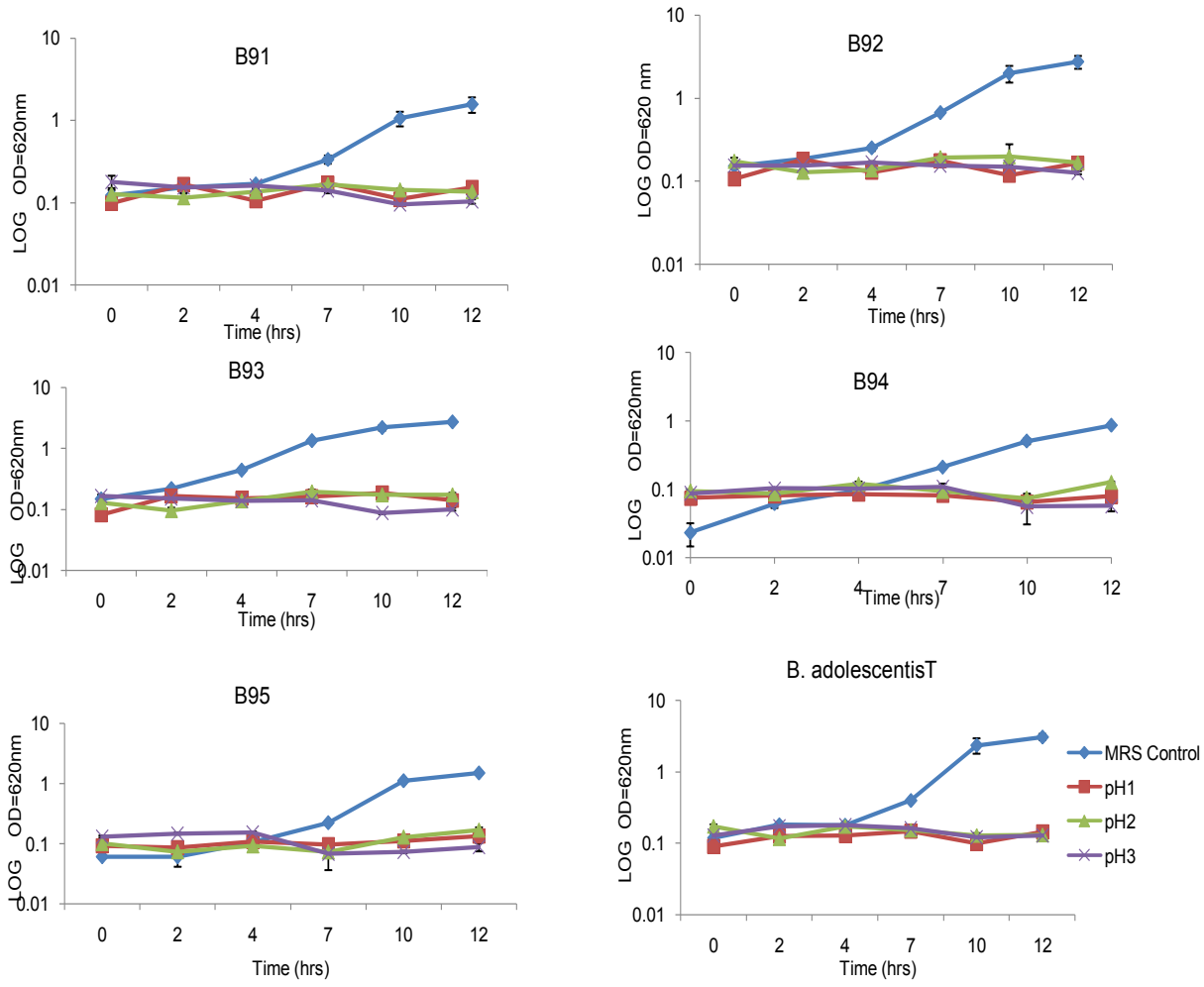


Figure 4.1 The effect of pH on the isolates and *B. adolescentis* ATCC 15703^T. The absorbance values at 620 nm were measured over a period of 12 hours of growth in media with adjusted pH of 1, 2 and 3. The MRS-cys broth with the unadjusted pH of 6.8 was used as a control.

It was clear that the cells were not growing in the low pH media, but whether the cells were viable, this could not be determined from the absorbance values. The state of viability of the isolates and *B. adolescentis* ATCC 15703^T were determined at the start of the experiment and after 5 hours by plating on MRS-cys agar. In the MRS-cys (pH 6.8), all the isolates and *B. adolescentis* ATCC 15703^T were viable and

growing after the 5 hours (Table 4.1). In the media with pH 2, all the isolates and *B. adolescentis* ATCC 15703^T were viable at the start of the experiment but isolates B91, B92, B93 and B94 did not show any viability after 5 hours of exposure. Isolate B95 and *B. adolescentis* ATCC 15703^T gave viable titres of 4.4 x 10^5 cfu/ml and 1 x 10^7 cfu/ml respectively, despite their inability to grow in broth at low pH.

Table 4.1 Viability in (cfu/ml) of the 5 isolates and *B. adolescentis* ATCC 15703^T before and after 5 hour of exposure to pH 2.

Strain	*MRS (time=0)*	*MRS (time=5 hrs)*	*pH=2 (time=0)*	*pH=2 (time=5 hrs)*
B91	8 x 10^5	9.2 x 10^5	4 x 10^5	NG
B92	9 x 10^5	1 x 10^6	1.2 x 10^6	NG
B93	6.8 x 10^7	1.6 x 10^8	5.1 x 10^7	NG
B94	4.2 x 10^6	5.2 x 10^6	3.94 x 10^6	NG
B95	1.86 x 10^6	4.85 x 10^6	1.6 x 10^6	4.4 x 10^5
BaT	1.57 x 10^7	5.36 x 10^7	1.26 x 10^7	1.02 x 10^7

(NG = No Growth)

4.3.2 Ability to tolerate bile salts

High concentrations of bile salts are also an obstacle for consumed probiotics on route to the lower GIT. The same approach used to test for the acid tolerance of the *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T, was used to test their ability to withstand exposure to bile salts. The *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T were incubated for 10 hours in MRS-cys media with 0.5% and 1% added bile salts. MRS-cys without any added bile salts was used as a control. After the incubation period, the highest growth was observed in the control media for all the isolates and *B. adolescentis* ATCC 15703^T (Fig 4.2). Isolates B92

and *B. adolescentis* ATCC 15703^{T} displayed moderate levels of growth, reaching OD values of approximately 1.0. Isolate B91 grew to a limited extent while isolates B93, B94 and B95 did not grow in media with bile salts. There were no notable differences in the growth of the isolates when grown in either 0.5% or 1% bile salts.

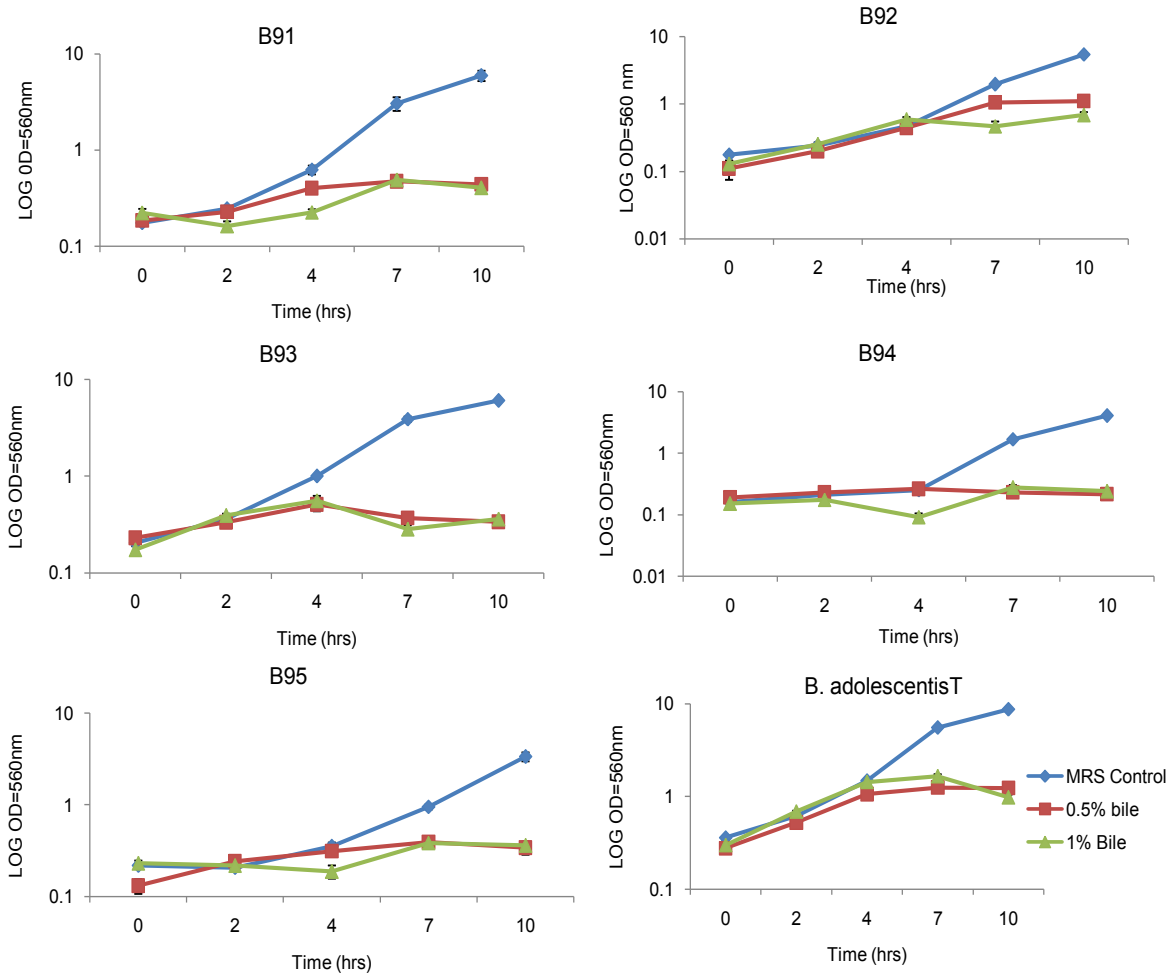


Figure 4.2 The effect of bile salts on the isolates and *B. adolescentis* ATCC 15703^{T}. The absorbance values at 560 nm were measured over a period of 10 hours of growth in MRS-cys broth with 0,5% and 1 % bile salts. The MRS-cys broth with no bile salts added was used as a control.

To test whether the cells were viable during the bile exposure, a sample was taken at the start of the experiment and after 5 hours of exposure and plated on MRS-cys agar. At the start of tests, all the isolates and *B. adolescentis* ATCC 15703^{T} were viable (Table 4.2). After 5 hours of exposure to the bile salts, isolates B92, B94, B95

and *B. adolescentis* ATCC 15703^T were still viable although reduced in number. Isolate B91 and B93 did not survive the 5 hours of exposure to bile salts.

Table 4.2 Viability (cfu/ml) of the 5 isolates and *B. adolescentis* ATCC 15703^T before and after 5 hour of exposure to 0.5 % bile salts.

Strain	*MRS (time=0)*	*MRS (time=5 hrs)*	*0.5% bile salts (time=0)*	*0.5% bile salts (time=5 hrs)*
B91	8×10^5	9.2×10^5	5.8×10^5	NG
B92	9×10^5	1×10^6	1.1×10^6	1.2×10^5
B93	6.8×10^7	1.6×10^8	5.2×10^7	NG
B94	4.2×10^6	5.2×10^6	4.37×10^6	1.82×10^6
B95	1.86×10^6	4.85×10^6	1.7×10^6	2.65×10^5
BaT	1.57×10^7	5.36×10^7	3.24×10^7	1.37×10^7

(NG = No Growth)

4.3.3 Ability to inhibit growth of pathogens

One of the proposed health benefits of probiotics is that their presence and products are able to inhibit pathogens in the GIT (Oh-Sik *et al.*, 2002). The ability of these isolates to inhibit pathogens would be an indication of their potential as probiotics. All of the isolates examined in this study,were able to prevent the growth of *E. coli, C. perfringens, E. faecalis* and *S. aureus* (Fig. 4.3). Only isolates B92 and B93 showed activity against *Clostridium botulinum* and only isolates B91, B92 and B95 showed activity against *Enterococcus faecium*. The rest of the isolates had activity against *S. typhimurium* except for isolates B92 and B95 which did not show any activity. *E. coli, S. typhimurium* and *S. aureus* were the most sensitive to the antimicrobial activities of the isolates as they showed the largest zones of growth inhibition.

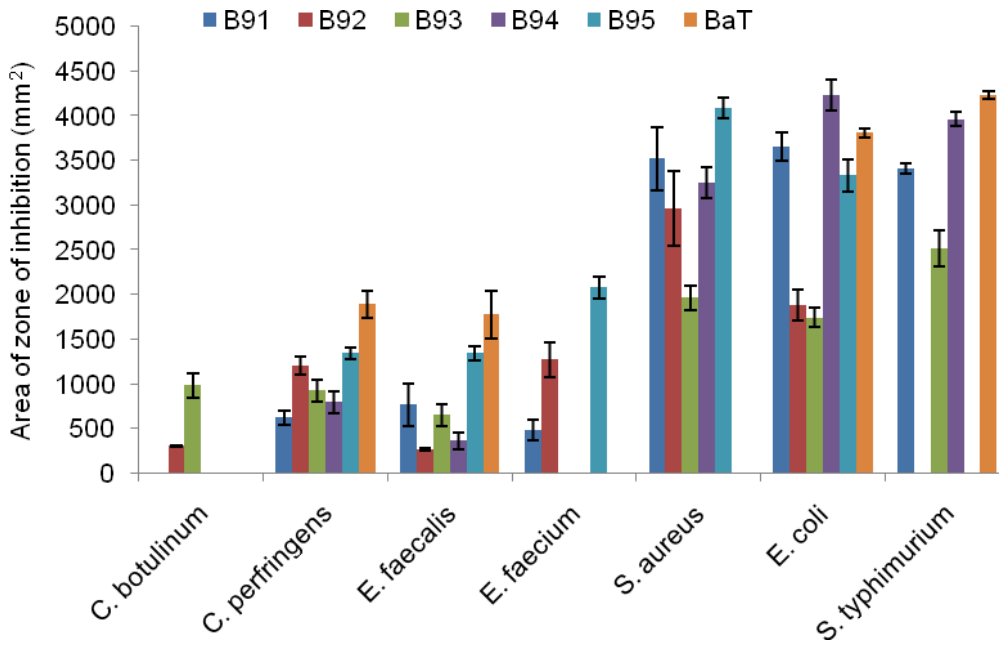


Figure 4.3 Antimicrobial profiles of isolates. The zones of growth inhibition clearance were measured in millimetres (mm).

4.3.4 Antibiotic resistance

Knowledge of antibiotic resistance and sensitivity is important in probiotics therapy and is also a useful tool for screening bacteria and minimizes contamination. The unknown resistance and sensitivity profiles of the 5 isolates were investigated as in section 4.2.5. After 48 hours of incubation, there were zones of clearance around all of the antibiotic disks for all the *B. adolescentis* isolates and *B. adolescentis ATCC 15703* except around the streptomycin and kanamycin disks (Fig. 4.4). This means that all the isolates and *B. adolescentis ATCC 15703* were resistant to streptomycin and kanamycin. The other exception was that only isolate B95 was resistant to vancomycin. All the isolates had the same antibiotic profiles but with varying degrees of sensitivity indicated by the areas of the zones of clearance.

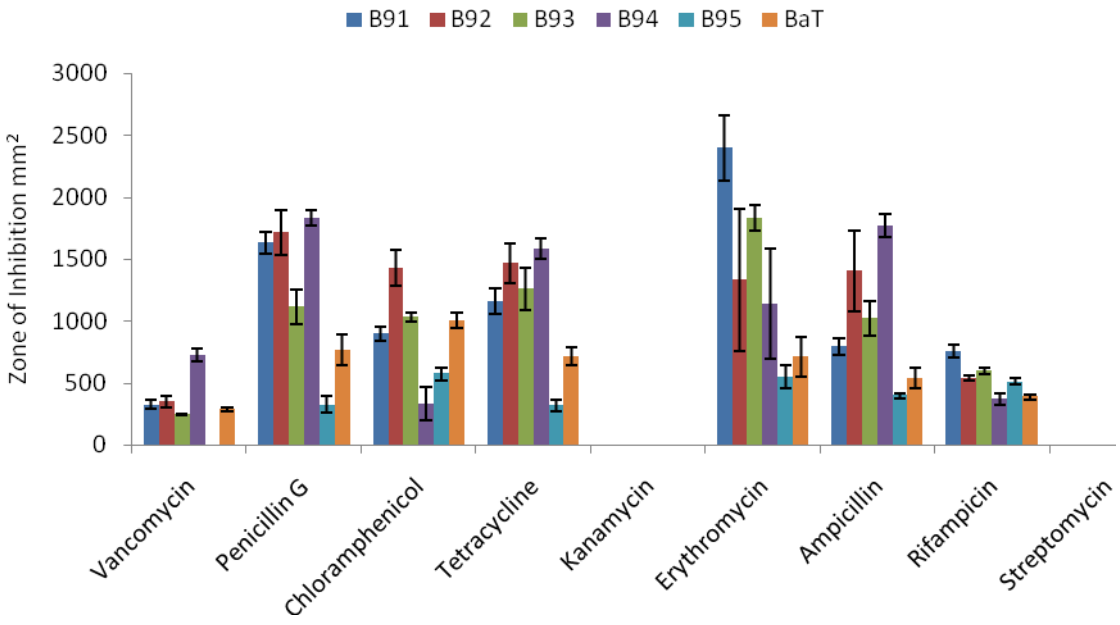


Figure 4.4 The antibiotic profiles of isolates. The isolates were plated on MRS-cys plates and antibiotic disks were placed on the plates and incubated for 48 hours. The zones of clearance were measured and used to interpret the antibiotic profiles of the isolates.

4.3.5 Adhesion assays

When bacterial cells have the ability to adhere to the intestinal lining, they have an increased chance of colonisation and a chance to contribute their health benefits. The auto-aggregation and yeast agglutination assays provide a preliminary assessment of the potential of the isolates to adhere to the GIT.

4.3.5.1 Auto-aggregation

Isolates B91 and B95 were the first to auto-aggregate settling to the bottom of the tube after 15 minutes (Table 4.3). Isolate B93 initiated settling after 30 minutes incubation with isolates B94 and B92 following with settlement times of 35 and 38 minutes, respectively. *B. adolescentis ATCC 15703* was the last to initiate settling with a settlement time of 70 minutes.

After 2 hours, the overall aggregation was assessed by comparing the clarity of the suspension and the amount of bacterial cells that had settled to the bottom of the tubes. The comparison was done by eye and a score given in the range of (+++++) for the best overall settlement and (+) for the poorest. In this study, the best overall aggregation was shown by isolate B95 followed by isolates B91, B92, B93 and B94. *B. adolescentis* ATCC 15703^T did not aggregate effectively at all (Table 4.3). The addition of the culture supernatant did not influence the initiation of settling nor the overall aggregation of any of the cells.

Table 4.3 The total time taken for the re-suspended isolates to settle in PBS buffer only or PBS buffer added with culture supernatant.

Strain	*Time taken for settling to initiate [Cells only] (minutes)*	*Time taken for settling to initiate [Cells + Supernatant] (minutes)*	*Overall aggregation after 2 hrs [Cells only]*	*Overall aggregation after 2 hrs [Cells + Supernatant]*
B91	15	15	+++	+++
B92	38	38	+++	+++
B93	30	30	++	++
B94	35	35	+	+
B95	15	15	+++++	+++++
***B. adolescentis* ATCC 15703**	70	70	+	+

4.3.5.2 Yeast Agglutination

Microscope images were taken to compare the different abilities of the isolates to agglutinate yeast cells (Fig. 4.5). Yeast cells suspended in the PBS buffer showed an even distribution in the microscope field and this was given a score of zero (Fig. 4.5A). When the bacterial cells were mixed with the yeast cells, various degrees of clumping occurred, with (+++++) being the highest possible scoring. Isolate B93 (Fig. 4.3B) and *B. adolescentis* ATCC 15703^{T} caused the yeasts cells to slightly increase their degree of agglutination when compared to the rest of the isolates (Table 4.4). The rest of the isolates caused very minimal agglutination of the yeast cells. When the isolates cells were suspended with the culture supernatant, the degree of agglutination of yeast cells caused by isolates B92, B93, B94 and B95 was the same as when suspended in yeast cells only. Addition of the supernatant, considerably increased agglutination of the yeast cells where isolate B91 and *B. adolescentis* ATTC 15703^{T} were added with isolate B91 causing the best overall agglutination of yeast cells (Fig. 4.5C and Table 4.4).

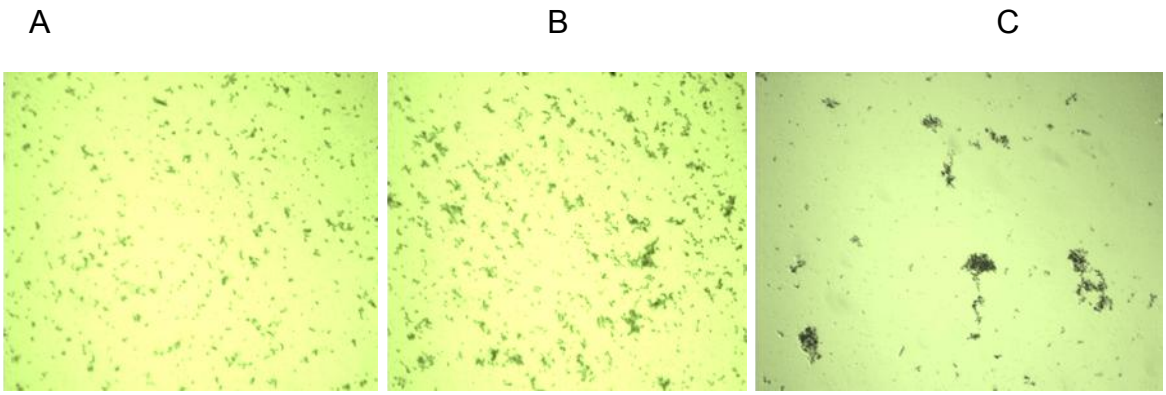


Figure 4.5 Images of the agglutination of some of the *B. adolescentis* isolates to safranin stained yeast strains. A) Yeast cells suspended PBS buffer score=0 B) Isolate B93 and yeast cells, score = [++] and C) Isolate B91 and yeast cells and the supernatant score = [+++++]. (*Only the brightness and contrast of the images was edited post the microscope viewing to improve quality).*

Table 4.4 The agglutination scores of the 5 isolates and *B. adolescentis* ATTC 15703^{T}

	Yeast cells in PBS only	*Yeast cells in PBS + supernatant*
B91	+	+++++
B92	+	+
B93	++	++
B94	+	+
B95	+	+
BaT	++	+++

4.3.6 Oxygen tolerance

4.3.6.1 The Relative Bacterial Growth Ratio

The relative bacterial growth ration (RBGR) compares the anaerobic growth of the isolates to the aerobic growth. Aerotolerant strains have higher RBGR because of the high aerobic growth. For example, Talwalkar *et al.* (2001), concluded that *B. infantis* was aerotolerant because it had RBGR as high as 0.78. All the isolates exhibited very low growth under aerobic conditions resulting in a low RBGR. Isolate B94 was the most sensitive to oxygen followed by isolate B91, B92, B93 and B95. *B. adolescentis ATCC 15703* showed the highest aerobic growth ability of the all the isolates hence had the highest RBGR (Table 4.5).

Table 4.5 RBGR which is the ratio derived from the measurements of aerobic growth of the isolates over the anaerobic growth.

	Relative Bacterial Growth Ratio
B91	0.129267
B92	0.157472
B93	0.223285
B94	0.114872
B95	0.227642
BaT	0.487567

4.3.6.2 Ability to grow after aerobic exposure

As expected, all the isolates and *B. adolescentis ATCC 15703* grew well in anaerobic conditions. However, after 24 hours of exposure to aerobic conditions, all 5 *B. adolescentis* isolates *and B. adolescentis ATCC 15703* could not resume growth in anaerobic conditions (Fig. 4.6). *B. adolescentis ATCC 15703* once again showed higher aerobic growth as compared to the rest of the isolates (Fig. 4.6). A few of the isolates and *B. adolescentis ATCC 15703 were* then tested for viability, and with the exception of isolate B92, the isolates were still viable after 2 hours of aerobic exposure (Table 4.6).

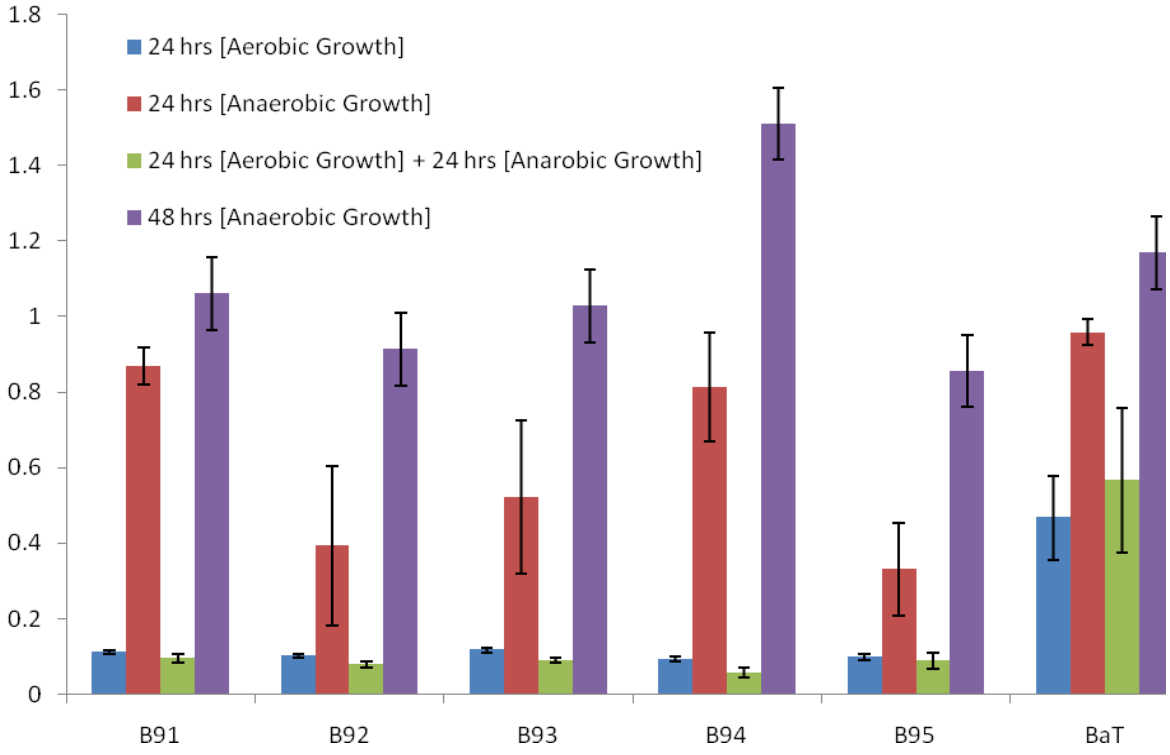


Figure 4.6 Ability of the 5 *B. adolescentis* isolates and *B. adolescentis ATCC 15703* to grow in aerobic conditions and to resume growth after 24 hours aerobic exposure.

Table 4.6 Total numbers of viable cells (cfu/ml) exposure to aerobic conditions.

Strain	*0 hrs*	*2 hrs*	*24 hrs*	*48 hrs*
B91	5.48×10^{7}	5.4×10^{6}	NG	N G
B92	4.7×10^{8}	NG	NG	NG
B93	3.5×10^{7}	4.6×10^{7}	NG	NG
BAT	1.67×10^{9}	1.7×10^{8}	NG	NG

(NG = No Growth)

4.4 Discussion

All 5 *B. adolescentis* isolates were found to have poor tolerance of low pH. This indicates that they will most likely be sensitive to the pH conditions found in the stomach during transit. Some bifidobacteria (Prasad *et al.*, 1999) and more specifically *B. adolescentis* strains have been shown to be generally sensitive to acidic conditions (Chung *et al*., 1999; Collado and Sanz, 2007*)*. The isolates were also sensitive to bile salts but were able to show low growth and survival in bile salts indicating some level of tolerance, with isolate B92 being the most tolerant to the bile salts.

Sensitivity to transit stress does not necessarily render strains useless as probiotics, but indicates that protective measures should be taken to guarantee delivery of the isolates to their desired location in the GIT. Using data from acid tolerance experiments, Vernazza *et al.* (2006) suggested that *B. adolescentis* DSM20083 (BaT) would need protective coating if used as a probiotic as it was very sensitive to low pH. There are a number of methods used to protect isolates to ensure that they reach the lower intestines viable (Ross *et al.*, 2005). The isolates can be encapsulated in gel beads made of alginate, pectin and whey proteins (Ross *et al.*, 2005). The other alternative is freeze-drying the isolates or using them as spray-dried powders. A number of bifidobacteria strains have been successfully dried and shown to be viable after delivery (Simpson *et* al., 2005; Ross *et al.*, 2005).

It has been shown that sensitivity to acid and bile salts can be reduced by pre-exposure of strains to sub-lethal conditions of acid and bile salts concentrations (Doleyres *et al.,* 2004; Collado and Sanz, 2007; Sleator and Hill, 2008). The heat tolerance *B. adolescentis* ATCC 15703 was increased 128-fold following pre-

exposure to lethal heat shock (Schmidt and Zink, 2000). This stress adaptation due to pre-exposure, may result in cross-protection of the organism against a variety of other types of stress such as heat, starvation and oxygen toxicity (Simpson *et* al., 2005; Sleator and Hill, 2008; Collado and Sanz*,* 2007).

The auto-aggregation assay predicts the ability of bacterial cells to clump hence increasing their chances of gut wall adherence (Conway *et al*., 1987) Yeast cells agglutination gives an indication of the affinity of the isolates to mannose-containing moieties of glycoconjugates on the mucosal surface (Pretzer *et al.* 2005). The correlation between colonization of probiotics in mice and the ability to agglutinate yeast cells was reported by Alderberth *et al.* (1996). In this study, the *B. adolescentis* isolates showed variable auto-aggregation and ability to cause yeast agglutination. Isolate B91 appeared to be the most likely to colonise the GIT because it showed the highest degree of auto-aggregation and yeast agglutination while isolate B94 was the poorest. Isolate B93 showed some potential to adhere to the GIT. The adhesion assays of isolate B92, isolate B95 and *B. adolescentis* ATCC 15703 were inconclusive as the auto-aggregation and yeast agglutination results were contradictory with one being high when the other was low. With all the isolates, the contents of the supernatant did not have an effect on the cells of adhering to each other but increased the ability of both isolate B91 and *B. adolescentis* ATCC 15703 agglutinating yeast cells. This implies that there is possibility, a secreted substance(s) that may enhance adhesion of the isolate B91 and *B. adolescentis* ATCC 15703 to the intestinal wall.

Compared to the *Lactobacillus* spp. results of similar adhesion assays (unpublished work from same laboratory as this study), the adhesion properties of all 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703 are generally poor. This is in

agreement with He *et al.* (2001), where several strains of *B. adolescentis* strains isolated from healthy adults were shown to have very low adhesion to human intestinal mucus.

Interaction with different types of bacteria has been suggested to increase the adhesion efficiency of *B. adolescentis* due to specific cell to cell interactions between the distinct bacterial cell types (Nagaoka *et al.,* 2008). This implies that these *in vitro* results could improve *in vivo* where there would be a higher chance of the occurrence of the specific interactions or if the right bacterial types are used for *in vitro* experiments. This could explain why *in vitro* adhesion results have been shown to be different to *in vivo* results (Tuomola *et al*., 2001). It is therefore important to carefully choose the methodology to test adherence. To get a more accurate analysis of the adherence of the 5 *B. adolescentis* isolates, alternative assessments using intestinal mucus or Caco-2 cells (Crociani *et al.,* 1995; Cepeljnik *et al.,* 2007).

The ability of some of the isolates to inhibit growth of certain pathogenic bacteria is a good indicator of their potential health benefits. All the *B. adolescentis* isolates had anti-microbial activity against *S. aureus* and *E. coli.* There were differences in activity between the isolates which could be an indication that antimicrobial activity is strain dependent. Several strains of bifidobacteria have been shown to have anti-microbial activity against *S. typhimurium* (Henriksson and Conway, 2001) and three of the isolates in this study, isolate B91, B93 and B94, had this activity.

In cases where patients suffer from antibiotic associated colitis, the knowledge of antibiotic profiles of bacteria to be used as probiotics can be useful for the re-establishment of the intestinal microbiota (Doleyres *et al.,* 2004) and can also be useful when patients are on both probiotics and antibiotic treatments. Evaluation of

the 5 *B. adolescentis* isolates revealed that all of them and *B. adolescentis ATCC 15703* were resistant to kanamycin and streptomycin which are amino-glycoside antibiotics (Lim *et al*., 1993). Yazid *et al.* (2000) found that *B. adolescentis* ATCC 11146 was sensitive to the same antibiotics. Charteris *et al*. (1998) also showed that human *Bifidobacterium* isolates were resistant to kanamycin, streptomycin and vancomycin. In this study, isolate B95 was resistant to vancomycin and the rest of the isolates showed only weak susceptibility. The sensitivity of the isolates to chloramphenicol, penicillin, ampicillin and to gram positive spectrum antibiotics, vancomycin and erythromycin, was in agreement with published bifidobacteria profiles (Yazid *et al*., 2000; Lim *et al*., 1993). It would be important to determine whether the whether the vancomycin resistance is due to the presence of vancomycin resistance genes and whether this would pose a health risk.

As strict anaerobes, bifidobacteria are expected to be sensitive to oxygen and oxygen species. There have, however, been reports of commercial bifidobacteria probiotic strains such as the *Bifidobacterium animalis ssp. lactis* USCC50051 that have the ability to withstand low levels of oxidative stress (Jayamane and Adams, 2006). This feature is crucial to the survival of strains in the event that probiotics are exposed to air during production or storage of probiotics. The results of the 5 *B. adolescentis* isolates in this study, are in agreement with previous studies that reported *B. adolescentis* to have a low tolerance to oxidative stress (Jayamane and Adams, 2006; Simpson *et* al., 2005). *B. adolescentis* ATCC 15703^{T}, however, was able to grow aerobically (Fig. 4.6) and the isolates were able to survive short periods of aerobic exposure (Table 4.5 and 4.6).

4.5 Conclusions

Although the isolates and *B. adolescentis ATCC 15703* showed low tolerance to pH, some of the isolates were able to remain viable in bile salts. With some additional measures, the transit tolerance of these isolates could be enhanced. The isolates also showed some adhesion properties but to a low degree. Further studies on the adherence properties of these isolates should be done using intestinal mucus and Caco-2 cells which are commonly used in the evaluation of adhesion of probiotics to the GIT walls (Crociani *et al.,* 1995; Cepeljnik *et al.,* 2007). Fluorescent and quantitative techniques should be used to further analyse the aggregation of the isolates. The isolates showed antagonistic activity against a number of pathogen indicator strains which is a beneficial property of a probiotic. As with the carbohydrate preferences in Chapter 3, there were differences in the profiles of the isolates which further suggest that although all of them were identified in chapter 2 as *B. adolescentis,* they differ from the Type strain of *B. adolescentis* and may be different strains of *B. adolescentis*, altogether.

Some of the isolates were able to remain viable for up to 2 hours of aerobic exposure while *B. adolescentis ATCC 15703* was able to grow. It was then decided to investigate the presence of a possible oxidative stress response mechanism responsible for the defence against oxidative stress. This is described in the following Chapter.

Chapter 5: Oxidative stress tolerance of the 5 isolates

5.1 Introduction

It is advantageous for probiotic strains to tolerate aerobic conditions so that they are viable when administered. In Chapter 4, it was shown that, the 5 *B. adolescentis* isolates were able to withstand brief aerobic exposure to varying extents. A search for a possible oxidative stress response system was, therefore, conducted in these strains and *B. adolescentis* ATCC 15703^T in order to understand the molecular basis of aerotolerance in these bacteria. Obligate anaerobes cannot derive energy from oxygen respiration and cannot survive atmospheric oxygen levels for long periods (Brioukhanov and Netrusov, 2007). Some anaerobes can, however, endure short-term aerobiosis and resume growth. Certain strains of *Bifidobacterium infantis, Bifidobacterium breve, Bifidobacterium longum* and *B. adolescentis*, are capable of growing under conditions of partial aeration (Brioukhanov and Netrusov, 2007). Different anaerobes have developed various mechanisms to deal with oxidative stress, which is defined as the imbalance between the production and disposal of active oxygen species (Takemoto *et al*., 1998). *Bacteroides fragilis*, for example, has a complex oxidative stress system that consists of at least 28 proteins (Rocha *et al*., 2007).

Thiol oxidation is one of the main problems that anaerobes have to adapt to in an aerobic environment. Thiol oxidation results in the production of reactive oxygen species (ROS) and these are extremely toxic to anaerobes (Rocha *et al*., 2007). The thioredoxin/thioredoxin reductase system is the redox system that deals with thiol oxidation in *B. fragilis* (Rocha *et al*., 2007) and in *Lactobacillus plantarum (*Serrano *et al*., 2007*)*. Thioredoxin is a small conserved and heat stable protein that has redox activity (Takemoto *et al*., 1998; Serrano *et al*., 2007). Some of the many roles of thioredoxin in the cell, are providing defence against oxidative stress by reducing

protein disulfide bonds produced by various oxidants (Rocha *et al*., 2007) through the scavenging of ROS (Takemoto et al., 1998; Rocha *et al.,* 2007) and by catalysing the reduction of H_2O_2 (Arner and Holmgren, 2000).

The above mentioned reduction of intracellular disulfides is mediated by NADPH, which operates together with flavin adenine dinucleotide-dependent thioredoxin reductase to convert oxidized thioredoxin to the free thiol form. Electrons from the cysteine rich site of the reduced thioredoxin are transferred to the substrates (proteins, disulfides, etc). This oxidises the thioredoxin, which is regenerated via thioredoxin reductase (encoded by the *trxA* gene) using NADPH as a cofactor (Serrano *et al*., 2007).

A number of studies have shown the role of thioredoxin reductase in oxidative stress defence in a number of organisms but such a study has, however, not been conducted in bifidobacteria. H_2O_2 and diamide were used to emulate the aerobic conditions that would be stressful to anaerobes. The abundance of H_2O_2 results in the production of OH^- species and these damage DNA, proteins and lipids (Takemoto *et al*., 1998). Diamide is a small thiol-oxidizing agent that induces disulphide stress and was used to cause thiol oxidation (Talwalkar and Kailasapathy, 2004). An *E. coli* thioredoxin reductase (*trxB*) mutant was shown to be hypersensitive to H_2O_2 (Takemoto *et al*., 1998). A *B. fragilis trxB* mutant was shown to be sensitive to both H_2O_2 and diamide and needed a reductant to grow (Rocha *et al*., 2007).

In this chapter, a search for the presence of several genes that are possibly involved in the oxidative stress response of *B. adolescentis* was conducted, with the main focus being on the thioredoxin reductase gene (*trxB*). PCR, cloning, gene expression

and analysis of the phenotypic contribution of the gene to oxidative stress response, were the approaches used.

5.2 Materials and Methods

5.2.1 Bioinformatics analysis of *Bifidobacterium* oxidative stress response genes

The genomes of *B. adolescentis* ATCC 15703 and *B. longum* NCC2705 lodged in the NCBI GenBank were screened for the presence of different genes that have been reported in the literature to be involved in the defence against oxidative stress. The gene sequences found were used to design primers using DNAMAN and primer blast on NCBI (Table 5.1). The sequence identities were obtained using the BLAST algorithm (Altschul *et al*., 1990).

5.2.2 Strains and growth conditions

The growth conditions for the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^T (BaT) were as described in section 2.2.1. *E. coli* JM109, *E. coli* JM110 and *E. coli* BHB2600 (Rainer *et al.,* 1989) were grown aerobically at 37°C in LB. Ampicillin (final concentration 100 µg/ml was used for screening *E. coli* JM109 transformants. *E. coli* BHB2600 was cultured on LB agar with 2 µg/ml chloramphenicol and 50 µg/ml erythromycin. The *Lactococcus lactis* MG1363 mutant was grown in M17 agar (Terzaghi and Sandine, 1975) that contained 5 µg/ml chloramphenicol and 1 µg/ml erythromycin. The *Lactococcus lactis trxB* mutant was grown in M17 agar (Oxoid) with 2.5 µg/ml erythromycin.

5.2.3 PCR conditions and Cloning

The PCR was performed using the same reagents and instruments as in 2.2.3 using primers shown on Table 5.1. The PCR program consisted of one initial cycle of 94°C

for 5 minutes and then 30 cycles of denaturation at 94°C for 30 seconds; annealing was at temperatures shown on Table 5.1 for 30 seconds; extension was at 68°C for 1 minute and 30 seconds and then one final cycle of extension at 68°C for 7 minutes. Genomic DNA extractions, PCR product purifications, gel electrophoresis, DNA gel extractions and the cloning into pTZ57/R were performed as in Chapter 2.

Table 5.1 Primers used to isolate genes from the genome of *B. adolescentis*

Name	Primer sequence	Annealing Temperature	Target	Reference
TxB1-f TxB1-r	GATTGTTCCGCACACCCTCT GACGCTATCCATGCAGTCTCC	52°C	*trxB*	This study
BaiC-f BaiC-r	GCATGGCAAAGTGAACAGTCC CGCCCGACTTCAGAATCTTC	52°C	*baiC*	This study
Bcp1-f Bcp1-r	GCCCACGGTCTCACTATTTGC CTGATTCAATGCCGGAACG	53°C	*bcp*	This study

5.2.4 Expression of *B. adolescentis* trxB in *E. coli* JM109

5.2.4.1 The agar plate well diffusion assay

The pB93TrxB and pBaTTrxB constructs were obtained from the cloning of *trxB* genes from isolate B93 and *B. adolescentis* ATCC 15703, respectively. pB93TrxB and pBaTTrxB were expressed in *E. coli* JM109 in media supplemented with 100 µg/ml ampicillin and 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). The *E. coli* plasmid without an insert (pTZ57) was used as a negative control. The recombinant strains were grown at 37°C in LB until OD_{600nm} of 0.6. Agar pour plates were prepared with 2.5 ml of the culture per 50 ml LB containing of 0.8% agar at a temperature of approximately 40°C. After the agar/culture plates hardened, two wells of 7 mm diameter were made in each plate using a sterile glass tube, and 30 µl of diamide (0.5 M and 0.25 M) or hydrogen peroxide (1M and 0.5 M) were added. Plates were incubated for 18 hours at 37°C. The zones of growth inhibition were

measured, and their diameters were used to calculate the area of inhibition (Serrano *et al*., 2007). The results represent the average of three biological replicates.

5.2.4.2 Hydrogen peroxide survival assay

The method of Takemoto *et al.* (1998) was used to evaluate the tolerance of the transformants to hydrogen peroxide. Broth cultures of *E.coli* JM109 (pB93TrxB), *E.coli* JM109 (pBaTTrxB) and *E.coli* JM109 (pPTZ57) were incubated for 16 hours. They were then inoculated into fresh LB medium (initial $OD_{600\ nm}$ of approximately 0.1) and grown at 37°C until the $OD_{600\ nm}$ reached approximately 0.6. The cells were collected, washed twice with phosphate-buffered saline (PBS) at pH 7.2, and re-suspended in PBS to an OD_{600nm} of 0.1. The cell suspensions were exposed for 60 minutes to 0.25 mM, 1.5 mM, 2 mM H_2O_2, diluted appropriately in PBS, and 100 µl plated on LB plates. Untreated cell suspension was used as the time zero control. The plates were incubated aerobically 18 hours at 37 °C, and colonies were counted to estimate the cell survival. A similar experiment was conducted as above except that the cell suspensions were exposed to 0.25 µM for 45 minutes. Serial dilutions and the plating on LB agar were carried out every 15 minutes. The incubation period and viable colony counts on the plates were done as before.

5.2.5 Expression of *B. adolescentis trxB* in *Lactococcus lactis*

5.2.5.1 Sensitivity of *L. lactis trxB* mutant to aerobic conditions

A *L. lactis* MG 1363 *trxB* mutant (Vido *et al*., 2005) was evaluated as an alternative to *E. coli* JM109. The mutant was made by the inactivation of the gene by single-crossover recombination (Vido *et al*., 2005). *L. lactis* would be a heterologous host for the *B. adolescentis trxB. L. lactis* MG 1363 *trxB* mutant and *L. lactis* NZ9700, an

isogenic strain of *L. lactis* MG 1363 with a functional *trxB,* were grown aerobically at 30°C in M17 broth (Oxoid). The growth was monitored over 24 hours with OD_{600nm} readings being taken every hour for 6 hours and then after 24 hours.

5.2.5.2 Cloning trxB into pGK12 shuttle vector

The pGK12 vector (Figure 5.1) (Kok *et al*., 1984) was extracted from *E. coli* BHB2600 (pGK12) and *E. coli* JM110 (pGK12) that had been incubated in LB broth, containing 2 µg/ml chloramphenicol and 50 µg/ml erythromycin, using the BioSpin plasmid extraction kit (according to the manufacturer's instructions).

The *trxB* gene was excised from pBaTTrxB and pB93TrxB by means of a double restriction enzyme digestion using *Bam*HI and *Ecl*611 (Fermentas) and cloned into pGK12, that had been double digested with *Bcl*I and *Hpa*I. In a second cloning strategy, pGK12 with *Cla*I, blunt-ended with T_4 polymerase (Fermentas) and further digested with *Bcl*I. A third cloning strategy involved digesting pGK12 with *Hpa*I and inserting a blunt-ended PCR product of the full *trxB* gene. *Hpa*I, *Bcl*I, *Nco*I and *Cla*I (Fermentas) were used to digest pGK12 (according to the manufacturer's instructions. T4 ligase (Fermentas) was used for the ligation (methods as stated by the manufacturer).

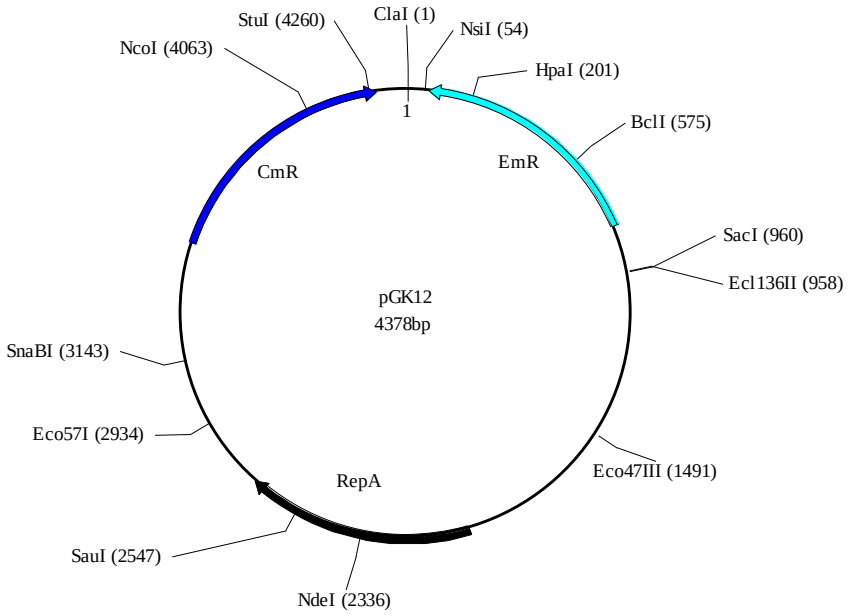


Figure 5.1 Shuttle vector, pGK12, used to express genes in *E. coli* and *L. lactis* (Kok *et al*., 1984)

5.3 Results

5.3.1 Bioinformatics analysis of oxidative stress response genes in bifidobacteria

A search for genes known to be involved in oxidative stress protection was conducted on the *B. adolescentis* ATCC 15703 (BaT) genome sequence on NCBI. The search was done by analysing annotated genes on the genome or by using deduced amino acid sequences from organisms shown to have an oxidative stress response to identify homologues in the genome. *AhpC* (encoding alkylhydroperoxide reductase)*, bcp (*encoding thioredoxin-dependent thiol peroxidise), *katB (*encoding catalase-peroxidase*), oxyR* (encoding the regulator of hydrogen peroxide-inducible genes)*, trxC* (encoding putative thioredoxin-like protein*), katG* (encoding catalase peroxidase)*, gorA* (encoding glutathione reductase), *msrA* (encoding for methionine sulfoxide reductase) and *nox* (encoding NADH oxidase) are some of the genes that were included in the search and were not found to have sequence homologues in

the genome of *B. adolescentis* ATCC 15703 (Zeller and Klug, 2006). A thioredoxin reductase gene (*trxB*), thioredoxin (*trxA2*) and a NADH-dependent flavin oxido-reductase (*baiC*) were, however, found in the type strain genome. The arrangement of the genes as annotated in the genome of *B. adolescentis* ATCC 15703 is shown in Fig. 5.2.

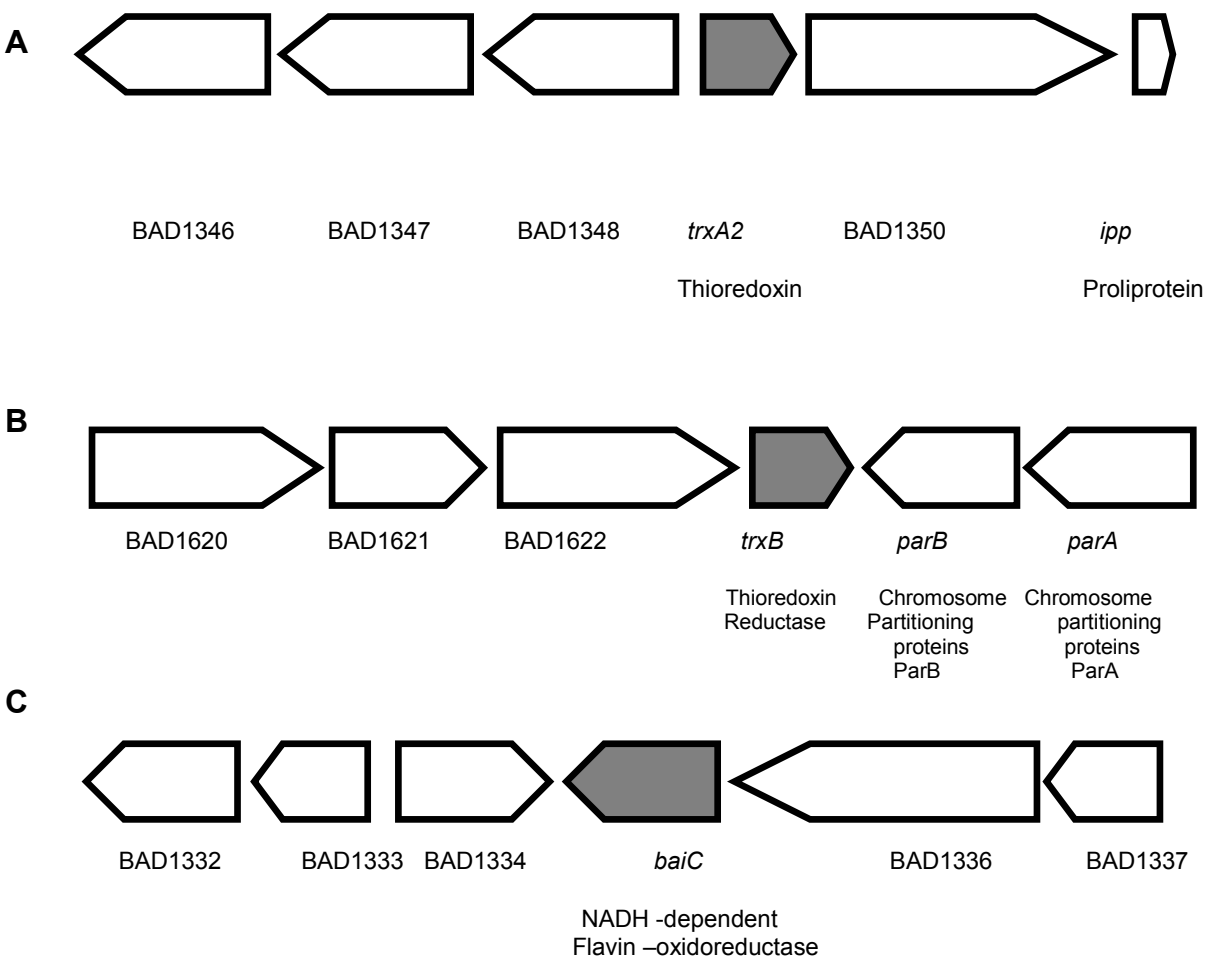


Figure 5.2 The arrangement of A) thioredoxin, B) thioredoxin reductase and C) NADH-dependent flavin oxido-reductase genes in the *B. adolescentis* ATCC 15703 genome. The genes of interest are shaded in grey.

The sequences of TrxB and BaiC proteins were aligned with sequences from other bifidobacteria available on NCBI using the BLAST algorithm (Altschul *et al*., 1990).

Both proteins appeared to have homologues in *B. adolescentis* L2-32*, B. dentium, B. animalis* subsp. *lactis* HN019 and *B. longum* NCC2705 as shown in Table 5.2.

The thioredoxin reductase of *B. adolescentis* ATCC 15703 is annotated as a nucleotide-disulphide oxidoreductase on the NCBI website and shown to be in the family that includes both class I and class II oxidoreductases and also NADH oxidases and peroxidases. All oxidoreductases have the conserved –CXXC- motif (Serrano *et al.,* 2007; Takemoto et al., 1998) and this motif was located in the protein sequence of the thioredoxin reductase in the *B. adolescentis* ATCC 15703.

Table 5.2 Deduced Amino acid percentage Identities of *B. adolescentis* ATCC 15703 TrxB and BaiC.

	Thioredoxin reductase (TrxB)	**Probable NADH-dependent flavin oxidoreductase BaiC**
***Bifidobacterium adolescentis* ATCC 15307**	100	100
***Bifidobacterium adolescentis* L2-32**	98	100
Bifidobacterium dentium	78	92
***Bifidobacterium pseudocatenulatum* DSM 20438**	77	84
***Bifidobacterium animalis subsp. lactis* HN019**	66	80
***Bifidobacterium longum* NCC2705**	62	82

A separate search for oxidative stress related proteins was conducted on the *B. longum* NCC2705 genome using information from Schell *et al.* (2002) and genes encoding for thioredoxin-dependent thiol peroxidase (*bcp)* (Fig. 5.3)*,* peptide methionine sulfoxide reductase *(mrsA),* NADH oxidase *(nox) and* alkyl hydroperoxide reductase *(ahpC)* were identified. The results of the alignments using the BLAST algorithm (Altschul *et al*., 1990) showed that these *B. longum* genes were not present in *B. adolescentis* ATCC 15703. The results also revealed a 70% deduced

amino acid identity of the *bcp* gene from *B. longum* NCC2705 to a sequence in *Bifidobacterium adolescentis* L2-32 genome.

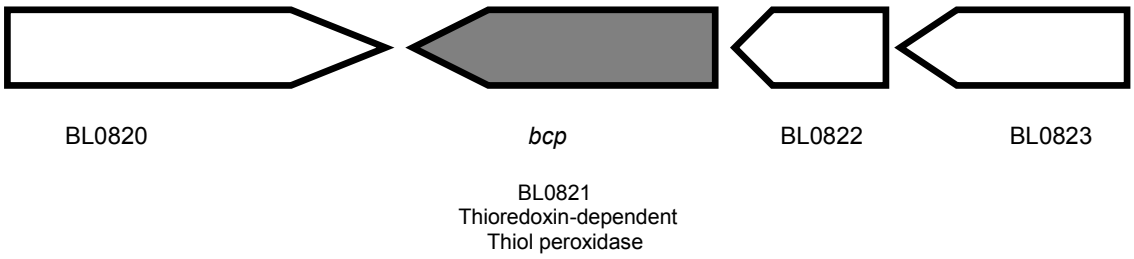


Figure 5.3. Arrangement of the thioredoxin-dependent thiol peroxidise gene (*bcp*) in the *B. longum* NCC2705 genome. The gene of interest is shaded in grey.

5.3.2 Gene isolation and identification

TxB1-f and TxB1-r primers (Table 5.1) were used to isolate *trxB* from *B. adolescentis* ATCC 15703 and the 5 *B. adolescentis* strains isolated in this study. The primers were designed using the published *B. adolescentis* ATCC 15703 genome (Accession number: NC008618) and were designed to bind outside of the start and stop codons of the gene. DNA fragments with an approximate size of 1 kb were isolated from all the *B. adolescentis* isolates and *B. adolescentis* ATCC 15703 (Fig. 5.4A)

The BaiC-f and BaiC-r primers (Table 5.1) produced a PCR band of approximately 1.15 kb in size, indicating the presence of the *baiC* in *B. adolescentis* ATCC 15703 and the 5 isolates (Fig. 5.4B). It was later noticed that an error in the reverse primer design had occurred. The primer annealed internal to the gene and did not include the stop codon. An attempt was made to re-design the primers so that the stop codon could be incorporated, but all the primer combinations showed non-specific binding to the genome under all of the conditions tested, and no *baiC* product was obtained.

The presence of a protein in *B. adolescentis* L2-32 that had 70% amino acid sequence identity to *bcp* from *B. longum* NCC2705 lead to the investigation of the presence of *bcp* in the 5 *B. adolescentis* isolates. Bcp-f and Bcp-r primers (Table 5.1) were designed using the *bcp* sequence from the published genome of *B. longum* NCC2705 (Accession number: NC004307). These primers were used to locate *bcp* in *B. longum* NCC2705 and the 5 *B. adolescentis* isolates. A PCR product of an approximate size of 600 bases was successfully isolated from *B. longum* NCC2705 by PCR (Fig. 5.4C).

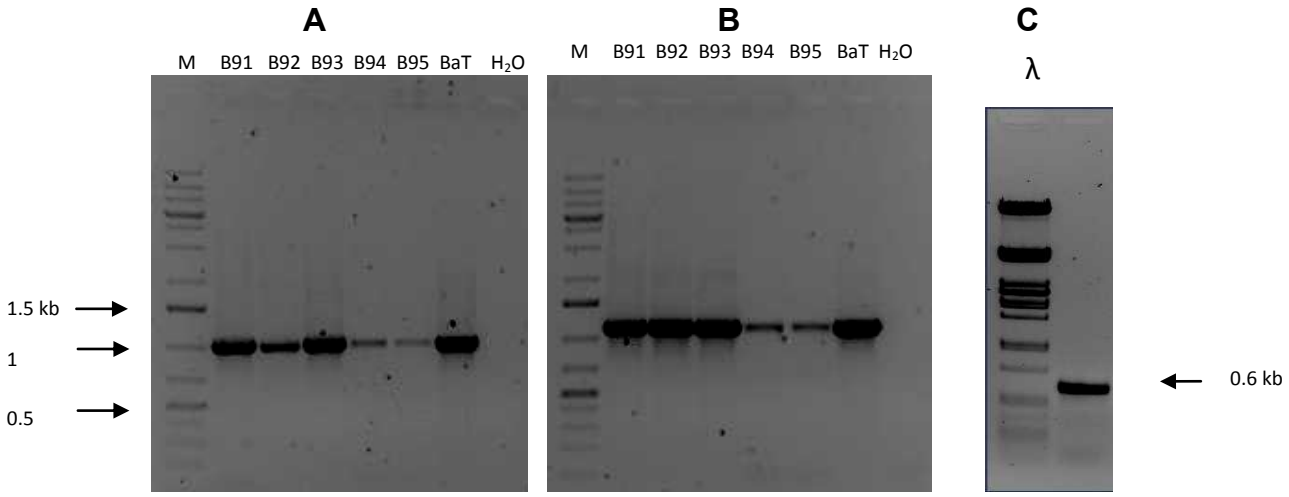


Figure 5.4 PCR products of oxidative stress response genes A) *B. adolescentis* trxB*;* B) *B. adolescentis baiC*; C) *B. longum* NCC2705 *bcp* gene. DNA markers M, O'GeneRuler^{TM} 1kb Plus DNA ladder; λ, Lambda DNA digested with *Pst*I.

The PCR *bcp* product from *B. longum* NCC2705 was purified and cloned into pTZ57/R using the same method as discussed in Chapter 2. The plasmid insert was sequenced using M13 primers (Chapter 2) and showed 99% identity to the *B. longum bcp* as lodged on NCBI. There was, however, extensive non-specific binding of the primers during PCR of genomic DNA of the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703 under all PCR conditions tested, and so no specific *bcp* gene could be isolated from these strains.

5.3.3 Expression of *B. adolescentis trxB* in *E. coli* JM109

The identification of the thioredoxin reductase (*trxB*) genes in *B. adolescentis* ATCC 15703 and all 5 *B. adolescentis* strains, by bioinformatics and by PCR, confirmed the presence of a thioredoxin/thioredoxin reductase system in these bacteria. Isolate B93 and *B. adolescentis* ATCC 15703 were selected for further study of the role of thioredoxin reductase in oxidative stress defence in these strains. Isolate B93 was selected out of the 5 *B. adolescentis* isolates because it was confirmed to be viable after 2 hours of aerobic exposure and also had a high RBGR (Chapter 4). The genes from both *B. adolescentis* ATCC 15703 and *B. adolescentis* B93 were successfully cloned into pTZ57/R and transformed into *E. coli* JM109. The presence of the inserts in the recombinant plasmids, their identity and orientation were confirmed by sequencing using M13 primers (Chapter 2). The *trxB* sequences for both isolate B93 and *B. adolescentis* ATCC 15703 were confirmed to be identical to the thioredoxin reductase gene of *B. adolescentis* ATCC 15703.

5.3.3.1 The agar plate well diffusion assay

Two colonies of *E. coli* JM109 transformants, one carrying pTZ57R with a *trxB* insert from *B. adolescentis* B93 (pB93trxB) and the other carrying pTZ57R with a *trxB* insert from *B. adolescentis* ATCC 15703 (pBaTtrxB) were exposed to H_2O_2 to evaluate whether the presence of thioredoxin reductase genes enhanced the survival of *E. coli. E. coli* JM109 (pTZ57) was used as a negative control.

The *E. coli* transformants carrying the *trxB* inserts did not show improved tolerance to 0.25 M and 0.5 M of diamide (Fig. 5.5A). In the event that the *B. adolescentis trxB* promoter was not being recognised in *E. coli*, IPTG was added to induce transcription from the *lacZ* promoter carried upstream of the gene on pTZ57R.

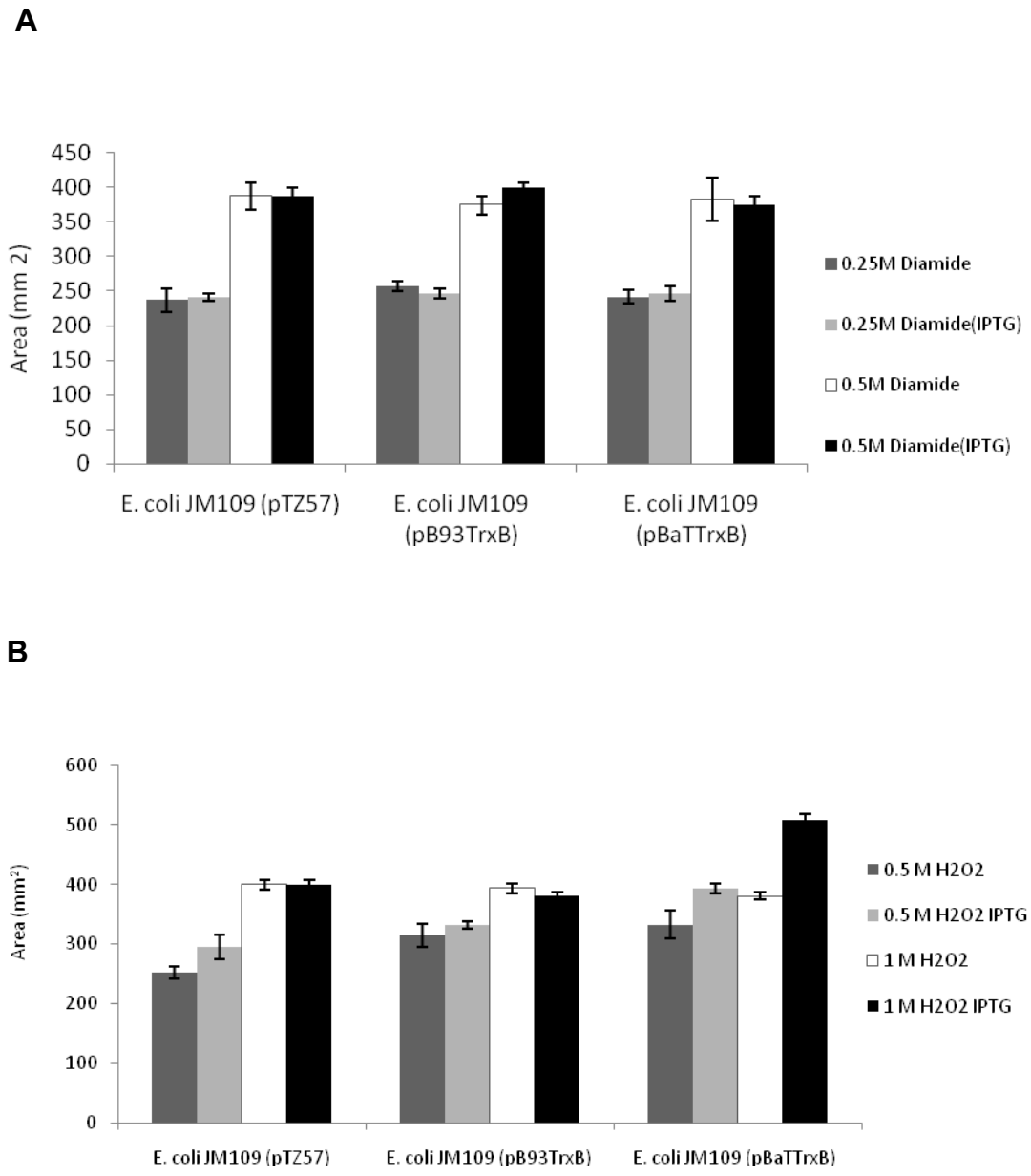


Figure 5.5. Zones of growth inhibition of the *E. coli* JM109 transformants after exposure to oxidative stress. A) Exposure to 0.25 M and 0.5 M diamide; B) Exposure to 0.5 M and 1 M H_2O_2 in the presence or absence of IPTG.

The zones of inhibition however, were the same size as when IPTG was not added. A similar pattern was observed when all the *E. coli* strains were exposed to 0.5 M and 1 M H_2O_2. *E. coli* JM109 (pBaTtrxB) however, did show increased sensitivity to H_2O_2 when IPTG was added.

5.3.3.2 Hydrogen peroxide survival assay

A different approach to assess the possible role of *trxB* was taken by performing survival assays. The transformants were exposed to different concentrations of H_2O_2 in PBS buffer and the number of surviving colony forming units per millilitre (cfu/ml) was calculated. After an hour of incubation in a 0 to 2 mM range of H_2O_2 concentrations, *E. coli* JM109 (pB93trxB) survived the exposure slightly better than the other 3 transformants up to the 1.5 mM concentration with *E. coli* JM109 (pBaTtrxB) being the most sensitive (Fig. 5.6).

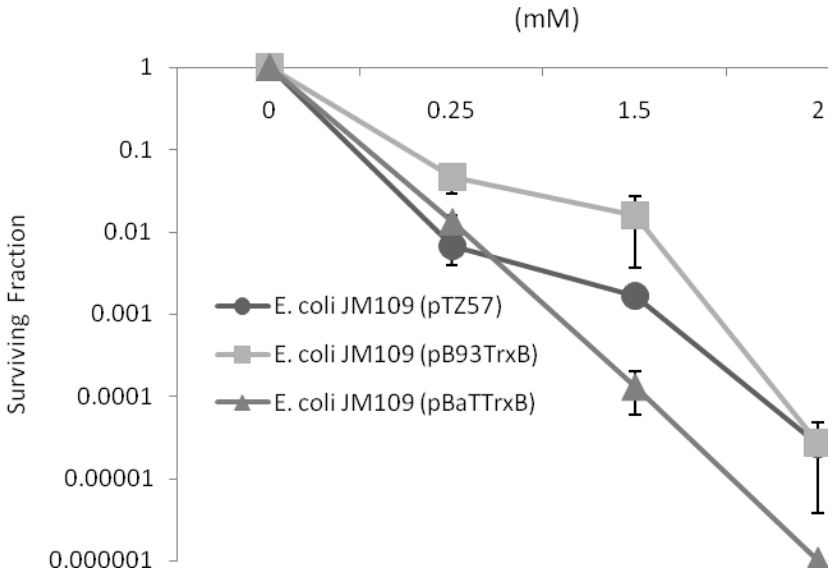


Figure 5.6 Survival curves of *E. coli* JM109 transformants after H_2O_2 treatment. *E. coli* JM109 (pTZ57), *E. coli* JM109 (pB93TrxB) and *E. coli* JM109 (pBaTTrxB) were exposed to 0.25, 1.5 and 2 mM H_2O_2 for 60 minutes.

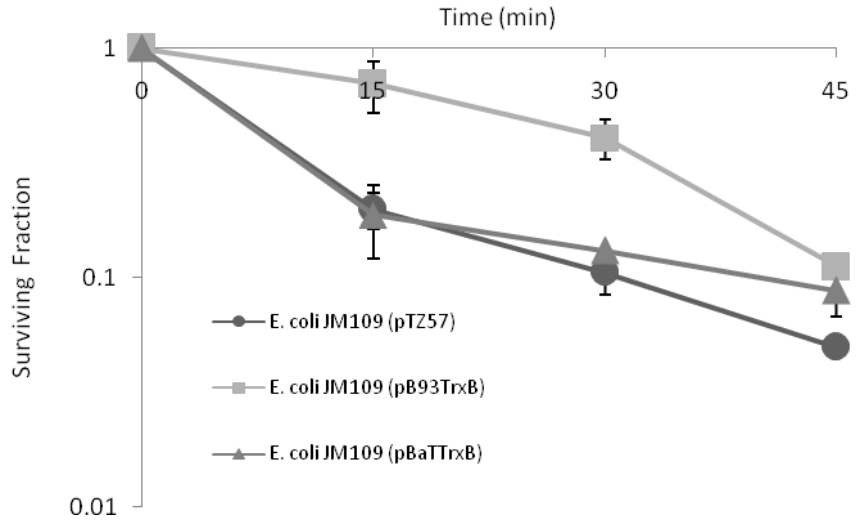


Figure 5.7. Survival curves of *E. coli* JM10 (pTZ57), *E. coli* JM10 (pB93TrxB) and *E. coli* JM10 (pBaTTrxB) transformants after 45 minutes exposure to 0.25 mM H_2O_2.

The survival of the isolates after exposure to 0.25 mM H_2O_2 over a period of 45 minutes was also evaluated in PBS buffer (Fig. 5.7). During the initial 30 minutes, *E. coli* JM109 (pB93TrxB) showed the best survival while there was no survival difference between *E. coli* JM109 (pTZ57) and *E. coli* JM109 (pBaTTrxB). At the end of the 45 minutes of exposure, all the transformants had the same number of cells that had survived the exposure.

5.3.4 Expression of *B. adolescentis trxB* in *Lactococcus lactis*

5.3.4.1 Sensitivity of *L. lactis trxB* mutant to aerobic conditions

The *L. lactis trxB* mutant was more sensitive to aerobic conditions than the *L. Lactis* NZ9700 with functional *trxB* as shown in Fig. 5.8. This therefore makes the *L. lactis trxB* mutant as suitable potential recipient for the *B. adolescentis trxB* gene.

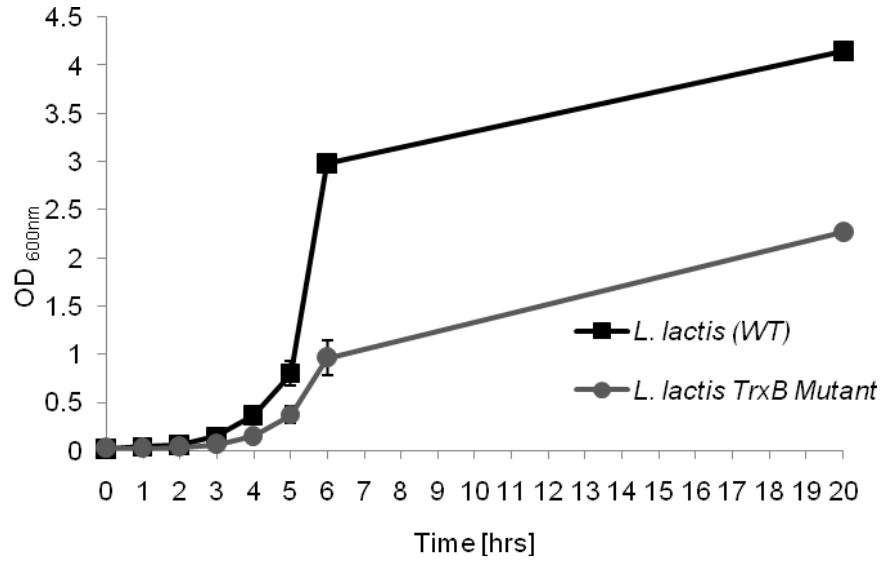


Figure 5.8 Aerobic growth of *L.lactis* (wild type) and *L. lactis trxB* mutant.

5.3.4.2 Restriction enzyme digestions

The cloned *trxB* gene was successfully excised from both pB93TrxB and pBaTtrxB using *Ecl*611 and *Bam*HI in order to subclone it into the *L. lactis* vector, (Fig. 5.9). The *Bam*HI site is compatible to *Bcl*I in the while *Ecl*611 created a blunt end.

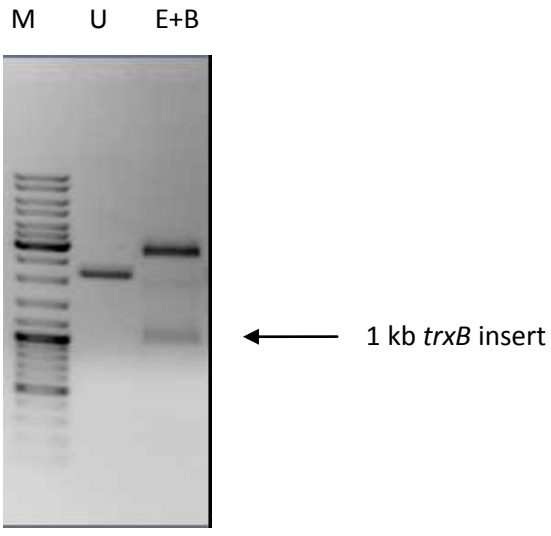


Figure 5.9 Isolation of the *trxB* gene by the restriction digests of the pB93TrxB. O'GeneRuler™ 1kb Plus DNA ladder was used as DNA marker (M), undigested pB93TrxB (U) and double–digest with *Ecl*136II and *Bam*HI of pB93TrxB.

The restriction enzyme digestion of pGK12 vector by *Bcl*I, however, did not occur using plasmid extracted from *E. coli* BHB2600. *Nco*I and *Hpa*I digested pGK12 to completion and were used as a control. It was decided that the partial digests by *Bcl*I, were possibly because the *Bcl*I restriction sites were blocked by DNA methylation. The pGK12 vector was, therefore, transformed into *E. coli* JM110, a *dam* and *dcm* methylase negative strain (Yanisch-Perron *et al.,* 1985), and extracted. Under these conditions there was partial digestion of pGK12 by *Bcl*I and *Cla*I (Fig. 5.10).

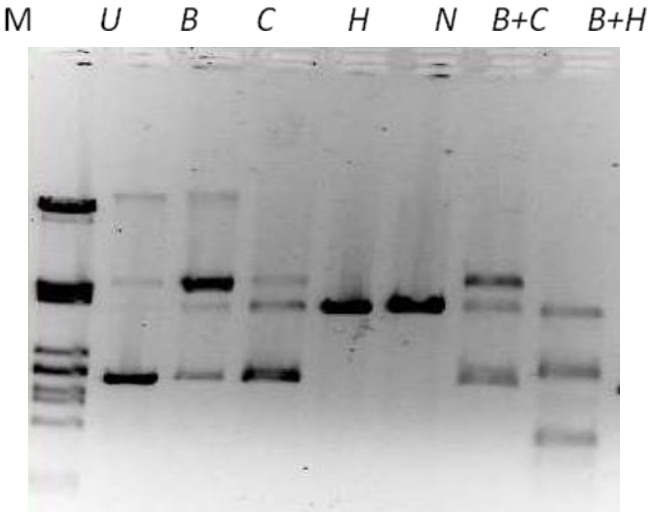


Figure 5.10 The restriction digests of the vector PGK12. The Lambda DNA digested with *Pst*I (M) was used as the molecular weight marker, and in the other lanes there was undigested pGK12 (U), pGK12 digested with *Bcl*I (B), *Cla*I (C), *Hpa* I (H), *Nco*I (N), *Bcl*I + *Cla*I (B+C) and *Bcl*I + *Hpa*I (B+H).

Even though the digests were only partial, attempts were made to first digest pGK12 with *Cla*I and purify the band corresponding to the linear size of the plasmid out of the gel. The gel-purified product was blunt-ended with T4 polymerase then digested with *Bcl*I. Another approach was a double digest of pGK12 with *Hpa*I and *Bcl*I directly. In both cases, most of the vector was lost during the multiple procedures of

digestion, blunt end formation and DNA purification steps, since the starting concentration of the vector was always limited due to low copy number (Kok *et al.*, 1984).

5.3.4.3 PCR approach

*Hpa*I, which creates blunt ends on the DNA, was the only enzyme which could digest pGK12 efficiently and within the erythromycin gene (Fig 5.10) The PCR product of the *trxB* gene was purified and blunt ends created with the T4 polymerase. The *trxB* insert was then ligated into the *Hpa*I site of pGK12 and the ligation mixed transformed into *E. coli* JM110.

As cloning into the *Hpa*I site in the vector disrupts the erythromycin gene, the transformants that showed the ability to grow on LB agar in the presence of chloramphenicol and not erythromycin, were selected. *E. coli* JM110 without the plasmid was sensitive to both chloramphenicol and erythromycin. Selected chloramphenicol resistant and erythromycin sensitive transformants were cultured and recombinant plasmid extracted. None of the plasmids extracted, however, showed the presence of inserts. In addition, sub-culturing of putative recombinant strains resulted in the reversion of strains to erythromycin resistance, suggesting plasmid instability. Time constraints did not allow further attempts at this cloning in the course of this research.

5.5 Discussion

It was reported in Chapter 4 that the isolates B91 and B93 were viable after 2 hours aerobic exposure and that *B. adolescentis* ATCC 15703 showed growth during aerobic exposure. These results suggested that there might be an oxidative stress

response system in *B. adolescentis.* Most of the genes and proteins shown in literature, to have a role in oxidative stress response, were, however, not found on the genome of *B. adolescentis* ATCC 15703. This may explain the general low oxygen tolerance of a number of *B. adolescentis* strains in this study and other previous studies (Jayamane and Adams, 2006; Simpson *et* al., 2005). The bioinformatic analysis and PCR, did, however, reveal the presence of *trxB* as well as *baiC*, both of which may play a role in the defence of *B. adolescentis* against oxidative stress. In this study, preliminary work was performed to investigate the role of thioredoxin reductase (encoded by *trxB*) in such a response system.

TrxB was successfully isolated from the genomes of *B. adolescentis* ATCC 15703 and isolate *B. adolescentis* B93, and cloned into *E. coli.* The role of *trxB* in the defence against oxidative stress could not be derived from the zones of growth inhibition data from the transformants carrying *trxB* and *E. coli* JM109 (pTZ57). There were no differences in the sizes of the zones of inhibition caused by the oxidative stress (Fig.5.5). Data from the survival curves of *E. coli* JM109 (pB93TrxB) however, suggested that *trxB* could be involved in eliminating the effects of H_2O_2 (Fig. 5.6 and 5.7) as the transformant showed better survival than *E. coli* JM109 (pTZ57). In contrast, *E. coli* JM109 (pBaTTrxB) was more sensitive to oxidative stress than *E. coli* JM109 (pTZ57) and *E. coli* JM109 (pB93TrxB). The role of *trxB* could, therefore, not be defined due to these contradictory results.

E. coli has a functional *trxB* and other genes responsible for defence against oxidative stress (Takemoto *et al*., 1998). Against this background, the thioredoxin reductase from the *B. adolescentis* strains might exert only a small effect that would not be revealed by the experiments used in this study. It is also possible that the inconclusive results are due to the lack of transcription or translation of the *B.*

adolescentis trxB in *E. coli* due differences in codon usage in the heterologous host, since the gene is from the Gram positive *B. adolescentis* while the *E. coli* is Gram negative. To eliminate this possibility, a *L. lactis trxB* mutant was thought to provide a more appropriate heterologous expression environment. The *L. lactis* mutant was also ideal to use as it had been shown to be sensitive aerobic conditions. Although *L. lactis* is still a heterologous host, both *L. lactis* and *B. adolescentis* are both Gram positives which may minimize differences in codon usage. The complementation of the non-functional thioredoxin reductase from the *L. lactis* mutant by that of *B. adolescentis* would mean that the gene was functional and was being expressed. The attempts to clone the *B. adolescentis* gene into pGK12 were, however, not successful mainly due to problems encountered with partial enzyme digestions of the limited restriction sites available on the pGK12 vector. There was also evidence of plasmid instability since the erythromycin sensitivity phenotype was only transiently expressed before the transformants once again became erythromycin resistant.

Future work on the *trxB* of *B. adolescentis* includes completing the ligation of the *trxB* PCR product to create stable transformants of *E. coli* JM110. To address the restriction digestion problem, different primers with restriction sites should enable the insertion of *trxB* into the sites of enzymes that efficiently digest pGK12. Transformants could also be grown at reduced temperatures to minimise loss of the inserts. The use of a thioredoxin reductase assay to quantify the activity of thioredoxin reductase would be useful as this would give an indication of gene expression as well as the thioredoxin reductase functionality. It would also be worthwhile in the future, to explore the link between the functions of thioredoxin and thioredoxin reductase as well as elucidating the role and regulation of this system. This can be done using the same approach as Takemoto *et al.* (1998) where by

either the thioredoxin or thioredoxin reductase gene was mutated at a time to study the effects of non-fuctional gene.

There are very few gene expression systems available in the public domain for use in *Bifidobacterium* species, and this limits genetic studies in these bacteria. For example, if such systems were available, it would be useful to mutate the *trxB* gene in *B. adolescentis* and study the effects of the mutation. The mutated gene could then be complemented with a functional *trxB* gene using a *Bifidobacterium* expression system. There have been reports in the literature of plasmid isolation from various *Bifidobacterium* species and the attempts to use these as vectors, but plasmid instability has hindered success (Shkoporov *et al.,* 2008). There are also alternative shuttle vectors which could be used as described by Bryan *et al.* (2000). These vectors can replicate in *E. coli* and in gram-positive bacteria. They contain a nisin-inducible promoter (P*nis*A) and the genes encoding NisR and NisK as well as the two component signalling mechanism required for activating transcription from P*nis*A when nisin is present (Bryan *et al.,* 2000)

Schell *et al.* (2002) linked thiol peroxidise (*bcp*) from *B. longum* with the oxidative stress response. In *E. coli*, *bcp* codes for bacterioferritin comigratory protein and is characterised as a thioredoxin dependent thiol peroxidase. An *E. coli* thiol peroxidase null mutant was found to be hypersensitive to H_2O_2 and grew slower than the wild type in aerobic conditions. All these effects were complemented by *bcp* expression (Jeong *et al*., 2000). This gene was, however, not found in the 5 *B. adolescentis* isolates and *B. adolescentis* ATCC 15703 using either bioinformatic tools or PCR. It was, however, identified in *Bifidobacterium adolescentis* L2-32 using bioinformatic analysis. The recently published genome sequence of *Bifidobacterium*

adolescentis L2-32 (Accession number: NZAAXD0000000) will be useful in designing PCR primers for the isolation of the *bcp* gene in the 5 *B. adolescentis* strains. Future work involving the expression of this *B. adolescentis* L2-32 *bcp* gene could lead to an understanding of the involvement of the gene in oxidative stress response.

Understanding the oxidative stress response of probiotics is important as proteins involved may provide the host with the additional health benefits such as the anti-oxidative effects resulting from scavenging of ROS (Mikelsaar and Zilmer, 2009). The other reason to understand stress response in probiotics is that exposure or sensitivity to certain stresses can cause the strains to develop sensitivity to stresses that the organism would normally not be sensitive to. Yoghurt exposes probiotic strains to acid, temperature, whey proteins, lactic acid, and other bacterial cultures (Talwalkar and Kailasapathy, 2004). Bile, acid and oxidative stresses have been shown to influence the antibiotic profiles of microorganisms. The inhibitory action of H_2O_2 is from the OH free radicals in the presence of iron and copper. These attack the polyunsaturated fatty acids directly in the cell membranes and this initiates lipid peroxidation which disrupts the membrane bound proteins and fluidity. This increases susceptibility to cell wall directed β-lactam antibiotics,chloramphenicol, erythromycin and tetracycline antibiotics (Kheadr *et al*., 2007). Understanding the stress defence mechanisms will enable the efficient preparation, delivery and storage of probiotics.

Chapter 6: General conclusions

Molecular methods used for bacterial identification rely on the ubiquitous and conserved nature of the DNA sequences. Sequence differences found in these sequences are then used for species or strain differentiation (Ventura *et al.* 2003). The use of 16S rRNA, 16S-23S rRNA, *hsp60* and *tuf* gene sequences as molecular tools, enabled the identification of the 5 isolates as *B. adolescentis* stains. All of the above mentioned genes showed the highest percentage identity to *Bifidobacterium adolescentis* ATCC 15703^T when the BLAST algorithm (Altschul *et al.,* 1990) was used. Data from the RAPD-PCR and the single base differences in the sequence alignments of some of the above mentioned genes, suggested that although all the isolates were all *B. adolescentis*, they are possibly different strains. Isolates B91, B93, B94 and B95 are closely related while isolate B92 was found to be very different from the other 4 isolates and from *B. adolescentis* ATCC 15703. It was recommended, however, that additional investigations be conducted using the combined data of sequences of the mentioned genes and additional genes, to verify that the isolates are different strains, to improve the low bootstrap values in the phylogenetic trees and to eliminate possible sequencing errors that may have influenced the identification of these isolates.

Bifidobacteria are able to utilize a wide range of substrates including mono-, oligo- and polysaccharides as carbon and energy sources. Previous pure culture growth studies have shown that, in addition to a wide spectrum of suitable energy sources, bifidobacteria show preferences to different molecules and exhibit varying growth rates when provided with these molecules (Hopkins et al. 1998; Vernazza *et al*., 2006). This was confirmed in this study as the carbohydrate utilisation preferences

of the 5 *B. adolescentis* isolates were found to be different for each isolate and different from *B. adolescentis* ATCC 15703^T. *B. adolescentis* ATCC 15703^T grew well in all of the carbohydrates tested. The carbohydrate substrate preferences was isolates was isolate dependent. Isolate B94 was the only isolate not able to grow in media supplemented with glucose and this will in the future, be investigated further as this has been reported to be rare in *Bifidobacterium* strains (Rad and Petr, 2000; Amaretti *et al*., 2007; Van der Meulen et al., 2004). It was clear then that if these novel isolates were to ever be used as probiotics, different prebiotics would have to be administered based on the carbohydrate preference profiles of each isolate.

Table 6.1 Summary of the probiotic potential evaluation

	Acid survival	**Bile survival**	**Yeast agglutination**	**Auto-aggregation**	**Antimicrobial activity (out of 7 pathogens)**	**Oxygen tolerance**
B91	NG	NG	+++	++++	6 out of 7	+
B92	NG	Viable	+	+++	6 out of 7	+
B93	NG	NG	++	+++	6 out of 7	++
B94	NG	Viable	+	++	5 out of 7	+
B95	Viable	Viable	+	+++++	5 out of 7	++
BaT	Viable	Viable	++	+	4 out of 7	++++

(NG= no growth)

Probiotic bacteria need to reach the lower GIT where they will colonize the epithelial and start utilizing these carbohydrates. Before this happens however, the bacterial strains have to tolerate the stresses they encounter during their transition to the lower GIT. The acidic environment of the stomach, and bile secretions into the small intestine have to be overcome. Previous studies have shown a high degree of

variability between strains in their tolerance to these conditions (Charteris *et al.*, 1998). In this study, only isolate B95 and *B. adolescentis* ATCC 15703^T were viable after 5 hours of exposure to pH 2 (Table 6.1) but were unable to grow under these conditions. The rest of the isolates appeared to generally have low tolerance to low pH as they could not grow or survive the low pH exposure. The isolates had higher bile tolerance as isolates B92, B94, B95 and *B. adolescentis* ATCC 15703^T were viable after 5 hours exposure to bile salts. To ensure that a significant number of probiotics reached the lower GIT, in the instance that these isolates are used as probiotics, protective measures such as gel bead encapsulation and freeze-drying would have to be taken (Simpson *et* al., 2005; Ross *et al.*, 2005).

The combined features of probiotic strains are useful in the evaluation of probiotic potential (Collins *et al.*, 1998). One of the positive features found in all of the isolates is their antimicrobial activity against pathogens such as *C. perfringens, E. faecalis, S. aureus* and *E. coli*. Some of the isolates were able to inhibit the growth of *E. faecium, S. typhimurium* and *C. botulinum* but this ability appeared to vary from isolate to isolate. These antimicrobial activities of the *B. adolescentis* isolates would be an additional health benefit that indicates some probiotic potential of the isolates.

All the isolates were resistant to kanamycin and streptomycin. Isolate B95 was resistant to vancomycin while the rest of the isolates showed very low sensitivity to it. This antibiotic resistance would have to be investigated further to assess the risks of transferable resistance. The general adhesion properties for all the isolates was generally low but isolate B91 appeared to be the most likely to adhere to the intestinal lining. This was evident from the results of the yeast adhesion and auto-aggregations assays. More experiments, such as mucous- binding or human cell lining adhesion assays, would have to be conducted to investigate the colonisation

properties further. It was therefore concluded that although some of the isolates appeared to be identical using the molecular tools, the 5 new *B. adolescentis* isolates and *B. adolescentis* ATCC 15703^{T} have different physiological properties. Future work will include the use of DNA sequence signatures that enable strain differentiation of these isolates in order to explain the physiological differences determined in this study.

More physiological differences were observed when the *B. adolescentis* isolates were exposed to aerobic conditions. The isolates were able to tolerate short periods of exposure to aerobic conditions which implied the possibility of the existence of an oxidative stress defence system. *B. adolescentis* ATCC 15703 was able to grow slightly in aerobic conditions and was still viable after 2 hours of aerobic exposure. The presence of thioredoxin gene (*trxB)* and NADH-dependent flavin oxidoreductase (*baiC*) was confirmed in all the isolates and in *B. adolescentis* ATCC 15703 by PCR. The *trxB* gene was successfully cloned into *E. coli* JM109 but the role of *trxB* with regard to oxidative stress response, could not established. It was decided that a *L. lactis trxB*$^{-}$ mutant would be an ideal alternative to *E. coli* JM109 for the expression of thioredoxin reductase of *B. adolescentis*. This would be done by complementing the mutated gene of *L. lactis* with the functional gene from *B. adolescentis* using the pGK12 vector. In this study, however, cloning attempts into the pGK12 shuttle vector were not successful. Different cloning and expression methods will also have to be explored and order to determine the role of *trxB* and other potential oxidative stress response genes.

In summary, all the isolates have good probiotic potential as they have the ability to inhibit the growth of some of the pathogens tested. When considering all of the tested properties, isolate B95 seems have the highest probiotic potential as it was

able to remain viable after 5 hours of acid and bile conditions tested, showed moderate oxygen tolerance and also has a high ability to autoaggregate (Table 6.1). More *in vitro* and *in vivo* experiments will have to be conducted to fully evaluate the probiotic potential of these new isolates.

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