London Metropolitan University

Probiotic Characterization of *Bifidobacterium* spp. Isolated From Commercial Fermented Milk in the UK

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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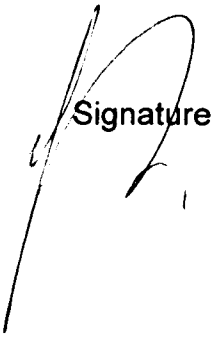
DECLARATION

I confirm that this is my work and the use of all material from other sources has been clearly acknowledged.



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ABSTRACT

The aim of this research was to study the identity and survival of probiotic bifidobacteria in fermented dairy products from the UK market throughout their shelf-life and carry out *in vitro* studies on some of the probiotic criteria and potential functional properties of the isolates including resistance to stomach-intestine conditions, antimicrobial activities, antibiotic resistance/susceptibility, antibiotic resistance genes and their transferability, biofilm formation and production of health-promoting secondary metabolites, such as conjugated linoleic acid (CLA).

The initial study for choosing the right selective medium for isolation and enumeration of bifidobacteria included a comparative study on selectivity of well known media transgalactosylated oligosaccharides-mupirocin lithium salt (TOS-MUP) and *Bifidobacterium* iodoacetate medium 25 (BIM-25). Considering selectivity of the medium, recovery of the bifidobacteria and ease of preparation, it was concluded that TOS-MUP is the medium of choice for determination of total viable counts of bifidobacteria in fermented milks.

The result of enumeration study indicated that most of the tested products (22 out of total 24) on TOS-MUP comply with the recommended minimum therapeutic level for probiotics (10^6 CFU/g) at the time of purchase and at the end of their shelf-life.

All presumptive *Bifidobacterium* isolates were identified as *Bifidobacterium animalis* subsp. *lactis* by analysis of partial sequences of the 16S ribisomal RNA gene. Further use of rep-PCR for differentiation of all isolates using two different primers (GTG-5 and BOXA1R) did not show any difference among tested isolates as all isolates presented the same band profile. As far as the claims on the label are concerned, most of the products (19 out of 24) failed to name the *Bifidobacterium* species at all or correctly.

In order to characterise the isolates for probiotic properties and/or probiotic measures, 10 isolates identified as *Bifidobacterium animalis* subsp. *lactis* were selected along with two commercial *Bifidobacterium animalis* subsp. *lactis* cultures from Chr. Hansen and Danisco and three different type strains (*Bifidobacterium bifidum* NCTC13001, *Bifidobacterium breve* NCTC11815, *Bifidobacterium longum* NCTC11818.).

The study on resistance of the isolates to acidic condition of the stomach and their survival in presence of bile salt (0.5, 1, 1.5 and 2% w/v) revealed that all tested isolates apart from type strains presented high resistance to low pH (2, 3, and 4) after 3 hours, and also they showed high resistance to bile concentration corresponding to the concentrations in the small intestine.

In order to mimic the stomach and small intestine conditions, the combined effects of pH, enzymes and bile salts on survival of one authentic isolate of *Bifidobacterium animalis* subsp. *lactis* (B6) using a batch culture fermentation system was tested. The results demonstrated a great resistance of *Bifidobacterium animalis* subsp. *lactis* under such circumstances. The results of this study confirmed that *Bifidobacterium animalis* subsp. *lactis* has the characteristic feature and ability to survive the low pH values and high concentrations of bile salts found in the upper digestive tract.

The above mentioned 15 isolates of *Bifidobacterium* species were also studied for their antibiotic resistance profile by determination of minimum inhibitory concentration (MIC). Their genetic background of antibiotic resistance genes, only in isolates that showed resistance above the break points, was further studied by PCR reactions. The acquired resistance to tetracycline, kanamycine. erythromycin and streptomycin was observed in all tested isolates. The potential of the isolates to transfer antibiotic resistance to other organisms, in particular the intestinal

pathogens, was performed through filter conjugation. The results illustrated the transferability of *tet*(W) to other bacteria, as one of the genes associated with tetracycline resistance.

The biofilm formation capacity of tested isolates in different conditions of nutritional stress was also studied. All tested isolates were able to produce biofilm in two rich media Reinforced Clostridia Medium (RCM) and MRS broth supplemented with cysteine (MRSC). The biofilm formation by all isolates was reduced as nutrients depleted as a result of diluting the medium (1:2 and 1:20).

All isolates exhibited antimicrobial activities against pathogenic bacteria including *Escherichia coli*, *Salmonella enteritidis* and *Salmonella typhimurium.* The key mechanism for antimicrobial activity was proved to be the production of organic acids which create inhibition by lowering the pH of the surroundings.

Screening the isolated for evaluating the competence of bifidobacteria to produce CLA attested to be species dependent and which entirely depends on the growth media.

In conclusion the results of this study revealed a considerable homogeneity in probiotic properties among the different isolates of *Bifidobacterium animalis* subsp. *lactis.* Although *Bifidobacterium animalis* subsp. *lactis* has many technological advantages, this superiority is not necessarily extended to the functional properties.

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Introduction

1.1 INTRODUCTION

An increasing public knowledge of healthy food has resulted in a growing demand for functional foods. Functional foods are known to contain biologically active compounds or microorganisms, which provide health benefits beyond their basic nutrition (Guarner and Schaafsma 1998).

Gut health is considered as general target for many functional foods. In developing nutritionally designed foods, the two main food ingredients that are offered for enhancing the health are probiotics and prebiotics (Puupponen- Piamia *et al.* 2003).

Probiotic fermented milks are one of the most important functional products. The meaning of probiotic is "for life" and the word is of Greek origin. Probiotic is the opposite of antibiotic, which means "against life" (Grady and Gibson 2005). Metchnikoff, who focused on lactic acid bacteria (LAB) studies, ascribed the longevity of Bulgarian people to the consumption of fermented milks. Henry Tissier found a relation between diarrhoea and low number of bifidobacteria in stool and suggested the consumption of high dose of bifidobacteria for recovery of gut microflora (Hickey 2005). Therefore, some probiotic strains, which could live to their technological expectations and provide great functional properties, are required.

Over the past few decades, an increased interest has existed in the isolation of novel *Lactobacillus* and *Bifidobacterium* strains, which can be applied for beneficial health effects (Yadav *et al.* 2007).

The definition by FAO/WHO (2002) describes probiotics as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host". Accordingly, probiotic products should comprise live microorganisms in high numbers to provide health benefits.

A large variety of microorganisms live in the human intestinal tract and probiotic bacteria are considered as one of the main inhabitants. Probiotic bacteria commonly consist of lactobacilli, enterobacteria and bifidobacteria. However, some other microorganisms, such as yeasts (*Saccharomyces boulardii*) have been recommended as a probiotic in spite of not being a normal constituent of human microflora (Crittenden *et al.* 2003). Also, *Bacillus clausii* a rod-shaped, Gram positive, motile and spore forming bacterium, which lives in soil has been suggested as probiotic. The bacterium is currently being studied in treatment of respiratory infections and some gastrointestinal disorders (Marseglia *et al.* 2007). It is now widely accepted that the health benefits of probiotic bacteria are strain dependent meaning that when a probiotic strain shows a particular health benefit, it is not necessarily applicable to other strains or species of this bacterium (Gurakan *et al.* 2010).

1.2 Microorganisms and the gut

The gastrointestinal tract (GIT) begins with the oral cavity and leads to the stomach, the small intestine (duodenum, jejunum and ileum) and the large intestine (cecum, colon and rectum). Each section of GIT has been inhabited by distinct bacterial community and bifidobacteria can be found in most part of the GIT except the stomach (Nielsen *et al.* 2003) (Figure 1.1).

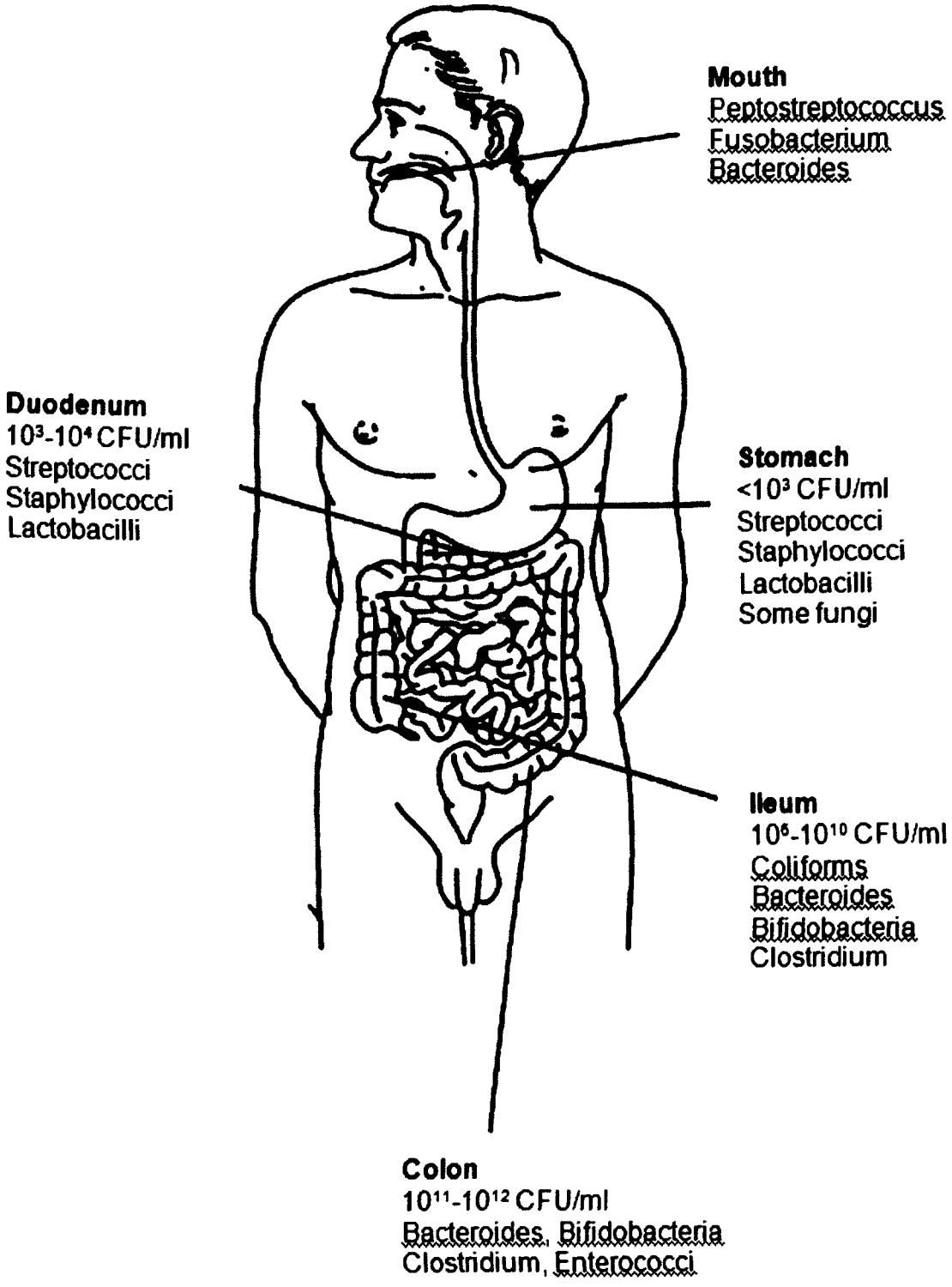

Figure 1.1 Diversity and number of microorganisms in the gastrointestinal tract

More than 10^{14} colony forming units (CFU) of bacteria consisting of over 400 different microorganisms live as normal inhabitants of the human GIT. The bacterial

number in the GIT varies from less than 10^3 CFU/g in the stomach due to the harsh acidic conditions, to greater than 10^{11} CFU/g in the colon (Figure1.1). Anaerobic bacteria, such as *Bifidobacterium* spp., *Bacteroides* spp. and *Eubacterium* spp. are major groups within the identified GIT microflora, which constitute 99% of bacterial population in the colon and also dominate at levels around 10^{11} CFU/g in the faeces (Angus *et al.* 2005; Holzapfel *et al.* 1998). The colon is considered a relatively stable ecosystem while small intestine is susceptible to modification (Bezkorovainy 2001).

For better understanding of microflora behaviour in gut ecosystem, normobiosis and dysbiosis are defined. Normobiosis is considered to exist when microorganisms with health benefits have the majority over harmful ones. Dysbiosis is defined as total reverse of normobiosis and usually generates disease (Roberfroid *et al.* 2010).

Normally, there is an associative growth relationship between host and bacteria in a healthy individual. More or less, the composition of gut microflora remains stable in spite of some variation which might alter the rate and ratio of the microorganisms in the GIT (Table 1.1), but in some conditions like antibiotic therapy, colon cancer and inflammatory bowel disease, the microbial composition in the gut can be affected and altered.

Many functions are ascribed to the human gut microbiota where some of the most important ones are nutrient synthesis, digestion and absorption, immune stimulation and the control of pathogens (Perdigon *et al.* 2002; Kelly *et al.* 2005). Also, their performance in certain pathological disorders including colon cancer, inflammatory bowel diseases have been studied and claimed to be significant (Salonen *et al.* 2010). Some evidence has started to emerge regarding their functions of gut microorganisms in obesity (DiBaise *et al.* 2008) as it has been reported that gut microbiota are able to enhance the extraction of calories from ingested food.

Turnbaugh *et al.* (2006) transfered the gut microbiota from obese mice to germ free mice, where an increase in fat mass in the recipients was observed.

Table 1.1 Factors affecting the microflora of the gastro-intestinal tract

Host mediated factors	• Stomach pH, bile salts, enzymes, Immunoglobulins • Motility, e.g. speed, peristalsis • Exfoliated cells, mucins, • tissue exudate
Microbial factors	• Adhesion • Motility • Nutritional flexibility
Microbial interactions	• Synergy • Growth factors and vitamin excretion • Short-chain fatty acids, amines • Antimicrobial components
Diet	• Non-digestible fibres, drugs, etc

Adapted from Holzapfel *et al.* (1998)

1.3 Bifidobacteria

Bifidobacteria have been considered as probiotics since they were first isolated from faeces of newborns by Henry Tissier in 1899. They were originally named *Bacillus bifidus communis* due to their branching morphology- *bifidus* in Latin means cleft in two parts (Ishibashi 1997). The name *Bifidobacterium* was proposed for *Bacillus bifidus* by Orla Jensen at 1924 (Russell *et al.* 2011).

Bifidobacteria are Gram positive, non motile, anaerobic, catalase negative and non-spore forming bacteria. They are found in several shapes like short, curved rods, Y shaped or bifid form. In addition they contain a high Guanine plus Cytosine (G+C)

content from 42 to 67 mol%. The optimum pH and temperature for their growth are 6-7 and 37-41°C, respectively (Gomes and Malcat 1999).

They are strictly anaerobes but in the presence of carbon dioxide some strains are oxygen resistant e.g. *Bifidibacterium minimum* (Simpson *et al.* 2004). Also they are able to produce organic acid by fermentation of a variety of sugars. Apart from *Bifidibacterium indicum* and *Bifidibacterium asteroides*, bifidobacteria are catalase negative (Felis and Dellaglio 2007).

Bifidobacteria with 42-67% G+C content are not considered to be member of LAB, However, due to their metabolic capacities they are included in this group by many scientists. According to phylogenetic grouping, they belong to actinobacteria (Tamime *et al.* 1995; Ishibashi 1997). Hexoses are catabolised by fructose-6-phosphoketolase enzyme which is specific to bifidobacteia and this metabolic pathway is known as the fructose-6-phosphate pathway or the so-called bifid shunt. Therefore the fructose-6- phosphoketolase is a taxonomic marker for identification of genus *Bifidobacterium*. Bifidobacteria are able to produce lactic and acetic acids without gas formation.

The principal end products of fermentation are acetate and lactate, produced in a 3:2 ratio, as shown below (Fooks and Gibson 2002)(See appendix 2).

$$2\ \text{Glucose} + 5\text{ADP} + 5\text{P} \longrightarrow 3\ \text{Acetate} + 2\ \text{Lactate} + 5\text{ATP}$$

Glucose, galactose, lactose and sometimes fructose are utilised as carbon sources by all *Bifidobacterium* spp. of human origin. In some cases, bifidobacteria are able to use complex carbohydrates, such as D-galactosamine, D-glucosamine, amylose and amylopectin (Crociani *et al.* 1994).

Some strains of bifidobacteria are able to grow in media which contains nitrogen in the form of ammonium, but some others use nitrogen from organic sources. It has

been shown that nutritional requirements of this genus are totally species dependent. De Man Rogosa Sharpe (MRS) agar fortified with 0.05% w/v cystein and Reinforced clostridial Agar (RCA) are two rich media in which bifidobacteria are able to grow better than in milk, but due to high costs and also because of contribution to off flavour in the final products, the organisms grown in these media cannot be used in dairy products. Thus to improve their growth in milk, fortifying milk with easily available nitrogen sources or redox potential-lowering compounds, such as hydrolysed casein and cystein, has been suggested.

In a review on different aspects of bifidobacteria including their taxonomy, Gomes and Malcata (1999) reported that the *Bifidobacterium* genus is composed of 30 different species, 10 of which are derived from human sources (dental, vaginal and faecal), 17 from warm blooded animal sources, two from waste water and one from fermented milk. However the latest updates indicate that there are currently 37 known species of bifidobacteria (Russell *et al.* 2011) which are listed in Table 1.2.

Regarding taxonomic issues on grouping of *Bifidobacterium longum* and *Bifidobacterium infantis* under *Bifidobacterium longum*, in 2008 they were reclassified into three biotypes named the *infantis* type, the *longum* type and the *Suis* type (Mattarelli *et al.* 2008). The relationship between *Bifidobacterium animalis* and *Bifidobacterium lactis* is also debatable, with the latter being reclassified as *Bifidobacterium animalis* subsp. *lactis* (Masco *et al.* 2004).

Table 1.2 Habitats of the species of the genus *Bifidobacterium*

Species	Habitat first identified from
Bifidobacterium actinocoloniiforme	Bumblebee intestine
Bifidobacterium adolescentis	Faeces of human adult, bovine rumen, sewage and human vagina
Bifidobacterium angulatum	Sewage, faeces of human adult
Bifidobacterium animalis subsp. *animalis*	Animal faeces
Bifidobacterium animalis subsp. *lactis*	Yogurt
Bifidobacterium asteroids	Honeybee intestine
Bifidobacterium bifidum	Faeces of human adult, infant, suckling calf and human vagina
Bifidobacterium bohemicum	Bumblebee intestine
Bifidobacterium boum	Bovine rumen, faeces of piglet
Bifidobacterium bombi	Bumblebee intestine
Bifidobacterium breve	Faeces of infant and suckling calf, human vagina and sewage
Bifidobacterium catenulatum	Faeces of infant and human adult and sewage
Bifidobacterium choerinum	Faeces of piglet and sewage
Bifidobacterium coryneforme	Honeybee intestine

Table 1.2 *continued*: Habitats of the species of the genus *Bifidobacterium*

species	Habitat first identified from
Bifidobacterium crudilactis	Raw milk and raw milk cheeses
Bifidobacterium cuniculi	Faeces of rabbit
Bifidobacterium dentium	Human dental caries and oral cavity; faeces of human adult
Bifidobacterium gallicum	Human faeces
Bifidobacterium gallinarum	Chicken caecum
Bifidobacterium indicum	Honeybee intestine
Bifidobacterium infantis	Faeces of infant and suckling calf
Bifidobacterium longum subsp. *longum*	Faeces of human adult
Bifidobacterium longum subsp. *infantis*	Faeces of infant
Bifidobacterium longum subsp. *suis*	Faeces of piglet
Bifidobacterium magnum	Faeces of rabbit
Bifidobacterium merycicum	Bovine rumen
Bifidobacterium minimum	Sewage
Bifidobacterium mongoliense	Fermented mare's milk product from Mongolia

Table 1.2 *continued*: Habitats of the species of the genus *Bifidobacterium*

species	Habitat first identified from
Bifidobacterium pseudolongum subsp. *pseudolongum*	Faeces of infant and suckling calf and sewage
Bifidobacterium pseudolongum subsp. *globosum*	Faeces of pig, chicken, bull, calf, rat, and guinea pig
Bifidobacterium psychraerophilum	Faeces of calf, rat, rabbit, lamb, sewage and bovine rumen
Bifidobacterium pullorum	Pig faeces
Bifidobacterium ruminantium	Faeces of chicken
Bifidobacterium saeculare	Bovine rumen
Bifidobacterium scardovii	Faeces of rabbit
Bifidobacterium stercoris	Human blood
Bifidobacterium subtile	Human faeces
Bifidobacterium thermacidophilum subsp. *theracidophilum*	Sewage waste water from anaerobic digester
Bifidobacterium thermophilum subsp. *porcinum*	Faeces of piglet
Bifidobacterium tsurumiense	Hamster dental plaque

Adapted from Russell *et al.* (2011)

1.4 Ecology of bifidobacteria

In general, *Bifidobacterium* species have been found to reside in various ecological places i.e. the human intestine, the human vagina, the oral cavity, food, the animal GIT, sewage and the intestine of honeybees (Russell *et al.* 2011)

A foetus is considered to be free of germs and the species composition in the colon is believed to be related to the method of birth (natural birth vs Cesarian section), breastfeeding and also hygienic condition of the hospital (Stark and Lee 1982; Marques *et al.* 2010). It is expected that in two weeks old infants, a stable microflora ecosystem is established, in which bifidobacteria constitute 40–60% of the total faecal microbiota. However, this is not a general rule and in some infants, bifidobacteria might appear with a significant delay (Russell *et al.* 2011). Also, research has shown that bifidobacteria species are the most numerous bacteria in breast fed infants. Bifidobacteria are estimated to be 75% in bottle-fed infants whereas in breast-fed, they are 95% of total microflora (Harmsen *et al.* 2000; Hadadji *et al.* 2005).

Bacterial colonization in the gut is completed in four stages. Childbirth would be the first stage of bacterial colonization in which the newborn initially encounters the maternal microflora. Second stage would be oral feeding, and then weaning period is considered to be the third stage. Throughout weaning period, bifidobacterial count gradually decreases and *Bacteroides* and *Eubacterium* become predominant. At the age of two years, adult microflora is acquired (Gurakan *et al.* 2010). Bifidobacteria comprise only 3% of the gut microflora in the adult GIT (Vaughan *et al.* 2005). Lay *et al.* (2005) stated that approximately 4.4% of the total faecal microbiota of adults in Northern Europe are bifidobacteria. With increasing age, further reduction arises in bifidobacterial count and the number of *Enterobacteriaceae* and clostridia increase. It

might be because of reduced gastric secretion in older people and also the effect of diet should not be neglected (Angus *et al.* 2005). It could partially explain the susceptibility of elderlies to several diseases (Hopkins *et al.* 2001 and 2002).

The 10 bifidobacteria species, which are most frequently recovered from human GIT, are *Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium gallicum, B. longum* subsp. *longum* and subsp *infantis*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium Scardovii* (Requena *et al.* 2002; Sakata *et al.* 2002). *Bifidobacterium longum, B. breve, B. bifidum, B. adolescentis* and *B. pseudocatenulatum* are representative of human intestinal origin.

In addition, at older age, the profile of bifidobacteria species changes and *B. adolescentis* becomes the dominant species replacing *B. infantis* and *B. breve* (two typical bifidobacteria in infants); however, *B. longum* continues to exist for a lifetime (Mitsuoka 1990).

1.5 Selecting probiotic strains: important aspects

Probiotic bacteria should fulfil certain requirements and basically the selection of probiotic bacteria is based on these criteria (Kailasapathy *et al.* 2008; Pan *et al.* 2009). Such criteria which include safety, functional and technological aspects are illustrated in Figure 1.2 and discussed in more detail.

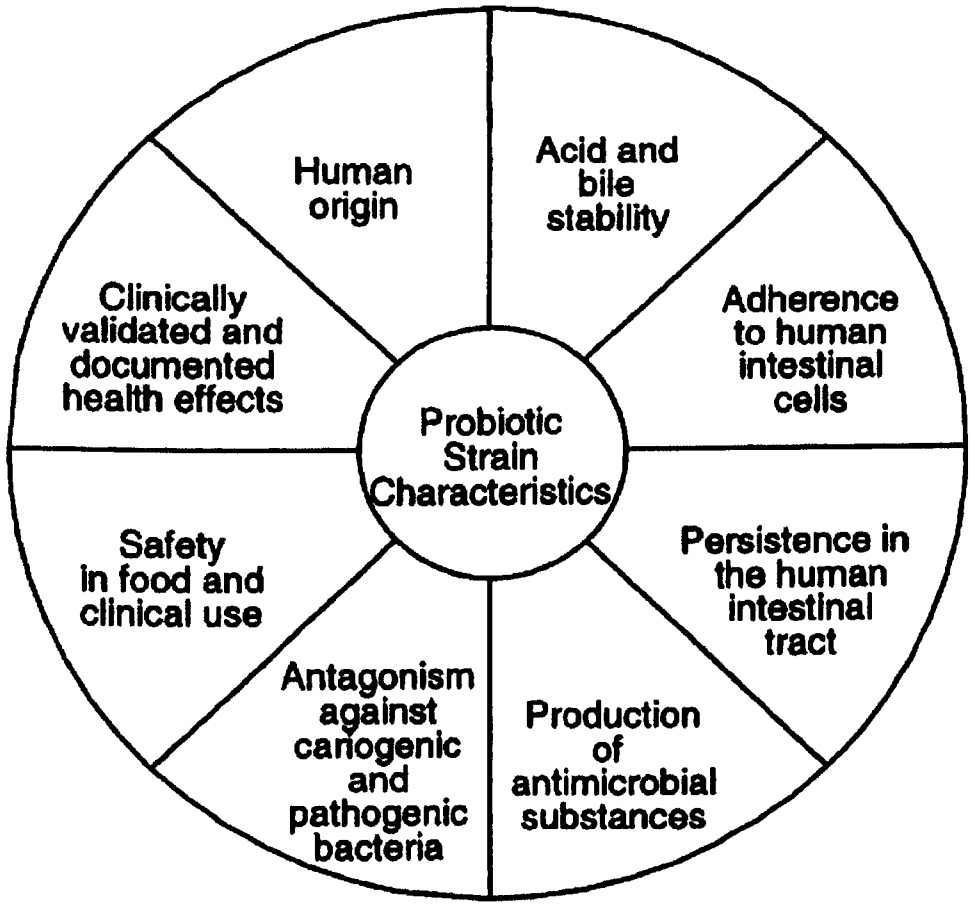

Figure 1.2 The theoretical basis for selection of probiotic microorganisms (Adopted from Saarela *et al.* 2000)

1.5.1 Safety of probiotics as a food ingredient

Bifidobacteria have a long safe history of use as probiotics in fermented foods (Russell *et al.* 2011). There are ample researches that signify the positive effects of bifidobacteria on health and nowadays they are used in various foods, such as yogurt, milk, infant formula and cheese (Zubillaga *et al.* 2001). In spite of all advantages of probiotic bacteria, their safety is still a matter of concern. The main interests with regard to the safety of probiotic bacteria are:

- Strains for human consumption need to be preferably from healthy human sources.
- They have no pathogenic history or association with diseases

- They do not carry transmissible antibiotic resistance genes

1.5.1.1 Origin of probiotic strains

It is of importance to emphasise that health promoting effects of probiotic bacteria are strain-specific: therefore, probiotic bacteria for human consumption are considered to be prepared from human origin strains which are more likely to have the potential for adaptation to the human GIT and have a better chance of colonisation in the gut. Although this criterion was taken into consideration as a requirement for selection of probiotic bacteria for sometime (Ammor *et al.* 2007), one strain of bifidobacteria with animal origin (i.e. *B. animalis* subsp. *lactis*) is currently widely used in fermented milk (Gueimonde *et al.* 2010). It has been debated that a probiotic strain can function better in a similar environment (e.g. human GIT) to where it was originally isolated from (Saarela *et al.* 2000). Over the years, however, it has been proven that bifidobacteria from human sources are more sensitive to gastric conditions than species isolated from animal sources. In addition, the probiotics are more considered as transient flora and as a result the colonisation in the gut is not measured as essential as it was initially thought. This, and perhaps more importantly the technological advantages of animal origin strain of bifidobacteria are the main reasons that has justified the application of *B. animalis* subsp. *lactis* in fermented probiotic products. Nevertheless, human origin bifidobacteria that could fulfil the technological requirements are preferred and the search for such strains needs to be continued. Likewise, some physiological manipulation, such as acid stress treatment may help with the elimination of physiological barriers for susceptible strains (Sanz 2007).

1.5.1.2 Consideration of pathogenic history

Equally important is that probiotic bacteria should be free from any pathogenic elements as another potential risk associated with probiotic bacteria is their likelihood of causing diseases, such as sepsis, endocarditis, and bacteremia (Land *et al.* 2005). Mucin, which is present on the surface of the intestine, acts as an important barrier for prevention of bacterial translocation. Translocation of probiotic bacteria by invasion of the intestinal wall has been of concern. Therefore, a condition for probiotics safety could be lack of mucin degradation activity. Abe *et al.* (2010) revealed that *B. longum* BB536, *B. Breve* M-16V *and B. infantis* M-63, have no mucin degrading activity and translocation ability.

Despite all the available evidence that probiotics are generally safe, there is at least one report that could question such a general statement.

In a study on treatment of acute pancreatitis with a multispecies probiotic preparation, carried out at the University Medical Centre in Utrecht between 2004 to 2007, 24 out of 298 volunteers who took part died. However, the cause of these deaths is not clear though some possible reasons were:

- The use of probiotic on intensive care patients
- Using the probiotic bacteria in the acute phase of disease

It was concluded that probiotic prophylaxis should not be administered in this category of patients (Besselink *et al.* 2008). Despite the fact that probiotics have largely a very good safety record, this incidence could serve as a warning that they should be used with prudence in certain patient groups-particularly neonates born prematurely or people with immune deficiency.

1.5.1.3 Consideration of antibiotic resistances

Probiotic bacteria are resistant to many antibiotics. Resistances may be either inherent to a bacterial genus/species (natural or intrinsic resistance) or acquired, either through one or more sequential mutations or by the incorporation of new genes which could be intrinsic or acquired (Ammor *et al.* 2007). The gene associated with antibiotic resistance should not be transferable to another species, but plasmids are spreadable and transfer to other genera easily (Adams 1999). Recently, microorganisms used in food fermentations have been associated with carriage of antibiotic resistance genes and some researchers have investigated the acquired antibiotic resistance by probiotic bacteria, such as *Lactobacillus* spp. (Ouoba *et al.* 2008). Therefore, before incorporation of a new probiotic strain into food, it should be assessed for such safety concerns. By correctly identifying each strain with PCR-based or similar methods and assessment of its safety, it should be possible to avoid such undesirable effects of probiotic bacteria

1.5.2 Some relevant functional aspects of probiotics

The functional properties of probiotics could be assessed using *in vitro* techniques; however, the results obtained of these studies should be verified by clinical studies. Some of the functional aspects of probiotics that in the context of this project seem pertinent are discussed below in more detail.

1.5.2.1 Survival of *Bifidobacterium* species in the gasterointestinal tract

The ability of probiotic bacteria to survive during passage through the upper GIT is considered as an important property. Probiotic microorganisms should be able to express a high resistance to the harsh conditions, such as the presence of acid and

bile salts, and also be able to adhere to intestinal mucosa. The main target for probiotic bacteria is the colon and during this transition, they are required to be resistant to gasterointestinal secretions.

The pH of empty stomach could decrease to lower than 2; also at the beginning of the small intestine (duodenum) bile salts are secreted to help digestion of fat. Bile salts are able to destroy bacterial cell membranes and reduce the survival of bacteria.

Therefore, probiotic bacteria should tolerate all these barriers and safely reach the colon which is supposed to be a place for their colonization (Lankaputhra and Shah 1995). The resistance of probiotic bacteria to acid and bile salts is studied by *in vitro* and *in vivo* experiments: however, it is believed that even a low level of tolerance in *in vitro* tests carried out with pure cultures is likely to be enough for *in vivo* resistance, because carrier food components, such as milk proteins, act as a supporter for probiotic bacteria and provide protection for them if the probiotics are dlivered in a food matrix, such as dairy product, as against the probiotic supplements. The health benefits of bifidobactera are expected even after they experienced acidic gastric conditions and decreased logarithmically.

1.5.2.2 *Bifidobacterium* species and protection against infection

The human natural defence system is composed of mucosa, epithelium and normal intestinal micro flora, which all together establish a protective system against pathogenic bacteria, such as pathogenic strains of *Escherichia coli*, *Salmonella* and *Helicobacter pylori*.

Generally, in some gastrointestinal diseases, which arise after altering of natural gut microflora, regular consumption of probiotic bacteria might be a helpful tool for

treating such disorders (Osullivan and Kullen 1998). Several *in vitro* studies confirmed the suppression of pathogenic bacteria by low molecular weight substances produced by probiotic bacteria such as short chain fatty. Also hydrogen peroxide presented similar effects of inhibition. Bacteriocins are another group of inhibitors, which are produced in the form of low or high molecular weight (Felley *et al.* 2001; Fooks and Gibson 2002; Oelschlaeger 2010).

Lactic acid and acetic acid are considered as the main organic acids produced by probiotic bacteria and other minor organic acids comprise citric acid, hippuric acid, orotic acid and uric acid. The function of organic acid is lowering of pH in the gut environment (Gurakan *et al.* 2010). At the same pH, acetic acid is more bacteriostatic than lactic acid. Accordingly, this might be the reason for the greater antimicrobial effect of bifidobacteria, because their metabolism results principally in production of acetic acid, compared to other bacteria e.g. the yogurt bacteria where only lactic acid is produced (Arunachalam 1999).

Bile acids are derivatives of bile salts deconjugated by probiotic bacteria.The antimicrobial effects of bile acids are much greater than bile salts. However, the mechanisms by which probiotic bacteria preserve themselves from these self made metabolites are still unclear (Oelschlaeger 2010).

Infectious diarrhoea is considered as fatal health problem, which causes illness and death among infants and children in many developing countries. Some of the most solid evidences for the use of probiotics are in the management of diarrheal diseases. Several randomized controlled trials have shown that many probiotics are effective in preventing antibiotic-associated diarrhea (D'Souza *et al.* 2002). The supplementation of bifidobacteria to infants and children has shown to be helpful to treat infectious diarrhoea (Vanderhoof *et al.* 1999; Thapar and Sanderson 2004).

1.5.2.3 *Bifidobacterium* species and bioactive metabolites

1.5.2.3.1 Conjugated linoleic aid (CLA)

Conjugated linoleic acid (CLA) is an essential fatty acid, which is naturally presented in ruminant milk and fat tissue. CLA is a mixture of positional and geometric conjugated isomers which the major biologically active isomers are cis-9, trans-11 CLA (c9, t11-CLA) and trans-10, cis-12 CLA (t10, c12-CLA)(Park *et al.* 2011).

Many physiological activities, such as anti adipogenic, anti diabetogenic, anti carcinogenic, and anti atherosclerotic activities, were ascribed to CLA (Russell *et al* 2011). Coakley *et al.* (2003) first reported the potential of *Bifidobacterium* species for CLA production. According to their experiments, different strains of *B. breve* converted linoleic acid to CLA at a high rate.

1.5.2.3.2 Short chain fatty acids (SCFA)

Short chain fatty acids (SCFA) are normally produced by anaerobic fermentation of non digestible carbohydrates in the large intestine (Cummings *et al.* 1987). Acetate, propionate and butyrate are considered as the major metabolic endproducts by probiotic cultures (Fooks and Gibson 2002). Non digestible carbohydrates are metabolised by bifidobacteria and produce phosphoenolpyruvate which is degraded to pyruvate. Pyruvate is further metabolised to form acetyl-CoA, this then leads to the formation of the SCFAs (Sela *et al.* 2008).

SCFAs are generally metabolised by colonic epithelial cells, liver and muscle cells. Different polysaccharides give different SCFA profiles. However, the major SCFA produced in the colon is acetate which is utilised by the liver and muscle cells (Roberfroid 2005). Acetate has been reported to provide the body an energy yield of 1.5–2 kcal/g (Russell *et al.* 2011).

It should be noted that the accumulation of SCFAs lead to decrease of the pH of their surrounding environment, and it is likely that this may act directly to inhibit the growth of harmful and pathogenic organisms (Fooks and Gibson 2002).

1.5.3 Technological aspects of probiotics

It is important for probiotic bacteria to fulfil several criteria to benefit human health. The technological properties are considered as major qualities for selection of probiotic bacteria. Even if a probiotic strain fulfils the required safety and functional measures, it needs to be included into food products without any risk of losing or reducing viability/functionality or even generating unpleasant flavours or textures. Due to technological limitations, sometimes probiotic bacteria with exceptional functional health properties are excluded (Mattila-Sandholm *et al.* 2002).

1.5.3.1 Stability of *Bifidobacterium* species in probiotic products

Good viability and activity of probiotics are considered as prerequisites for optimal functionality. Probiotic bacteria should remain alive and maintain their metabolic ativity in the vehicle medium during shelf-life. The role of food matrices in the survivability and health effects of probiotics is significant. Strain survival in the food environment depends on pH, oxygen, presence of other microorganisms, presence of food additives and preservatives, such as NaCl, sugar and H_2O_2 (Shah and Lankaputhrab 1997; Vinderola *et al.* 2002). Fermented milk products are the favourite carrier for probiotic bacteria due to healthy image of milk (Santo *et al.* 2011), the ability of LAB and most bifidobacteria to grow in milk and the fact that fermented milk carry substantial numbers of microorganisms already.

Russell *et al.* (2011) quoted 1×10^7 CFU/g of bifidobacteria in Indonesian powdered

formulas after 24 months storage at 30°C. It has been stated that survival rate would be diminished with higher water activity in the powder (Schoug *et al.* 2006).

Jayamanne and Adams (2009) reported a log-linear reduction in survival of *B. longum* in acidified skim milk (pH 4 and 4.25) over time, but *B. animalis* subsp. *lactis* presented better survival in aidic conditions significantly longer than other tested bacteria (i.e. weeks rather hours). Their study indicated the ability of *B. animalis* subsp. *lactis* to withstand environmental stress conditions, such as high oxygen and acidic environments.

1.5.3.2 Probiotic interaction with starter bacteria

Basically two types of starter cultures are available:

- Traditional cultures, which comprises complex and undefined strains.
- Selected and defined cultures, which contain identified pure cultures and their functionalities for acid production, flavour intensity and proteolysis, are known (Vasiljevic and Shah 2008).

The production of balanced starter and probiotic cultures, which include a high content of uninjured and viable cells, has always been of concern. Liquid and frozen cultures were popular in last few years, but new generation of starter cultures, which are prepared by freeze drying system, are quite common. Due to difficulties involved in propagation of probiotic bacteria at the production site, the use of highly concentrated direct-to-vat inoculation (DVI) cultures are quite recommended (Tamime and Robinson 2007). Usually DVI culture are presented in two very high concentrated types either frozen (contain more than 10^{10} cfu/g) or freeze dried (contain more than 10^{11} cfu/g) cultures.

Bifidobacteria alone, produce some acids in milk and also exhibit little growth during

fermentation. Besides, in order to multiply, they need particular anaerobic conditions and some growth promoting factors to be added to the milk (Modler 1994, Gomes *et al.* 1998). Therefore, they usually need a long incubation time. For a shorter fermentation time, the growth of bifidobacteria in sterilised milk needs to be improved. Some reports have stated that by fortification of foods with glucose as energy sources, protein hydrolysis as growth stimulator, and also suitable antioxidant, mineral and vitamin, the growth of probiotic bacteria would be improved (Dave and Shah 1998). However, by such adjustments only the performance of probiotic bacteria would be enhanced, but for overcoming of technological dilemma, it is strongly recommended to use starter cultures along with probiotic bacteria.

Acetic and lactic acids (theoretically, at the ratio of 3:2) are the final result of fermentation of milk by bifidobacteria. Sometimes with excessive fermentation, the taste of products turns vinegar-like, which is not acceptable (Gomes and Malcata 1999). Consequently, special attention should be paid to strain selection and also all process and procedures must be monitored carefully in order to produce a high quality fermented probiotic product. However, for reduction of fermentation time and improvement of sensory properties (like taste and texture), use of combined cultures is suggested.

This is why nowadays, bifidobacteria used as adjunct culture along with suitable starter culture bacteria in fermented probiotic products to contribute excellent sensory properties. *Sterptococcus thermophilus*, one of the yougurt cultures and mesophilic cultures could be used with *Bifidobacterium* spp. in fermented dairy products and the interaction between them might influence the final quality. It is quite common that starter culture manufacturers produce commercial probiotic culture by mixing LAB and bifidobacteria, which might consists of a single or a mixture of several

strains of each genus. It has to be taken into considertatin that the survival of bifido-bacteria might be affected by the metabolites formed by the starter cultures, such as lactic acid, hydrogen peroxide and bacteriocins. Therefore, to select a suitable starter, the negative impact on probiotic's survival should be examined.

1.5.3.3 Dairy products supplemented with bifidobacteria

Most common functional food products that exist in the world market are of dairy origin. More than 50 probiotic dairy products are produced in Japan, which have claimed to contain 10^7 CFU/g at the end of shelf-life. Similar trends could be seen in other developed countries, such as France, Germany and Sweden, where more than 25 percent of their fermented dairy products have been categorised as probiotic products. It is assumed that more than 80 products containing *Lactobacillus* and *Bifidobacterium* spp. exist in the world (Gomes and Malcata 1999), which has extended to even more in recent years. Nowadays, plenty of research has concentrated on fermented milks, yogurts and desserts (Shah 2000; Heenan *et al.* 2004), ice cream (Cruz *et al.* 2009), cheese (Kasımoglu *et al.* 2004), fermented soy milk and soy yogurt (Farnworth *et al.* 2007) as good carrier foods for probiotic bacteria. Several techniques are available for inoculation of probiotic strains into fermented products. The milk could be fermented with probiotics alone and mixed with milk fermented with other LAB (e.g. standard yogurt) at the final stage of product formulation or added to the fresh milk or milk product without fermentation (Gomes and Malcata 1999).

Recognition of such products may be facilitated by addition of the prefix "BIO" to the traditional names, so a new range of products were introduced into fermented dairy products' market (e.g. Biogurt, Biodrink).

Most studies on beneficial values of probiotics are carried out in dairy products. Depending on the type of probiotic bacteria in these products, beneficial features, such as reduced lactose level and an increase in free amino acids and vitamins, are expected. It has been reported that bifidobacteria and LAB strains increase the quantity of folate, vitamin K, vitamin B12, riboflavin, and thiamine when used in yogurt and fermented milks (Gurakan *et al.* 2010). Yogurt microorganisms and some probiotic cultures are able to synthesise some water soluble vitamins. In some commercial probiotic products like Actimel (*L. casei*), Pro Yogurt (*L. acidophilus*), Bio cultures (*L. casei* + *L. acidophilus* + bifidobacteria) and Bifidus (bifidobacteria + lactobacilli), considerable amounts of vitamins B1 (0.01mg/100g), B2 (0.17 mg/100g) and B6 (0.24 mg/100gr) were produced (Tamime and Robinson 2007).

Bifidobacteria are also able to produce L (+) lactic acid which is easily metabolised by humans, so may help to prevent metabolic acidosis in infants. In addition, L (+) lactic acid is considered as an energy source with an energy yield of 15 KJ/g (Shah 2006).

Since in real world and on commercial scale, the probiotic bacteria added to dairy products or other food products are not actually involved in the fermentation and only added at a very high dose, usually in the latter part of processing stage to promote higher survival (Saarela *et al.* 2000), the role of bifidobacteria in producing such nutrients is questioned.

1.5.3.4 Consumption dose of probiotic for optimal benefit

Probiotic bacteria should be consumed on a regular basis and at certain levels to exert their health effects. As soon as probiotic administration be discontinued, they would not be recovered in feces any more, which indicates that, although not

colonizing (i.e. they are transient in the GIT), these bacteria could continue to be metabolically active, thus providing health benefits to their hosts. Therefore, regular consumption of probiotic bacteria is strongly recommended for any expected beneficial effects (Saarela *et al.* 2000).

No agreement has been established about the effective dose yet, but a minimum dose of probiotic bacteria, to assure the therapeutic effect, have been suggested. For any probiotic expectation, the minimum concentration of probiotic microorganisms should be between 10^6 and 10^7 CFU/g of product at the time of consumption and it has been suggested that 100 g of fermented milk is required as daily serving portion (Samona and Robinson 1993; Shah 2000; Jayamanne and Adams 2006; Vasiljevic and Shah 2008).

1.6 Aims and objectives of the research

The main aim of this project was to screen commercially available fermented dairy products in the UK for the presence and diversity of bifidobacteria and evaluate their probiotic characteristics. Specific objectives included:

- Monitoring survival of the bifidobacteria in the carrier foods at the time of purchase and at the end of their shelf-life
- Isolation and identification of presumptive bifidobacteria with phenotypic and genotypic methods and differentiation of obtained isolates using of rep-PCR
- *In vitro* assessment of some of the requirements for probiotics as well as some of the functional properties of the isolates from fermented products particularly their
    - Resistance to acidic condition of stomach and the presence of bile salts in the small intestine

- Antimicrobial activities and potential for bacteriocin production and or other mechanisms involved
- Biofilm formation in different environments
- Production of secondary metabolite, such as CLA

- *In vitro* assessment of their safety by screening the isolates for antibiotic resistance, presence of antibiotic resistance genes and their possible transferability to some enterobacteria.

Chapter 2

Enumeration and identification of *Bifidobacterium* spp. in fermented dairy products available in UK market

2.1 INTRODUCTION

Bifidobacteria are Gram positive, curved or bifid, rod shaped bacteria, which vary in shape when growing in different media. They are one of the most important groups of probiotic bacteria that naturally inhabit in human and animal GIT and in several studies, their beneficial quality including stimulation of immune system, prevention of some diarrhoeal diseases, constipation and intestinal infections have been demonstrated (Reuter *et al.* 2002).

Nowadays, an increasing interest has been devoted to identification of new strains of bifidobacteria which might have functional properties and their addition to suitable products, in particular fermented milks, will confer dietary advantages (Jayamanne and Adams 2006). *Bifidobacterium bifidum*, *B. breve*, *B. longum* and *B. animalis* subsp. *lactis* are the most frequent bifidobacteria applied for preparation of probiotic products (Roy 2001). For health improvement effects, *Bifidobacterium* spp. should overcome the harsh conditions in GIT and remain alive to reach the colon and establish in the mucous layer properly (Gilliland 1978; Klaver *et al.* 1993; Lankaputhra and Shah 1995; Kailasapathy and Rybka 1997). Therefore, their survival in the food matrix and GIT should be guaranteed by the manufacturers.

However, so far no standard has been established for minimum number of viable probiotic bacteria in fermented products to confer a health effect. Some researchers suggest that it should be at least 10^6 CFU/g at the end of shelf-life (Samona and Robinson 1994; Jayamanne and Adams 2006), but others suggest a minimum of 10^7 CFU/g for a probiotic product (Gueimonde *et al.* 2004). Perhaps the only standard for the minimum level of the probiotic organisms in fermented milk is the standard proposed by FAO (2003) where a minimum of 10^6 CFU/g of the product has been recommended and only applies where a content claim is made in the labelling that

refers to the presence of a specific microorganism other than that of standard yogurt culture and some other lactobacilli.

In addition, several parameters like pH, acidity, oxygen, contamination and food additives are able to affect viability of probiotic bacteria in fermented food, and some studies on market products show low viability of probiotic bacteria during the shelf-life (Iwana *et al.* 1993; Micanel *et al.* 1997; Shah 2000).

In recent years, several elective and selective media have been studied which could be used for enumeration of bifidobacteria in presence of starter culture bacteria in a food matrix (Munoa and Pares 1988; Tabasco *et al.* 2007; Lima *et al.* 2009; Ghoddusi and Hassan 2011). One useful medium is Bifidobacterium Iodoacetate Medium (BIM-25) (Munoa and Pares 1988). The basal medium is Reinforced Clostridial Agar (RCA) with 4 anti-microbial agents added for selectivity; Nalidixic acid, Polymyxin β-sulfate, Kanamycin sulfate and Iodoacetic acid (sodium salt). Triphenyl Tetrazolium Chloride (TTC) serves as redox indicator. Iodoacetate inhibits glyceraldehyde-3-phosphate dehydrogenase, and reduces the growth of nonbifidobacterial colonies. Because of polymyxin β-sulphate presence in BIM-25, no Gram negative bacteria should grow on this medium (Munoa and Pares 1988). In order to isolate and identify probiotic microorganisms in fermented milk products, Scotti *et al* (2002) used BIM-25 and examined such products to substantiate or refute claims of probiotic organisms on their labels. Jayamanne and Adams (2006) also used BIM-25 for determination of survival and stress resistance of bifidobacteria in bio yogurt. There are, however, some concerns about inhibition of *Bifidobacterium* in this medium due to high concentration of anti-microbial agents. Gram positive cocci are also able to grow on BIM-25 under aerobic conditions and form small red colonies with a diameter of less than 2 mm. Under anaerobic incubation

Bifidobacterium spp. always produce large white colonies with a red centre and a diameter of more than 2 mm. Also some pink colonies might grow on BIM-25 which could be correspondent to cocci, bifidobacteria and other rods. Silvi *et al.* (1996) observed that BIM-25 is very selective and might inhibit the growth of some *Bifidobacterium* strains, but the advantages of this medium which are claimed by the authors are: firstly, BIM-25 presents a high selectivity compared to other media suggested for isolation of bifidobacteria. Secondly, the toxicity of BIM-25 is minimal for bifidobacteria (Munoa and Pares 1988; Roy 2001).

More recently, International Dairy Federation (IDF) specified a method for enumeration of bifidobacteria in milk products (ISO/IDF 2010). Transoligosaccharide propionate agar (TOS) with lithium mupirocin (MUP) supplement is a new medium for enumeration of bifidobacteria in presence of other lactic acid bacteria in probiotic dairy products. This medium comprises of galactooligosaccharide as a specific growth promoter for all bifidobacteria, magnesium sulphate for growth of injured bifidobacteria, sodium propionate as an inhibitor for other adjunct flora and finally lithium mupirocin which suppresses growth of lactobacilli, lactococci, streptococci and leuconostocs without any influence on bifidobacteria (Merk 2010).

Bacterial species in a probiotic product are required to be accurately identified and for health claims of probiotic products, accurate labelling is necessary. Some researches have shown that the recovered bacteria do not always match to what is claimed on the product label. Temmerman *et al.* (2003) evaluated 55 European probiotic products (30 dried food supplements and 25 dairy products) with regard to the identity and the antibiotic resistance of the recovered bacterial isolates. They reported mislabelling in 47% of the food supplements and 40% of the dairy products.

Therefore, proper identification of used strains in fermented products is very important.

Phenotypic assays are useful tools but not always adequate for identification of probiotic species especially bifidobacteria (Gueimonde *et al.* 2004). A more accurate and reliable identification of species could be genotypic assay like PCR based methods. For example, one accurate means among genotypic methodologies for identification of species is the sequencing of the 16s rRNA gene (Kaufmann *et al.* 1997; Gueimonde *et al.* 2004).

The aims of this part of the study were:

a) Evaluating some commercial probiotic dairy products for the number of bifidobacteria by enumerating at the time of purchase and at the end of shelf-life, using of BIM-25 and TOS-MUP media.

b) Isolation and storage of bifidobacteria from these commercial probiotic products.

c) Assessing the potential of phenotypic methods for identification of the bifidobacteria isolates.

d) Identification of the isolates using genotypic tests and in particular sequencing the 16s rRNA gene and discrimination by rep-PCR.

In addition, label accuracy in fermented milk products was assessed.

2.2 MATERIALS AND METHODS

2.2.1 General and selective media

The media used in this study were Bifidobacterium Iodoacetate Medium (BIM-25) and TOS-MUP as selective media. Maximum Recovery Diluents (MRD) (CM0733, Oxoid, Hampshire, UK) was used for serial dilutions. BIM-25 composition was as follows (g/l): Reinforced Clostridial Agar (RCA)(CM0151, Oxoid, Hampshire, UK); nalidixic acid, 0.02; polymyxin β sulphate, 0.0085; kanamycin sulphate, 0.05; iodoacetic acid (sodium salt), 0.025; and 2,3,5-triphenyltetrazolium chloride (TTC), 0.025. All reagents were purchased from Sigma. The autoclaved RCA basal medium was allowed to cool to 55 to 60°C, and then antibiotics were added through 0.2 µm syringe filter. Reinforced Clostridial Media and MRD were prepared according to manufacture's instructions and sterilised by autoclaving at 121°C for 15 min.

For preparation of TOS-MUP, dried ready medium was suspended in water according to manufacture's instructions to reach a 190 ml of basal medium and autoclaved at 115 °C for 15 min. Immediately before use, 50 mg of mupirocin was dissolved in 50 ml of water and filter sterilized (pore size 0.2 µm), and 10 ml of mupirocin supplement solution was added to 190 ml portion of the basal medium.

2.2.2 Probiotic products

Twenty four commercial yogurts and yogurt drinks from different manufacturers, claiming to contain bifidobacteria were purchased and carried straight away to laboratory and kept in the fridge at 4 °C until analysed. Among the products, some were plain, while the others were fruit and/or flavoured products. The range of measured pH for all tested samples was between 4 and 4.5 during the sampling

period. All samples were named from A to Y. Table 2.1 shows details of tested products.

Table 2.1 Probiotic products tested for presence of *Bifidobacterium* spp.

Sample code	Product description	Days to expire	Claimed probiotic culture (s)
A	Stirred yogurt	19	*Bifidus acti regularis**
B	Stirred yogurt	28	*Bifidobacterium*
C	Drinking yogurt	25	*Bifidobacterium*
D	Stirred yogurt	21	*Bifidobacterium*
E	Stirred yogurt	14	*Bifidobacterium*
F	Stirred yogurt	19	*Lactobacillus acidophilus, Bifidobacterium*
G	Goat milk yogurt	13	*Lactobacillus acidophilus, Bifidobacterium*
H	Organic Greek-style natural yogurt	12	*Bifidobacterium*
I	Honey and ginger yogurt	12	*Lactobacillus acidophilus, Bifidobacterium*
J	Vanilla fat free yogurt	17	*Bifidobacterium*
K	Passion and apricot low fat yogurt	14	*Lactobacillus acidophilus, Bifidobacterium*
L	Whole milk yogurt	12	*Lactobacillus acidophilus, Bifidobacterium*
M	Fat free yogurt	17	*Bifidobacterium*

Table 2.1 *continued*: Probiotic products tested for presence of *Bifidobacterium* spp.

Sample code	Product description	Days to expire	Claimed probiotic culture (s)
N	Custard style yogurt	9	*Bifidobacterium*
O	Fat free fruit yogurt	13	*Bifidobacterium*
P	Fruit yogurt	12	*Bifidobacterium*
Q	fruit yogurt	19	*Bifidobacterium*
R	Organic bio pouring yogurt	7	*Bifidobacterium*
S	Chocolate drink	15	BB12*
T	Probiotic yogurt drink	15	*Bifidobacterium*
U	Goat milk yogurt	12	*Bifidobacterium longum*
V	Fat free yogurt	13	*Bifidus* and *Acidophilus*
W	Fruit yogurt	26	*Lactobacillus acidophilus, Bifidobacterium longum*
Y	Fruit yogurt	12	*Bifidobacterium*

* Commercial name/code for *Bifidobacterium* spp.

2.2.3 Determination of the viable cell count of *Bifidobacterium* spp. in fermented milks

All products were tested on arrival in the laboratory and a second sample, purchased at the same time, at their expiry date. One gram of each sample was suspended in 9 ml of MRD and vortexed. The uniform suspension was decimally diluted in MRD up to 10^{-8} and 25 µl of all dilutions was spread on to quartered BIM-25 and TOS-MUP plates in duplicate. The plates were incubated at 37 °C for 3 to 5 days in anaerobic cabinet (Don Whitley, Skipton, UK) in an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Plates containing 8 to 80 colonies per 25µl volume were enumerated by a Quebec colony counter and the colony forming units per gram (CFU/g) of the product were calculated and log CFU/g was reported.

2.2.4 Isolation and storage of the isolates

After verifying the purity of the colonies grown on BIM-25 and TOS-MUP by Gram staining and catalase test, one typical colony was harvested and cultured on RCA. Following overnight incubation at 37 °C, the colonies were transferred aseptically into a sterile vial (MicroBank, Wirral, UK) containing porous beads which serve as carriers to support microorganisms. After bonding of microorganisms to beads by gentle aspiration, the excess cryopreservative was poured off and vials kept at - 20 °C for future use.

2.2.5 Identification of the isolates

2.2.5.1 Phenotypic tests

2.2.5.1.1 Gram staining

Bifidobacterium spp. are Gram positive, curved or bifid and rod shapes. Gram staining was carried out using Biomerieux reagents, according to the recommended procedure (Biomerieux, Basingstoke, UK).

2.2.5.1.2 Catalase test

Catalase is a common enzyme in most living organisms which are aerobes or facultative aerobes. If the organisms possess the catalase enzyme, it splits H_2O_2 into water and oxygen. Formation of bubbles of oxygen with immersion of some colonies into hydrogen peroxide was considered as positive. Due to absence of catalase, *Bifidobacterium* spp. are expected to give a negative catalase reaction.

2.2.5.1.3 Screening the fermentation profile of isolates using API systems

API systems (Analytical Profile Index; Biomerieux, Basingstoke, UK) are a miniaturised way of obtaining a large quantity of data about the phenotypic characteristics of microorganisms. Each microtube on the card contains a different dried substrate. When a liquid suspension of the organism is added, the substrate rehydrates and the organism can grow and utilise the substrate (or not). The reactions can be read after a given incubation time.

2.2.5.1.3.1 API 50 CHL

This system is principally for LAB. However, it was used for bifidobacteria to obtain the carbohydrate fermentation profiles. Overnight RCA culture of each isolate was

utilised as inoculum. All the colonies were harvested from the surface of the agar and placed into 2 ml sterile distilled water. This gave a very dense suspension and 8 drops of this suspension was added into the vial containing 10 ml of API 50CHL suspension medium. The 50CHL test strips were inoculated with this suspension. After inoculation all compartments were filled with sterile mineral oil and incubated at 37°C for 48 h.

2.2.5.1.3.2 API Rapid ID 32 A

API Rapid ID 32 is a standardised system for the identification of anaerobes in 4 h, which uses 29 miniaturised enzymatic tests and a data base.

Overnight RCA culture of each isolate was utilised as inoculum. All the colonies were harvested from the surface of the agar and placed into 2 ml ampoule of API suspension medium. Then this suspension was inserted into each of the microtubes in the strip. After covering with 2 drops of sterile mineral oil to maintain anaerobic conditions, strips were incubated at 37°C for 4 h. Reading was performed according to manufacture's instructions.

2.2.5.2 Genotypic tests

2.2.5.2.1 Identification by sequence analysis of 16s ribosomal RNA gene

An important procedure for identification of *Bifidobacterium* spp. could be genotyping test which has been undertaken by partial sequence analysis of the 16S rRNA gene. The presumptive *Bifidobacterium* spp. isolates which have been kept on cryobeads at -20°C were grown on RCA anaerobically at 37°C for 24 h.

2.2.5.2.1.1 DNA Extraction

A fresh 24 h colony was resuspended in 1 ml of sterile water in a sterile Eppendorf tube and centrifuged for 1 min at 6708 × *g*, then supernatant was discarded. After addition of 100 µl of Instagene matrix (BioRad, Paisley, UK) mixture was incubated at 56 °C (in heating block) for 30 min. After vortexing for 10 sec, incubation was repeated at 100°C for 10 min. The tubes were then vortexed for 10 s and centrifuged at 9660 × *g* for 3 min and supernatant was collected in a steriled Eppendorf tube and stored at -20 °C for future use as DNA template.

2.2.5.2.1.2 Sequencing 16s ribosomal RNA gene, amplification by PCR (1st PCR)

The amplification of 16s rRNA gene was undertaken using the primers pA (5`-AGA-GTT-TGA-TCC-TGG-CTC-AG-3`) and pE (5`-CCG-TCA-ATT-CCT-TTG-AGT-TT-3`; Sigma, UK). The polymerase chain reaction (PCR) was carried out using a reaction mixture consisting of:

Water (autoclaved high purity)	37.75	µl
10 X PCR Buffer (with MgCl2)	5	µl
dNTP (1.25 mM)	5	µl
Primer pA (100 µM)	0.5	µl
Primer pE (100 µM)	0.5	µl
Taq DNA Polymerase (5U)	0.25	µl
Chromosomal DNA	1	µl

Amplification of DNA was performed using a thermo cycler (GeneAmp PCR 2700 system) under the following conditions, 5 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72 °C and finally 5 min at 72°C.

2.2.5.2.1.3 Agarose Gel Electrophoresis

The PCR products were analysed using agarose gel electrophoresis, followed by staining with ethidium bromide and visualisation by examination under UV light of the stained gel. Small part of PCR products was run on the 1% w/v agarose gel to check the quality of PCR products and to check if amplification has been successful and to get an approximate size and concentration. A volume of 5 µl of PCR products was mixed with 2 µl loading dye (Sigma, UK) and loaded in to a 1.5% w/v agarose gel (Bio Rad, UK). DNA molecular marker (Sigma, UK) was also run to give an indication of the size of the bands. The gel was run in 1X TBE (Tris Borate-EDTA) buffer (Sigma, UK) for half an hour at 120V. The gel was stained by placing in an aqueous solution of ethidium bromide 0.5 µl/ml (Sigma, UK) for 20 min and the excess stain was rinsed off with distilled water. PCR products were viewed under a UV light at 260 nm.

2.2.5.2.1.4 Purification of PCR products

The PCR products were then purified using a QIA quick PCR purification kit (Qiagen Ltd, UK) according to manufacturer's instructions.

2.2.5.2.1.5 Second PCR Cycle Sequencing

The second PCR was undertaken to generate 550 base pair (bp) of nucleotides. This was done with a reaction mixture which consisted of:

PCR product	4	µl
Primer pD (5`-GTA-TTA-CCG-CGG-CTG-CTG-3`)	2	µl (20 ng/µl)
ABI Big Dye Terminator Reaction	4	µl

Amplification was performed under the following conditions: an initial denaturation at 95°C for 2 min, 35 cycles consisting of 96°C for 15 s, 40°C for 1 s and 60°C for 4 min.

2.2.5.2.1.6 Precipitation

Secound PCR products were precipitated with 1µl of 3 M sodium acetate (pH 4.6) and 50 µl of absolute ethanol and centrifuged at 9660 × *g* for 20 min. Pellets were rinsed with 250 µl of 70% v/v ethanol and centrifuged for 10 min at 9660 × *g*. Supernatant were discarded and pellets were air dried and sent for sequencing (Gene servicing, Oxford, UK).

Sequences of each isolate were compared to the GenBank database (National Centre for Biotechnology Information, USA) using the online Basic Local Alignment Search Tool (BLAST) program. Identities of the isolates were confirmed when a particular sequence showed similarity to those in the database.

2.2.5.2.2 Differentiation of the isolates by rep-PCR genomic fingerprinting

Rep-PCR was applied for differentiation of all isolates by the method of Ouoba *et al.* (2008). Rep-PCR was undertaken in 25 µl of reaction mixture containing 2 µl of DNA template, 2.5 µl of 10 X PCR buffer (MP2797; Applied Biosystems, 58002067-01, UK), 4 µl of dNTP (1.25 mmol l^{-1}; Promega, U151A, UK), 2 µl of $MgCl_2$ (25 mmol l^{-1}:,Applied Biosystems, LP0693, UK), 4 µl of primer GTG5 (5'-GTG GTG GTG GTG GTG-3') (5 pmol μl^{-1}), 2.5 U of Taq polymerase (5 U/µl; Applied Biosystems, N11912, UK) and 10.25 µl of autoclaved high purity water (Sigma, UK). Amplification consisted of 30 PCR cycles in a thermocycler (GeneAmp PCR 2700 system). The cycling was programmed as follows: initial denaturation at 94°C for 4 min followed by

30 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min and elongation at 65°C for 8 min. Also, final extension at 65°C for 16 min ended the rep-PCR and the amplified product cooled at 4°C. The DNA fragments were separated by applying 10 µl of each PCR product with 2 µl of loading buffer (G7654-5ML, Sigma, UK) to 1.5% w/v agarose gel (BioRad, 161-3101, UK). A DNA molecular marker (D7058-1VL, Sigma, UK) was included as standard for the calculation of the size of the fragments. The gel was run in 1 X TBE buffer (Tris, Borate-EDTA buffer, Sigma, UK) for 2 h at 120 V and photographed using an UV transilluminator.

2.3 RESULTS

2.3.1 Survival of probiotic bifidobacteria in commercial fermented milks

In this research, survival of *Bifidobacterium* spp. in 24 fermented milk products was studied during their shelf-life at refrigerated conditions. Twenty three out of 24 fermented milks showed more than 10^6 CFU/g at the time of purchase on the both tested media (Figure 2.1 a, b, c and d). The counts are average of duplicate.

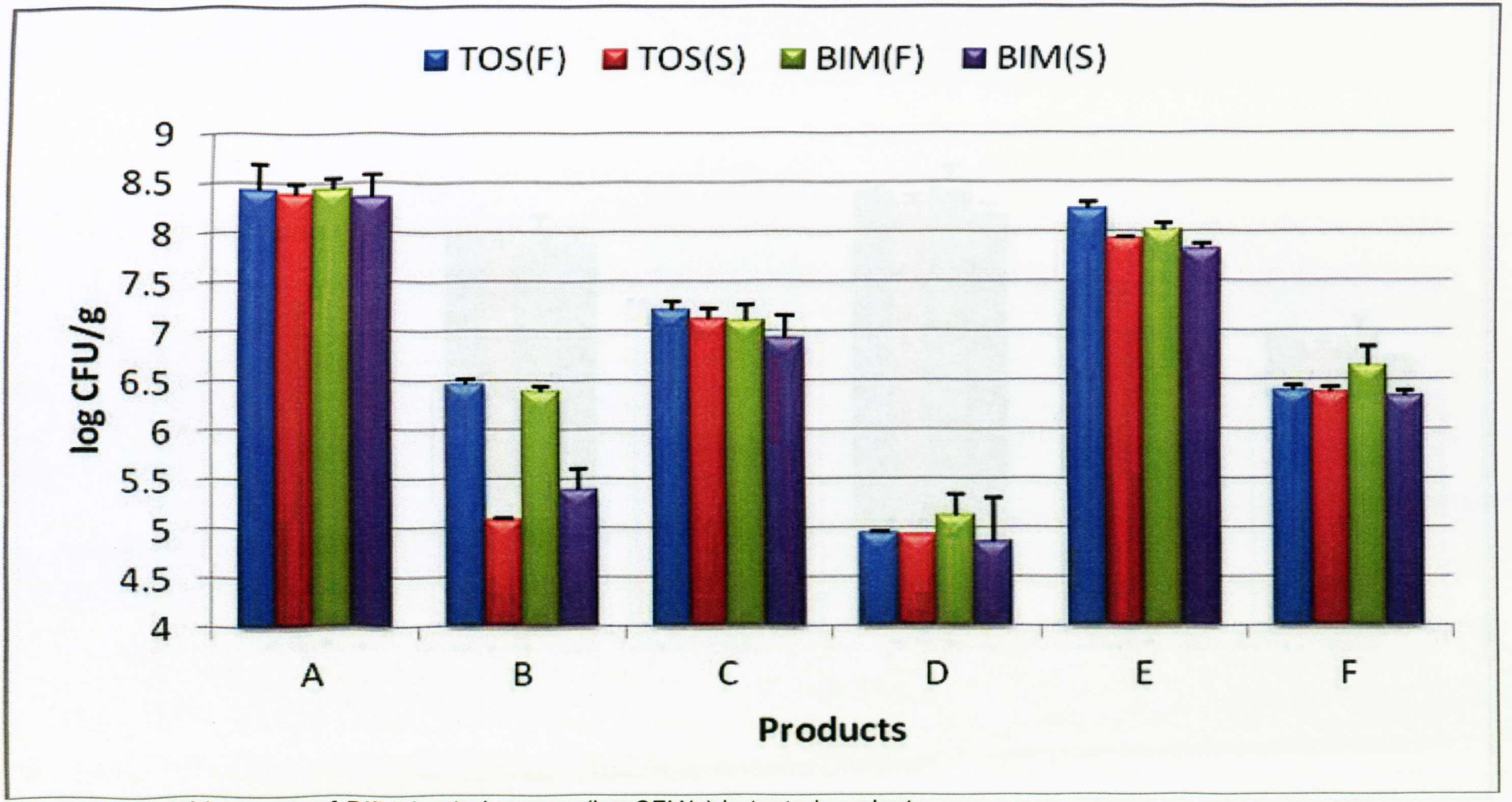

Figure 2.1a Viable counts of *Bifidobacterium* spp. (log CFU/g) in tested products.
F: fresh products, S: stored products, Error bars are standard deviations (n=4),
For number of storage days of each products see Table 2.1

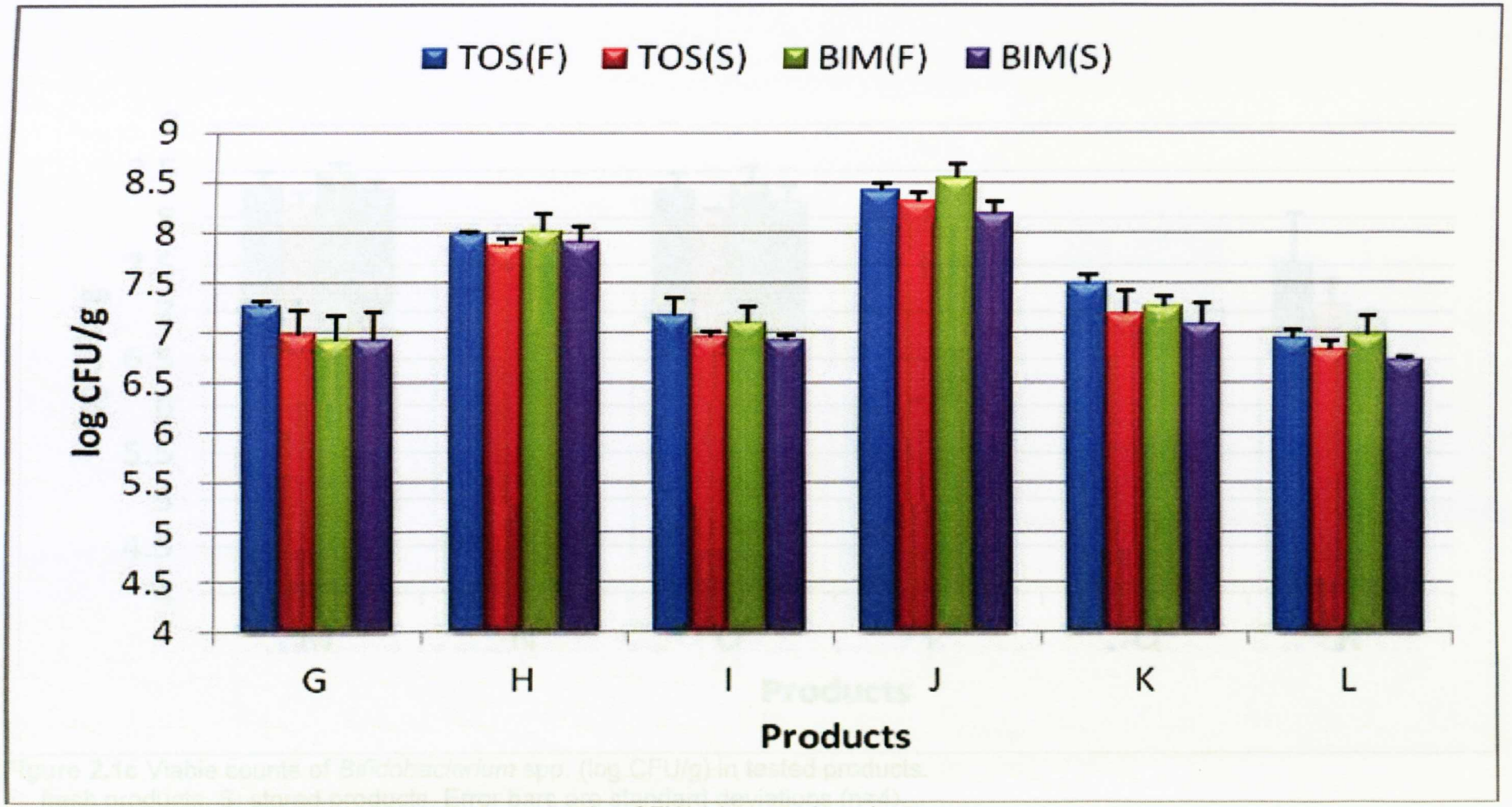

Figure 2.1b Viable counts of *Bifidobacterium* spp. (log CFU/g) in tested products.
F: fresh products, S: stored products, Error bars are standard deviations (n=4).
For number of storage days of each products see Table 2.1

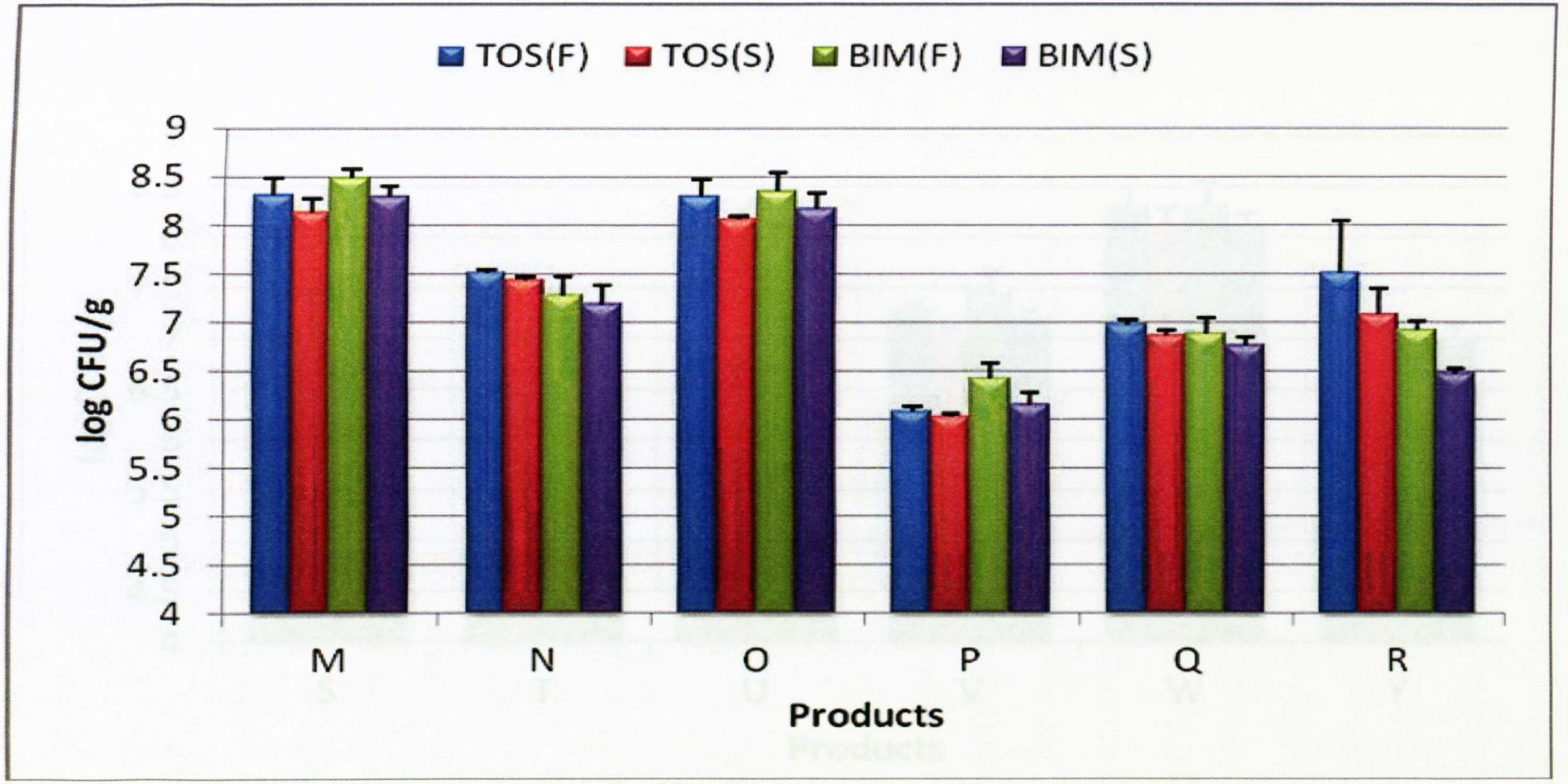

Figure 2.1c Viable counts of *Bifidobacterium* spp. (log CFU/g) in tested products.
F: fresh products, S: stored products, Error bars are standard deviations (n=4).
For number of storage days of each products see Table 2.1

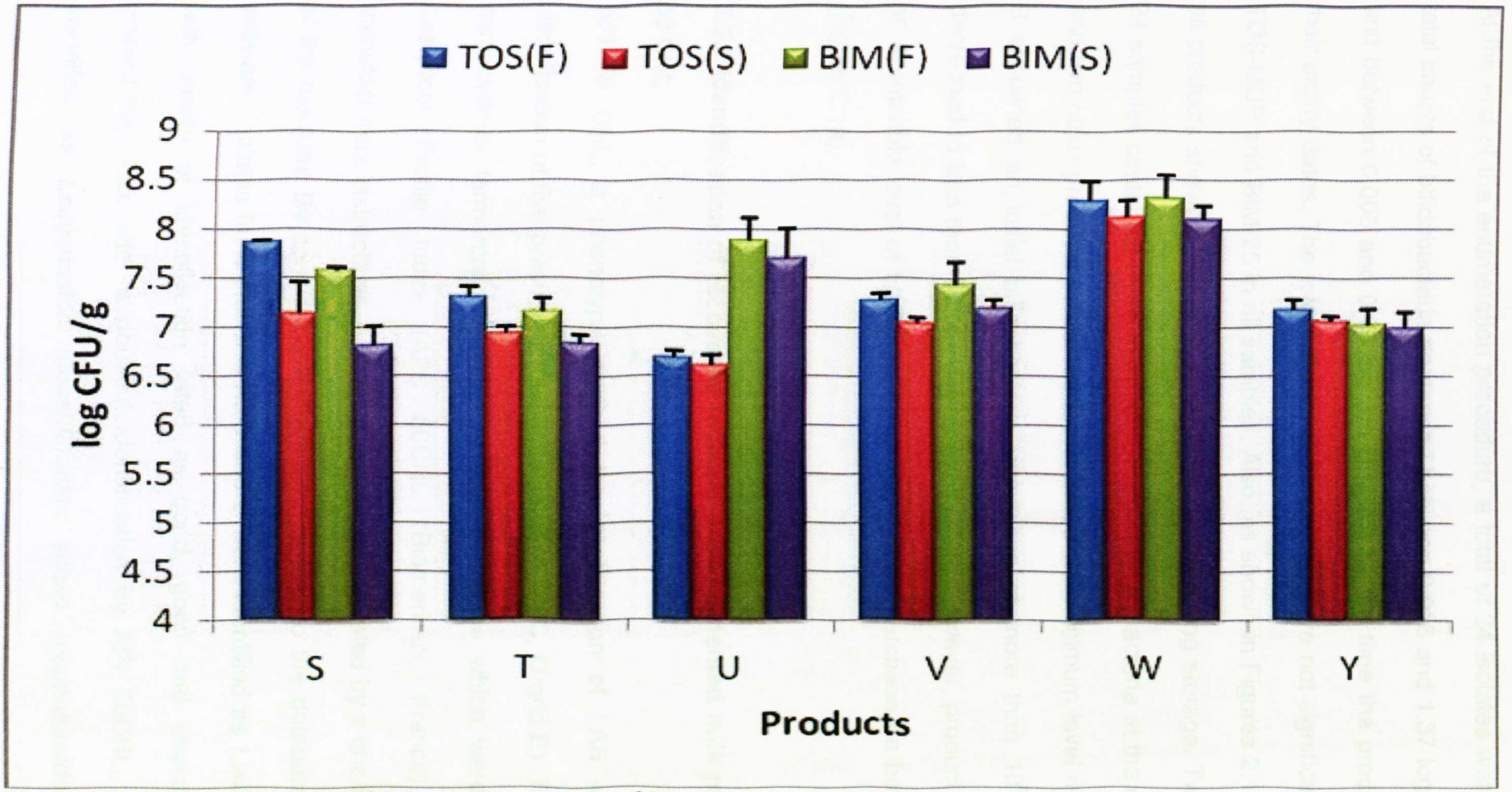

Figure 2.1d Viable counts of *Bifidobacterium* spp. (log CFU/g) in tested products.
F: fresh products, S: stored products, Error bars are standard deviations (n=4).
For number of storage days of each products see Table 2.1

At the end of the enumeration procedure, a total of 24 isolates were obtained. The total counts of bifidobacteria decreased between 0.018 and 1.37 log (on TOS-MUP) and between 0.006 and 0.99 log (on BIM-25) by the time the products reached to their expiry dates. The total bifidobacterial counts were not significantly different on TOS-MUP and BIM-25 in all samples. Also, as shown in Figures 2.1 (a, b, c and d), all products showed a decline in viable numbers during storage. Twenty two out of 24 samples contained more than 10^6 CFU/g of bifidobacteria at the end of shelf-life, and two other products contained levels below the minimum level required. Product B contained an initial bifidobacteria population of more than 10^6 CFU/g, which decreased to less than 10^5 CFU/g on expiry date, however, product D did not show an acceptable level of bifidobacteria at the time of purchase on both tested media (Figure 2.1a).

2.3.2 Identification of recovered isolates from fermented milk products by API 50 CHL

API 50 CHL, a phenotypic method for identification of LAB was applied for identification of five presumptive bifidobacteria (A, B, C, D and E). Table 2.2 reveals carbohydrate fermentation pattern of tested isolates which were performed by Analytical Profile Index (API) 50CHL (Biomerieux, France) following the manufacturers instructions. A positive result was indicated by a change in the colour of the medium. By comparing fermentation profiles to the database using the API software, isolates from products A, C and E were identified as *Lactobacillus brevis* with quality of identification rated as good, good and unacceptable profile, respectively. Also with a doubtful identification by API 50CHL, product B was identified as *Leuconostoc mesenteroides* subsp *mesenteroides*. API 50CHL

identified product D isolate as *Streptococcus thermophilus* with a very good quality of identification.

Table 2.2 Carbohydrate utilisation profiles API 50 CHL of 5 presumptive bifidobacteria isolated from commercial dairy products

Substrate	**A**	**B**	**C**	**D**	**E**
Glycerol	–	–	–	–	–
Erythritol	–	–	–	–	–
D-Arabinose	–	–	–	–	–
L-Arabinose	+	+	+	–	+
D-Ribose	+	+	+	–	+
D-Xylose	–	+	+	–	+
L-Xylose	–	–	–	–	–
D-Adonitol	–	–	–	–	–
Methyl-βD-Xylopyranoside	–	+	–	–	–
D-Galactose	+	+	–	–	–
D-Glucose	–	+	+	+	+
D-Fructose	–	+	–	–	–
D-Mannose	–	+	–	–	–
L-Sorbose	–	–	–	–	–
L-Rhamnose	–	–	–	–	–
Dulcitol	–	–	–	–	–
Inositol	–	–	–	–	–
D-Mannitol	–	–	–	–	–
D-Sorbitol	–	–	–	–	–
Methyl-αD-Mannopyranoside	–	–	–	–	–

Table 2.2 *continued*: Carbohydrate utilisation profiles API 50 CHL of 5 presumptive bifidobacteria isolated from commercial dairy products

Substrate	A	B	C	D	E
Methyl-αD-Mannopyranoside	+	+	+	–	+
N-AcetylGlucosamine	–	+	–	–	–
Amygdalin	+	+	+	–	+
Arbutin	–	+	–	–	–
Esculin	+	+	+	–	+
Salicin	–	+	+	–	+
D-Cellobiose	–	+	+	–	–
D-Maltose	+	+	+	–	+
D-Lactose	+	+	+	+	–
D-Melibiose	+	+	+	–	+
D-Sucrose	+	+	+	+	+
D-Trehalose	–	+	–	–	–
Inulin	–	–	–	–	–
D-Melezitose	–	–	–	–	–
D-Raffinose	+	+	+	–	+
Amidon	–	–	–	–	–
Glycogen	–	–	–	–	–
Xylitol	–	–	–	–	–
Gentiobiose	+	+	+	–	+
D-Turanose	–	+	+	–	+
D-lyxose	–	–	–	–	–
D- tagatose	–	–	–	–	–

Table 2.2 *continued*: Carbohydrate utilisation profiles API 50 CHL of 5 presumptive bifidobacteria isolated from commercial dairy products

Substrate	A	B	C	D	E
D-Fucose	–	–	–	–	–
L-Fucose	–	–	–	–	–
D-Arabitol	–	–	–	–	–
L-Arabitol	–	–	–	–	–
Potassium Gluconate	–	–	–	–	–
Potassium 2-cetogluconate	–	–	–	–	–
Potassium 5-cetogluconate	–	–	–	–	–

2.3.3 Identification of recovered isolates from fermented milk products by API Rapid ID 32 A

API Rapid 32 A was applied to four isolates (A, B, C and D) and the results are presented in the Table 2.3.

Three out of four isolates (A, B and C) were identified as *Bifidobacterium* spp. However, in case of isolate D, with unacceptable quality of identification, no result was obtained. To identify the isolate at species level, some of the fermentation profiles by API 50 CHL needed to be combined with rapid ID 32 A results. The identification results of these isolates are summarised in Table 2.4.

After including the complementary test which was resulted from API 50 CHL, isolate B matched three out of four suggested species namely; *B. adolescentis*, *B. breve* and *B. infantis* which is unusual and raise doubt about the suitability of such procedure.

Table 2.3 API Rapid ID 32 results of 4 presumptive bifidobacteria isolated from commercial dairy products

Substrate	A	B	C	D
Urea	–	–	–	+
L-arginine	+	–	–	–
4-nitrophenyl- αD-galactopyranoside	+	+	–	–
4-nitrophenyl- β D-galactopyranoside	+	+	+	+
4-nitrophenyl- β D-galactopyranoside-6-phosphate-2CHA	–	–	–	–
4-nitrophenyl- αD-glucopyranoside	+	+	+	–
4-nitrophenyl- β D-glucopyranoside	+	+	+	–
4-nitrophenyl- αL-arabinofuropyranoside	+	+	+	–
4-nitrophenyl- β D-glucoronide	–	–	–	–
4-nitrophenyl- N-acetyl- βD-glucosaminide	–	–	–	–
D-mannose	–	+	+	+
D-raffinose	+	+	+	+
Potassium nitrate	–	–	–	–
L-tryptophan	–	–	+	+
2-naphthyl-phosphate	–	–	+	+
L-arginine- β-naphthylamide	+	+	+	+
L-proline- β-naphthylamide	+	+	+	+
L-leucyl-L-glycine- β-naphthylamide	+	+	+	+
L-phenylalanine- β-naphthylamide	+	+	+	+
L-leucine- β-naphthylamide	+	+	+	+
Pyroglutamic acid β-naphthylamide	–	+	–	–
L-tyrosine- β-naphthylamide	+	+	+	+
L-alanyl-L-alanine- β-naphthylamide	+	+	+	+
L-glycine- β-naphthylamide	+	+	+	+
Glutamic acid	–	–	–	+

Table 2.3 *continued*: API Rapid ID 32 results of 4 presumptive bifidobacteria isolated from commercial dairy products

Substrate	A	B	C	D
4-nitrophenyl-αL-fucopyranoside	–	–	–	–
L-histidine- β-naphthylamide	+	+	+	+
L-glutamyl-L-glutamic acid- β-naphthylamide	+	+	+	+
L-serine- β-naphthylamide	+	+	+	+

Table 2.4 Identification results of 4 presumptive bifidobacteria isolated from commercial dairy products with Rapid ID 32 A

Product	Identified by Rapid ID 32 A	Quality of identification	Comments
A	*Bifidobacterium* spp.	Unacceptable profile	-
B	*Bifidobacterium* spp.	Good identification	Perform Complementary test
C	*Bifidobacterium* spp.	Unacceptable profile	-
D	None	Unacceptable profile	-

2.3.4 Identification of recovered probiotic isolates from fermented milk products by partial sequencing of 16s ribosomal RNA gene

Table 2.5 presents the results of identification which have been acquired using 16S rRNA gene partial sequencing of bifidobacteria strains, isolated from fermented milk products.

Sequences from all isolates were more than 97% similar to sequences held in GenBank for microorganisms classified as *B. animalis* subsp. *lactis* (Table 2.5).

Table 2.5 Identification of probiotic bifidobacteria isolated from commercial fermented milks by sequence analysis of 16S rRNA V1–V2 gene region

Product	Claimed bifidobacteria	Identified by 16S rRNA gene sequencing	Similarity
A	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
B	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%
C	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
D	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
E	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%
F	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
G	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
H	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	97%
I	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%
J	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
K	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%
L	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
M	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	100%
N	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
O	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%
P	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%
Q	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%
R	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	97%
S	BB12	*B. animalis* subsp. *lactis*	98%
T	*Bifidobacterium* spp	*B. animalis* subsp. *lactis*	99%
U	***B. longum***	*B. animalis* subsp. *lactis*	96%
V	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	98%

Table 2.5 *continued*: Identification of probiotic bifidobacteria isolated from commercial fermented milks by sequence analysis of 16S rRNA V1–V2 gene region

Product	Claimed bifidobacteria	Identified by 16S rRNA gene sequencing	Similarity
W	***B. longum***	*B. animalis* subsp. *lactis*	98%
Y	*Bifidobacterium* spp.	*B. animalis* subsp. *lactis*	99%

According to BLAST results, all isolates from fermented milk products were more than 97% identical to sequences in GenBank for *B. animalis* subsp. *lactis,* therefore, these isolates are identified as *B. animalis* subsp. *lactis*. Yogurt U and W claimed to contain *B. longum,* but the isolated colonies from these two products were identified as *B. animalis* subsp. *lactis* too.

2.3.5 Differentiation of isolates by rep-PCR

All commercial isolates and three different type strains (*B. bifidum* NCTC13001, *B. breve* NCTC11815 and *B. longum* NCTC11818) (T1, T2 and T3, respectively) were differentiated by rep-PCR (repetitive extragenic palindromic polymerase chain reaction). As Figure 2.2 and 2.3 show, the pattern of the bands of all isolates exhibited the same rep-PCR banding profile and were characterized by several constant bands with molecular sizes between 4000 and 400 bp. However, all three type strains (T1, T2 and T3) showed different bands profile, which were not the same as tested isolates.

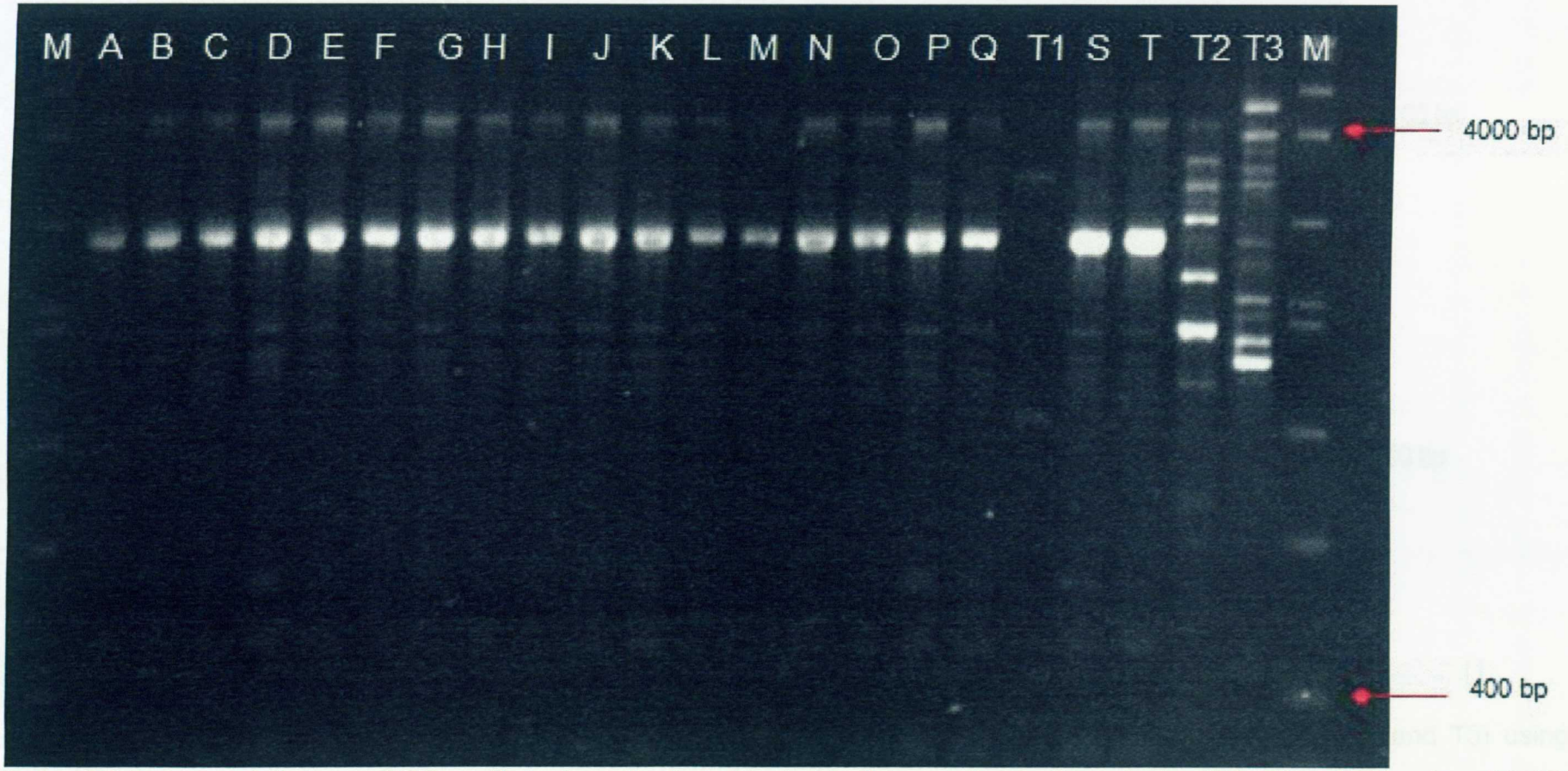

Figure 2.2 Representative rep- PCR profiles of a selection of tested isolates and pure type strains (T1, T2 and T3) using of GTG5 primers
M: DNA marker,T1: *B. bifidum* NCTC13001, T2: *B. breve* NCTC11815, T3: *B. longum* NCTC11818
All other isolates are *B. animalis* subsp. *lactis*

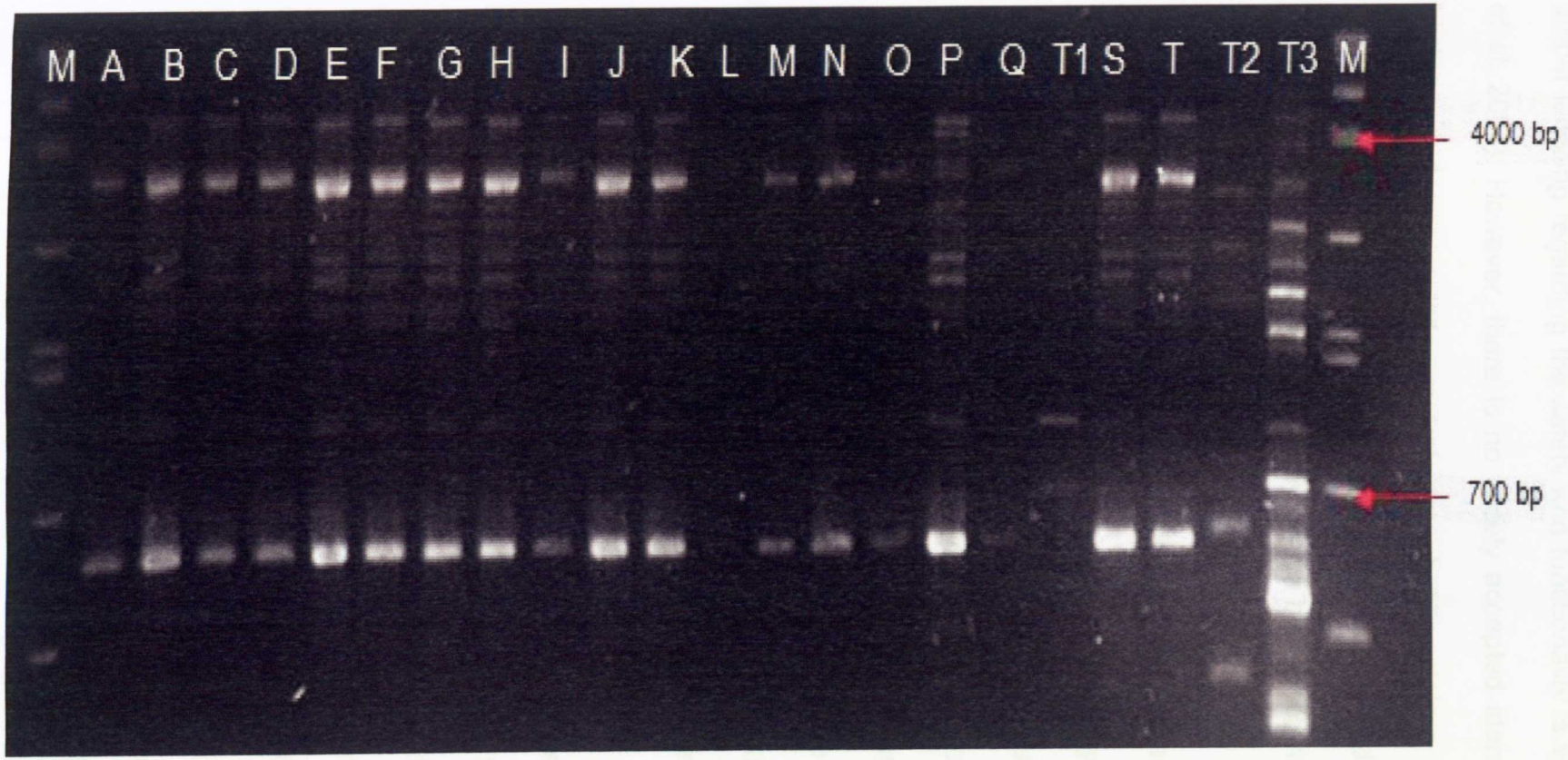

Figure 2.3 Representative rep- PCR profiles of a selection of tested isolates and pure type strains (T1, T2 and T3) using of BOXA1R primers
M: DNA marker, T1: *B. bifidum* NCTC13001, T2: *B. breve* NCTC11815, T3: *B. longum* NCTC11818
All other isolates are *B. animalis* subsp. *lactis*

2.4 DISCUSSION

With respect to sharp increase in consumption of probiotic products in the world, proper labelling regarding the safety and functionality is very important (Gueimonde *et al.* 2004). However, there is no widely accepted standard for level of probiotic bacteria and no or little control over health claims which can be made by manufacturers. However, to have any health benefits and functional properties, it is suggested that at least 10^6-10^7 CFU/g of live bifidobacteria should be present in the probiotic product at the expiry date.

Bifidobacteria are usually counted on selective medium and we have evaluated two of these media, BIM-25 and TOS-MUP, and their reliability was verified by molecular techniques. We found that BIM-25 and TOS-MUP media are both reliable in their ability for accurate enumeration of bifidobacteria in fermented milk. TOS-MUP, however, has the advantage of being simple (containing only one antibiotic) and easier to prepare. In addition, it has proved to be more selective than BIM-25 as some LAB, such as *Streptococcus thermophilus,* could still grow on BIM-25 (Munoa and Pares 1988), which are relatively difficult to differentiate from colonies of bifidobacteria.

These findings showed that at the time of purchase almost all tested products (apart from product D) contained the minimum therapeutic level of 10^6 CFU/ml, but afterward, a reduction in viable counts of bacteria in tested products was noticed and not all were in the range of minimum value required for probiotic products.

Some researchers have also reported losses of viable bifidobacteria between 0 and 3 logs during the shelf-life of fermented milk (Iwana *et al.* 1993; Medina and Jordano 1994; Nighswonger *et al.* 1996; Dave and Shah 1997; Micanel *et al.* 1997; Rosenthal and Bernstein 1998; Schillinger 1999; Shin *et al.* 2000; Gilliland *et al.* 2002).

Among the products tested, the poorest bifidobacteria survival was for yogurt D, which showed the lowest count of bifidobacteria. However, products B and D did not fulfil the minimum requirement for level of probiotic. These variations in the viable cell count could be attributed to the lack of resistance of strains to some factors, such as pH, oxygen and additives in the food as a probiotic vehicle (Dave and Shah 1997; Shah 2000). Another valid reason could be the interaction of bifidobacteria with other auxiliary and complementary starter cultures. *Bifidobacterium* spp. are not able to produce a palatable taste and most manufacturers prefer to use a secondary starter culture to cover this failure. As a result there might be an antagonistic effect which could contribute to the low survivability of *Bifidobacterium* spp. in some fermented milk products. Therefore, use of proper carrier for probiotic delivery and greater initial inoculums levels could be helpful for preventing the probiotic loss during shelf-life.

From these results it appears that, there is a greater chance for achieving higher numbers of probiotic when these products consumed earlier than their expiry date and these finidings are in agreement with Jayamanne and Adams (2006).

This study also showed that API 50 CHL alone is an unreliable method for identification of *Bifidobacterium* spp. because of poor identification obtained by the software and the fact that bifidobacteria is not included in the list of organisms that can be identified by this kit as it is intended for identification of lactobacilli. It can, however, be used to assess the carbohydrate fermentation profiles and thus differentiate/characterise bifidobacteria. However, along side API 50 CHL, rapid ID 32 A might be a useful method for identification of bifidobacteria, but not at species levels. Collado *et al.* (2006) reported that the identification of strains with commercial kit API 50 CHL is ambiguous for differentiation of bifidobacteria and they were not

able to identify the isolates at species level by API 50 CHL kit. However, Vlkova *et al.* (2004) characterised bifidobacteria by using of API 50 CHL and RAPID ID 32 A kits. Screening of 24 isolates with PCR-based methods revealed that *B. animalis* subsp. *lactis* was the only recovered bifidobacterial species from tested fermented milk products. Due to superior technological properties *B. animalis* subsp. *lactis* is used in most fermented dairy products and other *Bifidobacterium* species seem to be less suitable for these purposes (Reuter *et al.* 2002; Gueimonde *et al.* 2004).

Ventura and Zink (2003) demonstrated that *B. lactis* and *B. animalis* belong to the same species and suggested that they could be unified as *B. animalis* subsp. *lactis*. There are some claims that *B. animalis* subsp. *lactis* is of animal origin (Ishibashi and Shimamura 1993) but it is isolated from fermented milk product (Meile *et al.* 1997) and its origin is not clear.

Some researchers have claimed that rep-PCR genomic fingerprinting is able to differentiate bacteria at species, subspecies and even strain level (Rademaker *et al.* 2005). In addition, many repetitive sequence primers are available, and a different primer could generate different band patterns, which would assist identification. Masco *et al.* (2003) evaluated the ability of primer BOXA1R, GTG 5, REP1R-I / REP2-I and ERIC1R/ERIC2 to discriminate between the genotypic characteristics of *Bifidobacterium* species, and found that BOXA1R has the highest discriminatory power and can generate a wide range of molecular weight amplicons. In this project, seven different DNA patterns for tested isolates were obtained with rep-PCR. However, differentiation of tested isolates by rep-PCR with two different primers (GTG-5 and BOXA1R) could not reveal relative genetic differences between the tested isolates from fermented milk products, and all isolates had the same band profile. The results of identification showed that majority of tested products were

inadequately labelled (only refering to genus or giving commercial codes) and also two of them (U and W) were not matched with the real identity of the incorporated strains. Despite earlier reports regarding mislabelling of the probiotic products, this study indicates that the situation still needs to be improved.

Generally speaking and compared to human origin of bifidobacteria, it has been established that *B. animalis* subsp. *lactis* is less sensitive to stressful conditions (e.g. exposure to oxygen, acid and bile) encountered during the production of fermented milk and/or the passage through upper gastrointestinal tract. Therefore, *B. animalis* subsp. *lactis* is commonly added to commercial products due to its better tolerance to the above mentioned conditions. Such practice has been reported from around the world including North America and Europe (Prasad *et al* 1998; Masco *et al* 2005).

From safety, consumer rights and quality assurance point of view as well as the future of probiotic industry it is strongly recommended to use correct labelling.

During the course of this study it was found that in many cases the names of probiotic organisms on product labels are incomplete (do not identify species e.g. "bifidobacteria" or "*Bifidobacterium*"), invalid ("Bifid" or "Bifidus") or misidentified (*B. animalis* subsp. *lactis* instead of *B. longum*). Although it is mentioned elsewhere in this thesis that the propiotic characteristics are strain dependent, at the very least, it is expected that the added organism should be accurately named on product labels according to its species..

2.5 CONCLUSION

The potential of BIM-25 and TOS-MUP as selective medium for isolation of *Bifidobacterium* spp. from commercial fermented milks was examined. BIM-25 medium can be used successfully as a selective medium for the isolation of *Bifidobacterium* spp. from fermented products if a special attention was given to appearance of colonies on the BIM-25, but TOS-MUP is the medium of choice.

Bifidobacteria is not included in the list of organisms that can be identified by API 50 CHL. Hence, this method by itself is not a reliable method for recognition of *Bifidobacterium* spp. but in combination with rapid ID 32 A it might be a helpful technique for discrimination or characterisation of bifidobacteria at genus level, even so only a limited number of *Bifidobacterium* spp. is listed in the API database and therefore, it is not the recommended method for identification of bifidobacteria. The total viable count of *Bifidobacterium* spp. in most tested products remained over 10^6 CFU/g at the end of shelf-life. This could mainly be due to technical processing and strain selection improvement. Also, all *Bifidobacterium* spp. were isolated from commercial fermented milks were identified as *B. animalis* subsp. *lactis* by analysis of partial sequences of the 16S rRNA gene. However, differentiation of all isolates by rep-PCR with two different primers did not show any difference among tested isolates.This could be due to high degree similarity in size, organisation and sequence of genome between the strains of this subspecies.

Chapter 3

In vitro tolerance assessment of isolates to acid and bile salt

3.1 INTRODUCTION

Viability is one of the most important criteria in determination of probiotic properties, otherwise any therapeutic properties of probiotic food would not exist. The survivability of probiotic bacteria in a food matrix could be affected by several factors, such as strain of incorporated probiotic bacteria, the adjunct starter cultures used and any interaction between the species present, fermentation time and storage conditions, pH of the fermented milk, NaCl and sugar concentration (osmotic pressure), milk solids content, availability of nutrients, the presence of hydrogen peroxide, dissolved oxygen content (especially for *Bifidobacterium* spp.) (Shah and Lankaputhra 1997; Dave and Shah 1998). In addition, an important criterion for selection of a strain as probiotic is resistance to harsh conditions in the gastrointestinal tract (GIT) (Goldin and Gorbach 1989).

The primary function of the digestive system is to break down food components. Digestion breaks large food molecules into smaller molecules (amino acids, fatty acids and glucose) that can be absorbed across the wall of the GIT and into the circulatory system for dissemination around the body.

Probiotic cultures meet acidic conditions during transition through stomach. Therefore, probiotic functionality would be related to tolerance of low pH.

The average estimated remaining time (entry to release) for probiotic bacteria in the stomach is about 1.5 h (Berrada *et al.* 1991). The stomach has a variable pH environment ranging from 1.5-3.5 depending on fasted or fed states (Vandamme *et al.* 2002). This low pH environment poses the first hurdle to the survival of probiotic bacteria in their journey through the GIT. Therefore, an effective probiotic must be able to withstand the pressures exerted by the stomach's harsh environment (Lankaputhra and Shah 1995).

Another obstacle encountered by probiotic bacteria during the journey to the lower intestinal tract is bile salts, which are secreted in the small intestine. The anatomy of human GIT is shown in Figure 3.1. In order to break down the large macromolecules of lipids, bile is released into the duodenum from the gall bladder and it facilitates the emulsification and absorption of lipids and plays an important role in further breakdown of liquefied food, which is then absorbed in the jejunum and ileum (Sanchez *et al.* 2007).

Bile salts are able to destroy bacterial cell membranes and reduce the survival of bacteria. Bacterial cell walls are composed of lipid bi-layers, which can be dissolved by bile acids, thus resulting in the death of the bacteria (Gilliland *et al.* 1984; Gilliland 1987). Depending on its concentration, bile salts can inhibit the growth of Gram positive bacteria, but its suppressive effect against Gram negative bacteria, such as *Escherichia coli* is limited (Ding and Shah 2007). Thus, probiotic bacteria should be able to withstand acid and bile salts in the upper digestive tract (Zhu *et al.* 2000; Hirayama and Rafter 2000). Deconjugation of bile salts in the intestine is a vital activity carried out by LAB and bifidobacteria, and bile salt hydrolase (BSH) is identified as the key enzyme for hydrolysis of bile salts into amino acids, such as taurine and glycine and deconjugated bile salts (Tanaka *et al.* 2000).

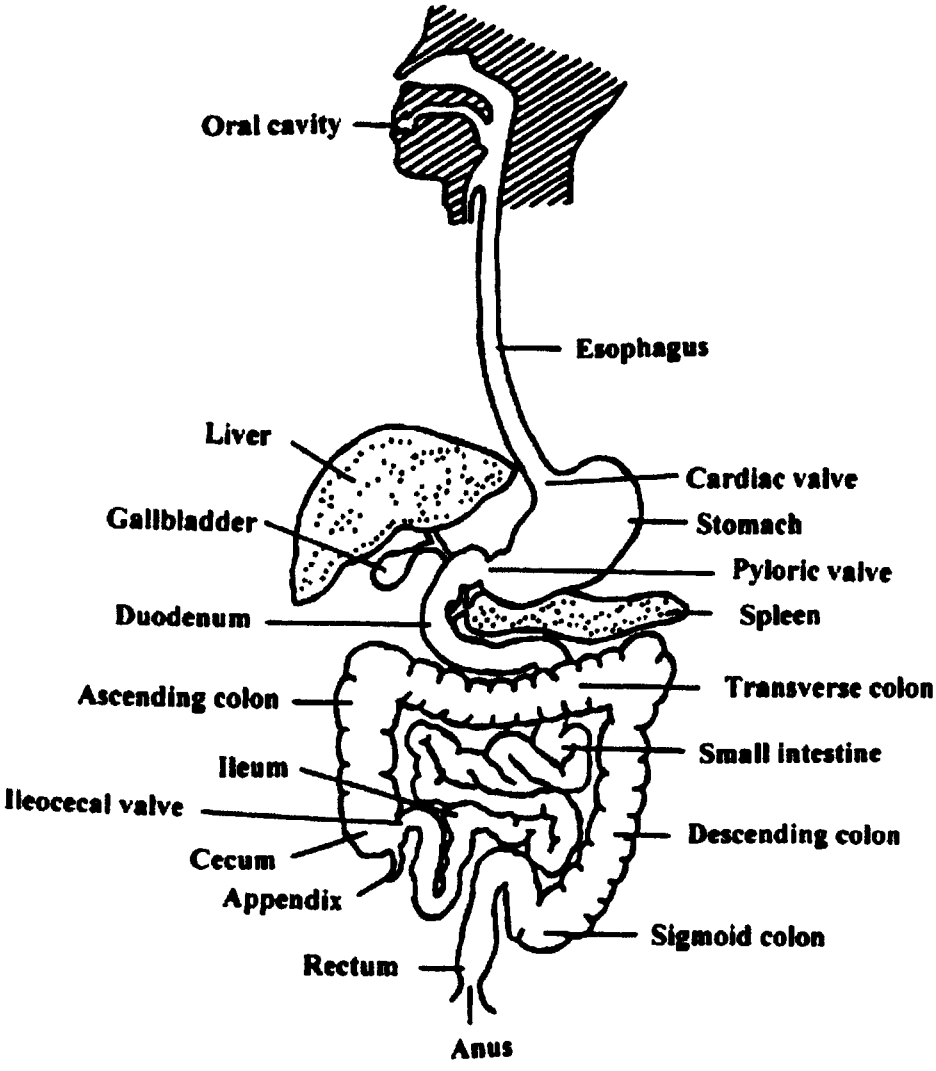

Figure 3.1 Anatomy of human gasterointestinal tract (Adapted from Vandamme *et al.* 2002)

One useful tool in the selection of a probiotic strain would be *in vitro* tests to determine the survivability of these bacteria in the upper gastrointestinal conditions. Clinical studies might not be applicable to screening of survival, due to high costs and ethical and safety regulations. Instead *in vitro* studies are recommended which are much easier and more feasible (Ritter *et al.* 2009).

Therefore, in this research viability of some *Bifidobacterium* spp. at different levels of pH and in presence of different concentrations of bile salt was determined. Also in a further step, the simulation of GIT was carried out using stirred batch culture system.

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms

Out of 24 identified isolates of probiotic bifidobacteria, 10 were selected to be screened for functional properties. These 10 isolates were carefully chosen to include all major brands of probiotic products, products with different levels of fat content, products with different types of milk and fermented and non fermented products. These isolates were used throughout.

The bacteria which were tested in this experiment were as follows:

a) Ten *Bifidobacterium* spp. which were isolated from yogurt and other fermented milk products and were identified as *B. animalis* subsp. *lactis* (B1 to B10).

b) Two commercial cultures of *B. animalis* subsp. *Lactis,* Bb-12 and vital probiotic yogurt were kindly provided by two starter culture suppliers, Chr. Hansen and Danisco, respectively (B11 and B12).

c) Three different type strains of bifidobacteria purchased from National Collection of Type Cultures (Salisbury, UK) *B. longum* NCTC11818, *B. breve* NCTC11815, *B. bifidum* NCTC13001(B13, B14 and B15, respectively).

3.2.2 *In vitro* assessment of acid tolerance

Acid resistance of the above bacteria was studied in phosphate buffered saline (PBS) (BR0014G, Oxoid) with pH of 7 as control and PBS with adjusted pH to 4, 3 and 2 using 2M hydrochloric acid (37%, Fisher Scientific, UK). It should be stated that acid tolerance at pH 2.0 was determined with and without 3 g/l of pepsin (P7000, Sigma, Poole, UK).

Acid resistance test was performed according to the method of Prasad *et al.* (1999) and Matto *et al.* (2006). Overnight colonies of isolates grown on RCA were harvested

and diluted in MRD to make a standard suspension (final concentration of ca 10^7 CFU/ml). For this purpose, after two consecutive streakings of culture, individual colonies from RCA were suspended in 1 ml of MRD (stock solution). The working inoculum suspension was prepared by diluting stock inoculum in 5 ml MRD in glass boiling tube using a calibrated sensititre nephelometer (TREK, Diagnostic Systems Ltd., UK). The final concentration of cells per tube was adjusted to 0.5 MacFarland standards (~10^7-10^8 CFU/ml). This microbial suspension was used as inoculum for further experiments. An aliquot of each standardised inoculum (0.5 ml) was inoculated into control tube containing 5 ml PBS pH 7) and experimental tubes containing 5 ml of PBS (adjusted to pH values of 4, 3 and 2). The tubes were incubated at 37 °C for up to 3 h in anaerobic conditions to simulate the environment of the stomach. The resistance to acid was assessed by enumeration of incubated samples every 0.5 h on RCA, and the results were recorded after anaerobic incubation at 37 °C and expressed as log CFU/ml.

3.2.3 *In vitro* assessment of bile tolerance

Bile salt tolerance of isolates was studied in MRS broth (control) and MRS broth containing 0.5, 1, 1.5 and 2% w/v bile salt (Ox-bile, B3883, Sigma UK), which represented the physiological condition of small intestine (Collado and Sanz 2007). The tubes were incubated at 37 °C for 1, 2, 3, 6 h which mimics the transit time in small intestine, as described by Prasad *et al.* (1999). All inocula were prepared as described in section 3.2.2 and inoculated into control tube (MRS) and experimental tubes (with added bile salts). Viable cells were enumerated on RCA after 48 h incubation at 37 °C in anaerobic cabinet and expressed as log CFU/ml.

3.2.4 *In vitro* viability assays in the gastrointestinal tract model

In order to study the resistance of the isolates to upper GIT condition under a more realistic condition, the tolerance of *B. animalis* subsp. *lactis* (B6), which showed good resistance to both pH and bile acid in previous experiments, was studied in a sequential model, involving exposure first to gastric conditions, followed by exposure to conditions in the different parts of the intestine.

In this part of the experiment, a batch fermentation system (Figure 3.2 and 3.3) was used. The system consisted of sterile, magnetically stirred, water jacketed batch fermentation vessels (300ml, Soham Scientific, UK). The temperature was maintained at 37°C by means of a circulating water bath (Grant, type GD 120, UK) and pH was screened in each vessel using a pH meter (FerMac 260, Electrolab, UK) which was set to correspond to each fermentation vessel.

The fermentation vessels were filled with 150 ml PBS and autoclaved at 121°C for 15 min.

The vessels were continuously sparged with O_2-free N_2. Fermentations were run over a period of 10.25 h and samples were obtained at the start of the experiment and then at the end of the transit/incubation time through each section.

The conditions in the vessels were set according to the method described by Gbassi *et al.* (2011). Five compartments of upper GIT with their specific pH and transit (incubation) time was studied. The conditions of GIT were simulated as shown in Table 3.1. Three glass vessels were used to mimic the GIT condition and one vessel served as control which contained PBS at pH 6.5.

Figure 3.2 A single batch fermentation system used for studying the resistance to bile and acid

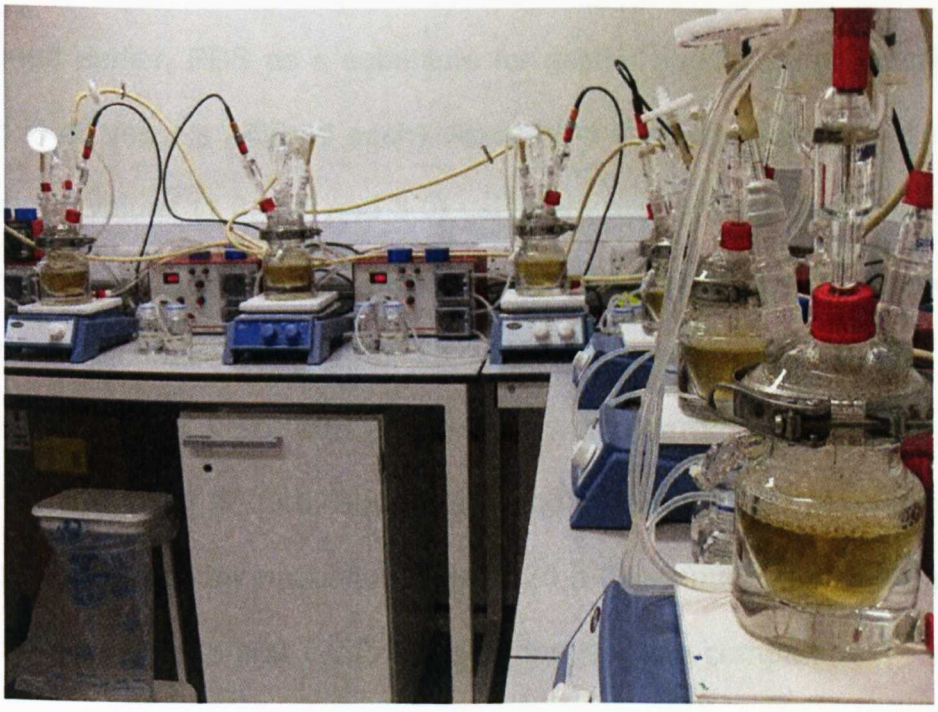

Figure 3.3 The series of batch fermentation system used for studying the resistance to bile and acid

Table 3.1 *In vitro* experimental condition in the gastrointestinal tract model

		Stomach	Duodenum	Jejunum	Ileum	Cecum
Incubation/ transit time		2h	0.25h	3h	4h	1h
pH values	No food	1.8	4.0	6.0	7.0	7.0
	Standard food	2.5	5.5	6.5	7.5	7.5
	Excess food	3.0	6.0	7.0	8.0	8.0

Adapted from Gbassi *et al.* (2011)

As mentioned earlier, PBS as a substitute for gastric and intestinal secretions was introduced in all vessels (150 ml each). Apart from control vessel, pH was adjusted to stomach pH values (1.8, 2.5 and 3.0) using 2M hydrochloric acid. Inoculum suspention (%1 v/v) and pepsin (3 g/l) were added into the gastric fluid in the begining of stomach transit/incubation time. After 2h, pH was slowly increased to the values of duodenum (4.0, 5.5 and 6.0) with 2M sodium hydroxide (Fisher Scientific, UK) and also pancreatin (10 g/l) (P7545, Sigma, UK) and bile salts (3 g/l) were added at the begining of the duodenum incubation. After 0.25 h duodenum incubation, pH values were changed to 6.0, 6.5 and 7.0 to simulate the jejunum compartment and held for 3h. These conditions followed by those of ileum (7.0, 7.5 and 8.0) and incubation continued for 4 h and cecum (7.0, 7.5 and 8.0) where the incubation only lasted for 1 h.

Sampling of gastric and intestinal fluids was carried out at the end of average transit (incubation) time. One ml of each sample was suspended in 9 ml of MRD and the

uniform suspension was decimally diluted, and 25 µl of all dilutions was spread on to quartered RCA plates in duplicate. The plates were incubated at 37 °C for 48h an-aerobically. The colony forming units per ml (CFU/ml) of the sample were calculated and log CFU/ml was reported.

3.3 RESULTS

Probiotic survival during passage through GIT is an essential criterion for their selection. Therefore, acid resistance and bile tolerance, as prerequisities for satisfactory activity as probiotics, was studied.

3.3.1 Acid resistance

The *in vitro* survival studies of probiotic bacteria may help to clarify *in vivo* survival in GIT conditions. Bifidobacteria strains were examined for their survival at low pH. Different pH values in this study were selected to represent the pH range of the human GIT. As Figure 3.4 shows, the viability of all isolates and also commercial pure starter cultures was maintained after 3 h of exposure to pH 4, however, only two out of three type strains showed fair survivability in this condition (up to an hour for *B. longum* and *B. breve*). The number of viable *B. bifidum* fell to undetectable level (log 1.6) after exposure at pH 4. At pH 3, all tested isolates showed good resistance even after 3 h but *B. bifidum*, *B. longum* and *B. breve* declined to an undetectable level after half an hour in this pH (Figure 3.5).

According to the results at pH 2, isolates and also commercial cultures were able to withstand this pH for at least an hour. After 2 h, 5 out of fifteen isolates (B1, B2, B5, B6 and B8) retained their viability but others showed a significant reduction in counts. However after 3 h of incubation at pH 2, the number of studied isolates decreased to undetectable level. *B longum, B. breve* and *B. bifidum* died very rapidly at the beginning of the incubation at pH 2 and were below the detection limit (Table 3.2).

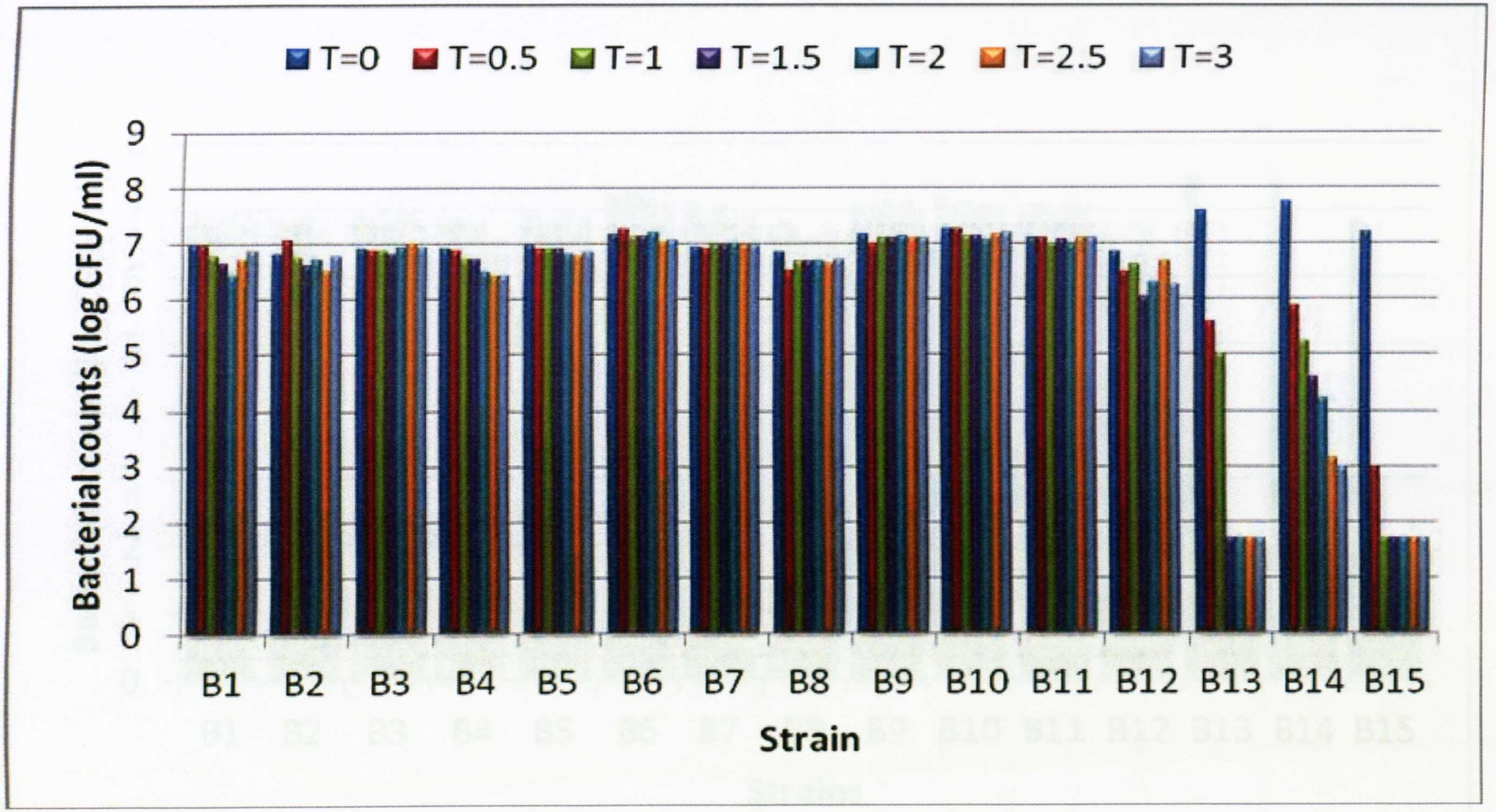

Figure 3.4 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), pure commercial cultures (B11 and B12) and type strains (B13, B14 and B15) under acidic condition up to 3 h at pH 4. T: time

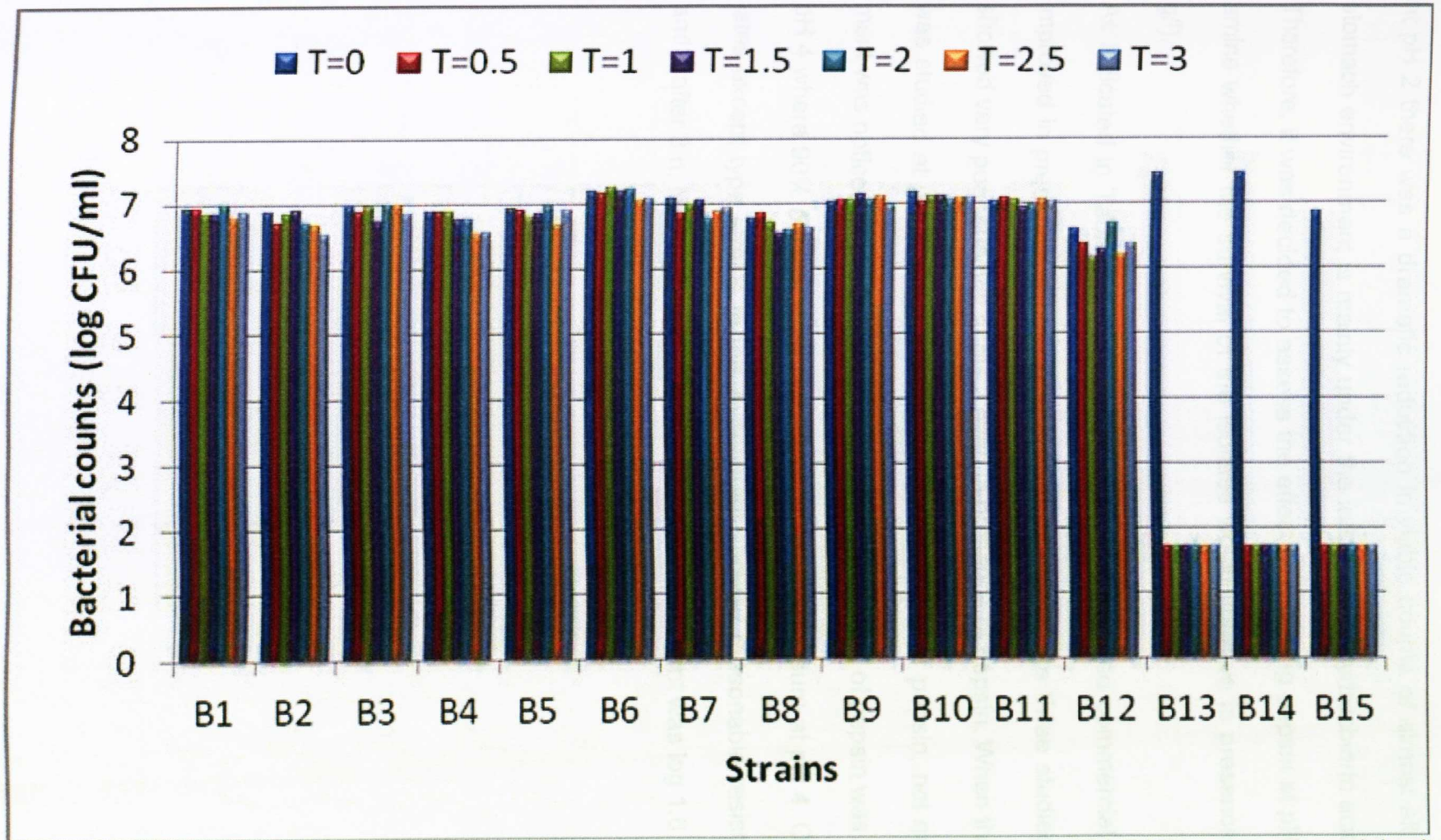

Figure 3.5 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), pure commercial cultures (B11 and B12) and type strains (B13, B14 and B15) under acidic condition up to 3 h at pH 3. T: time

3.3.2 Protective ability of pepsin

All studied *B. animalis* subsp. *lactis* isolates survived pH 3 and 4 very well; however, at pH 2 there was a dramatic reduction in viable counts of almost all isolates. The stomach environment is mainly under the influence of hydrochloric acid and pepsin. Therefore, it was decided to assess the effect of including pepsin at pH 2 and to examine whether the survival of the isolates would improve in presence of pepsin (3 g/l).

As indicated in Table 3.1 the survival of the isolates and commercial cultures was improved in presence of pepsin at pH 2.0. However, the three studied type strains showed very poor survival at pH 2 even in presence of pepsin. When the same effect was studied at pH 3 and 4 with and without addition of pepsin, not much improvement was noticed at pH 3; however, the protective effect of pepsin was highlighted at pH 4 where 90% of cells were recovered after 3 h exposure at pH 4. Overall, all isolates except type strains in this experiment showed reasonable resistance to pH 3 and 4 after 3 h. Minimum detection limit for this experiment was log 1.6 CFU/ml.

Table 3.2 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), commercial cultures (B11 and B12) and type strains (B13, B14 and B15) at pH 2 and presence of pepsin up to 3 h

	pH 2 without pepsin				pH 2 with pepsin (3 g/l)		
Time / Isolates	0 h	1 h	2 h	3 h	1 h	2 h	3 h
B1	6.29	6.34	5.80	3.17	6.41	6.42	6.19
B2	5.99	6.31	5.19`	2.84	6.14	6.17	5.99
B3	6.10	5.99	3.74	1.60	5.99	5.99	6.19
B4	5.99	6.00	3.64	1.6	6.07	5.90	6.14
B5	6.29	6.29	5.29	2.75	6.20	5.80	6.10
B6	6.25	6.37	6.1	3.19	6.53	6.59	6.38
B7	5.90	5.66	2.1	2.1	6.07	6.05	5.49
B8	6.05	6.25	5.29	2.6	6.26	5.99	5.90
B9	6.19	6.29	4.03	2.60	6.05	6.38	4.40
B10	6.03	5.98	3.85	2.6	5.98	5.88	4.68
B11	5.75	6.12	3.05	1.60	5.99	5.57	6.25
B12	5.99	5.20	1.60	1.60	6.44	6.25	6.14
B13	6.57	1.60	1.60	1.60	1.60	1.60	1.60
B14	6.05	1.60	1.60	1.60	1.60	1.60	1.60
B15	6.23	1.60	1.60	1.60	1.60	1.60	1.60

3.3.3 Bile tolerance

Tolerance to bile salts allows bacteria to survive in the small intestine. At the beginning of the experiment, initial cell population ranged from 6 to 8 log CFU/ml; however, all tested isolates showed very good resistance to bile salts even in presence of 2% w/v bile and their viability was maintained with little or no loss. Figures 3.6, 3.7, 3.8 and 3.9 illustrate the tolerance of tested *Bifidobacterium* strains in presence of 0.5, 1, 1.5, and 2% w/v of bile salts.

The survival of all tested *Bifidobacterium* strains at four different concentrations of bile salts for a period of 6 h exposure, were very similar to the control group. However, *B. bifidum* showed the lowest resistance to bile salts, especially at 2% w/v bile salts and there was about 1.5 log reduction in initial count after 6 h (Figure 3.9).

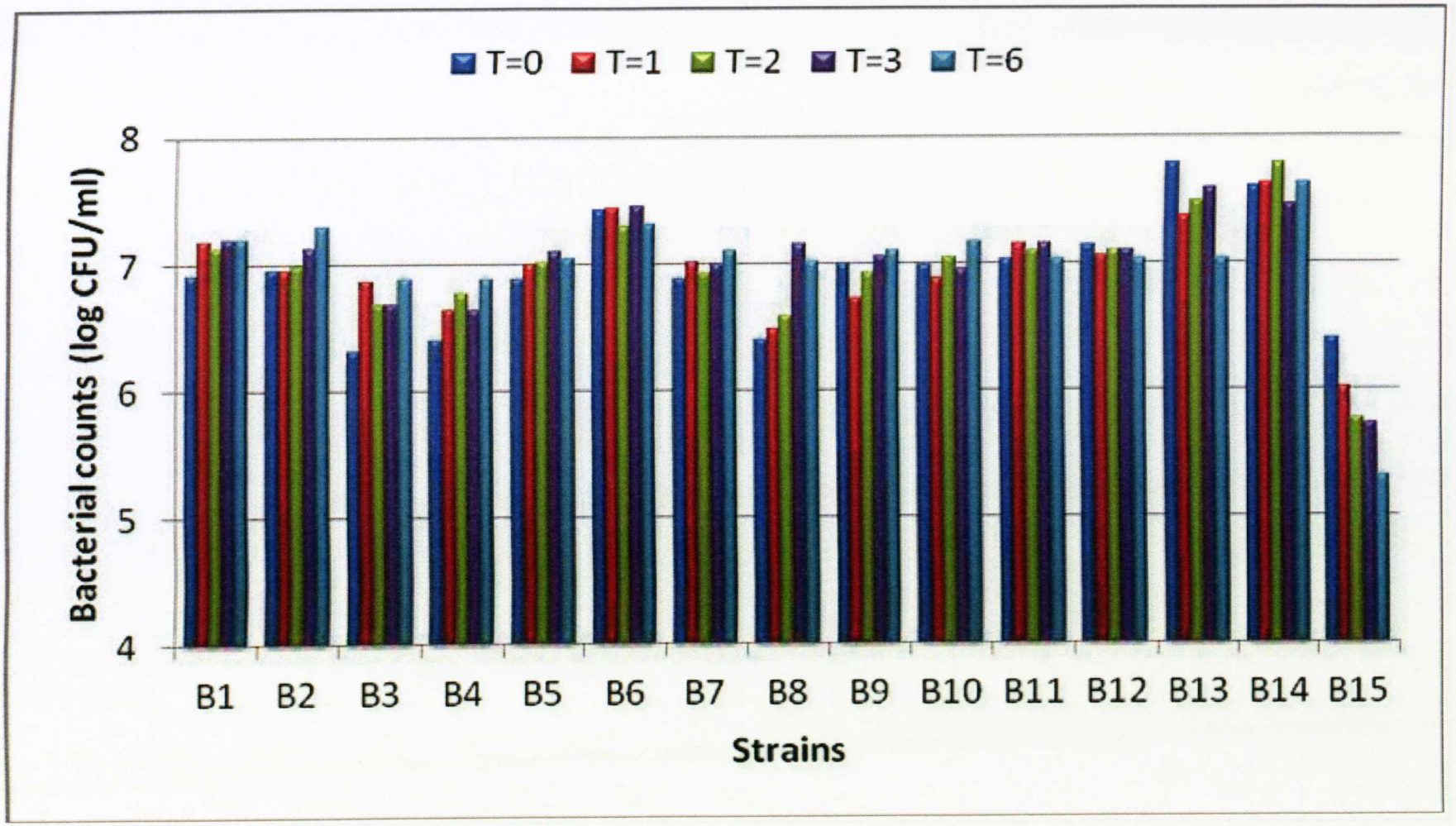

Figure 3.6 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), pure commercial cultures (B11 and B12) and type strains (B13, B14 and B15) in presence of 0.5% w/v of bile salt up to 6 h. T: time

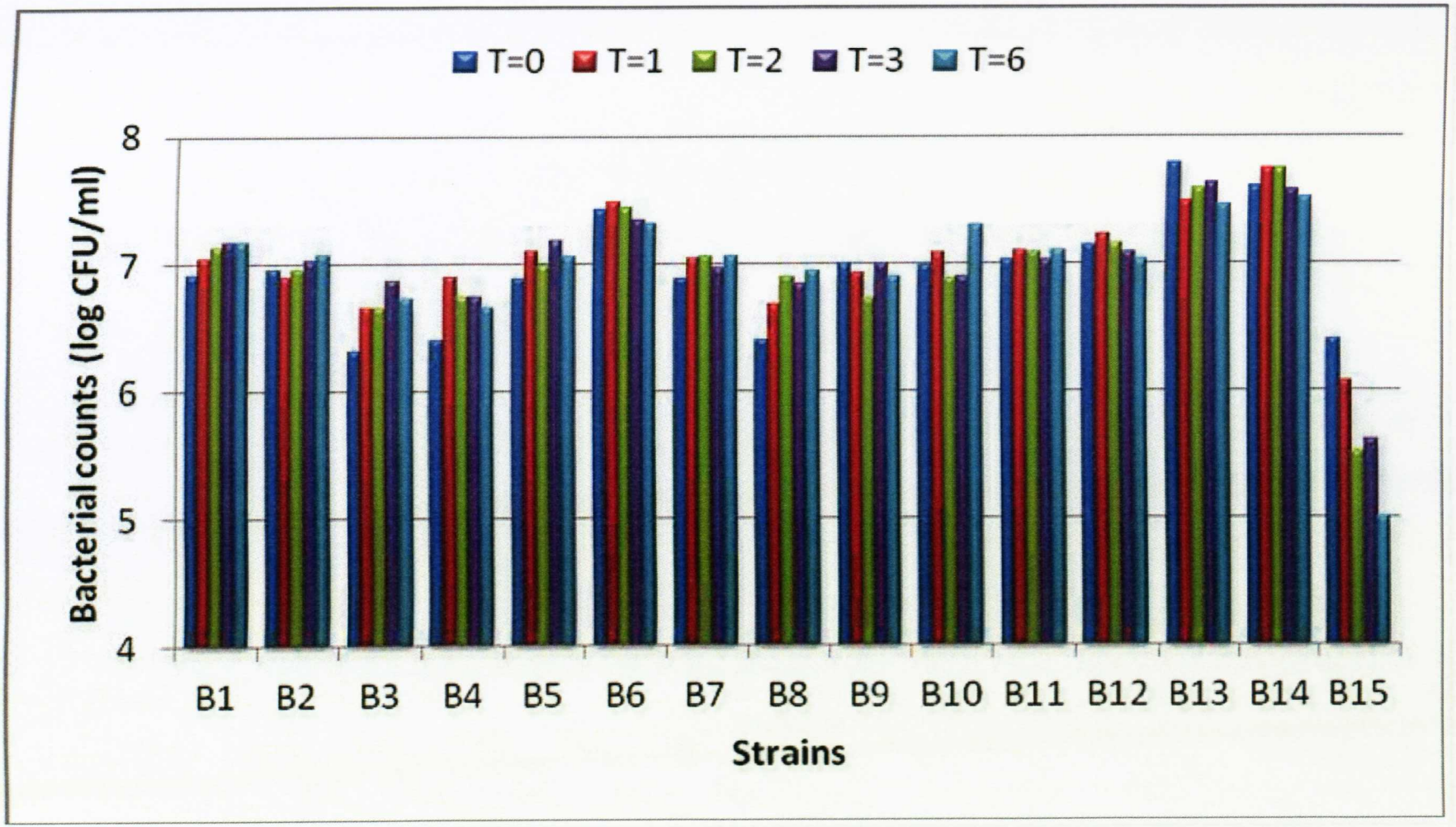

Figure 3.7 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), pure commercial cultures (B11 and B12) and type strains (B13, B14 and B15) in presence of 1% w/v of bile salt up to 6 h. T: time

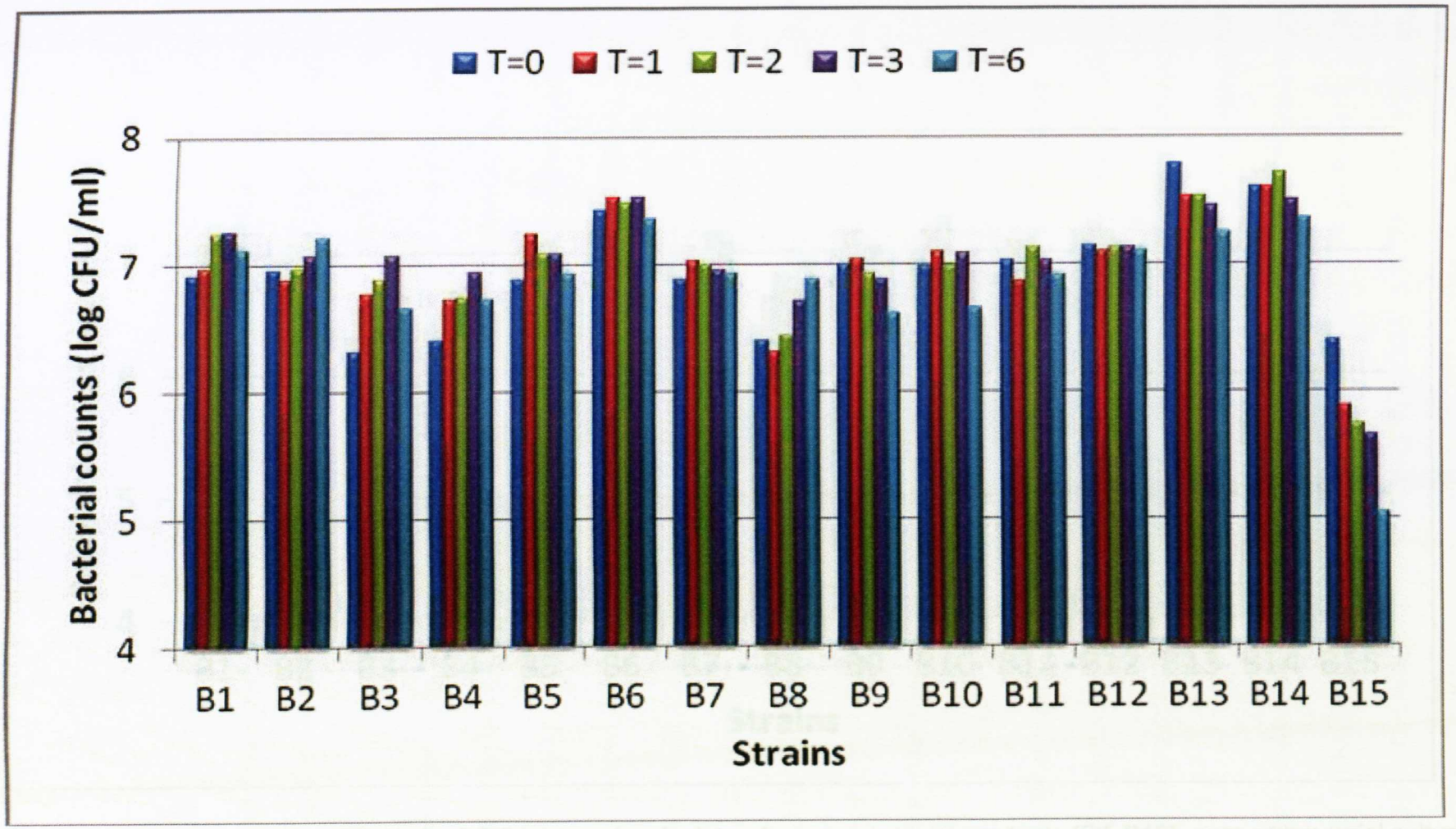

Figure 3.8 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), pure commercial cultures (B11 and B12) and type strains (B13, B14 and B15) in presence of 1.5% w/v of bile salt up to 6 h. T: time

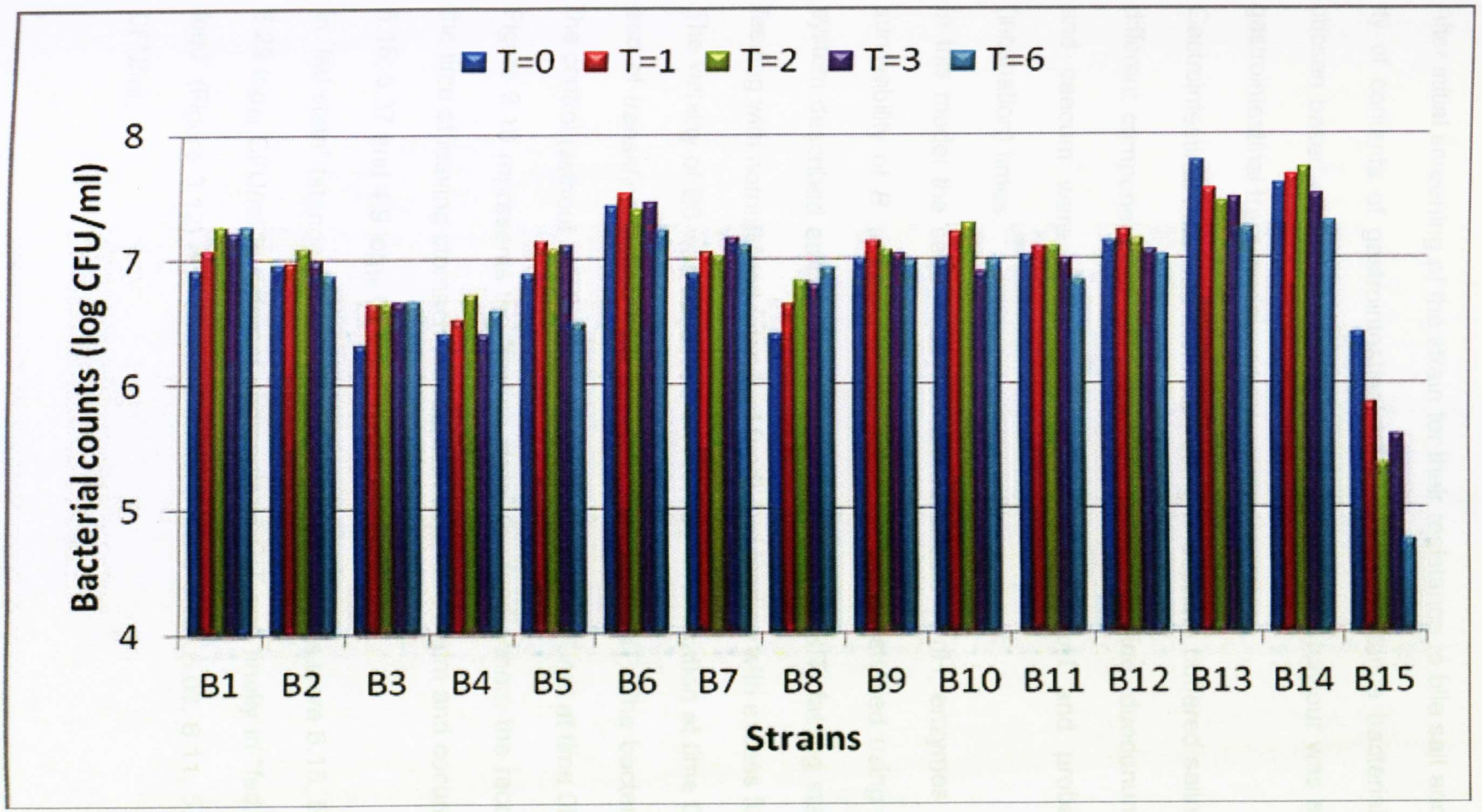

Figure 3.9 The survival (log CFU/ml) of *Bifidobacterium* isolates from commercial products (B1-B10), pure commercial cultures (B11 and B12) and type strains (B13, B14 and B15) in presence of 2% w/v of bile salt up to 6 h. T: time

3.3.4 Effects of sequential treatment with pH, bile salts and enzymes on the viability of tested bacteria

After initial screening of the strain for their resistance to bile salt and due to complexity of contents of gastrointestinal tract, a representative bacterial strain (B6) was chosen based on the above experiments and its behaviour was studied by *in vitro* gastrointestinal tract model.

Gastrointestinal fluid was mimicked using phosphate buffered saline (PBS) and also different components of GIT; stomach, small intestine; duodenum, jejunum, ileum and caecum were simulated, considering their pH and probable food transit (incubation) times.

In this model the sequentially combined effects of pH, enzymes and bile salts on survivability of *B. animalis* subsp. *lactis* (B6) were studied using the batch culture system described earlier. Three possible states of GIT; fasting state (without food), feeding with normal food (standard food) and feeding with excess food were studied. The viability of B6 was assessed on RCA by enumeration at time 0h and then at the end of transit/incubation time for each section of GIT. The bacteria enumerated in the control (without any treatment) was 6.26 $\log_{10}$ CFU/ml at time 0h.

Figure 3.10 represents the "fasting state" (no food) where the recovered bacteria at the time of leaving stomach, duodenum, Jejunum, Ileum and cecum were 6.26, 5.45, 5.16, 5.37 and 4.9 $\log_{10}$ CFU/ml, respectively.

In "fed state" (standard food) (Figure 3.11), B6 counts were 6.15, 5.9, 5.89, 5.37 and 5.29 $\log_{10}$ CFU/ml in different segments of GIT and finally in "fed state with excess food" (Figure 3.12), the counted bacteria were 6.29, 6.06, 6.11, 5.25 and 5.34 $\log_{10}$ CFU/ml.

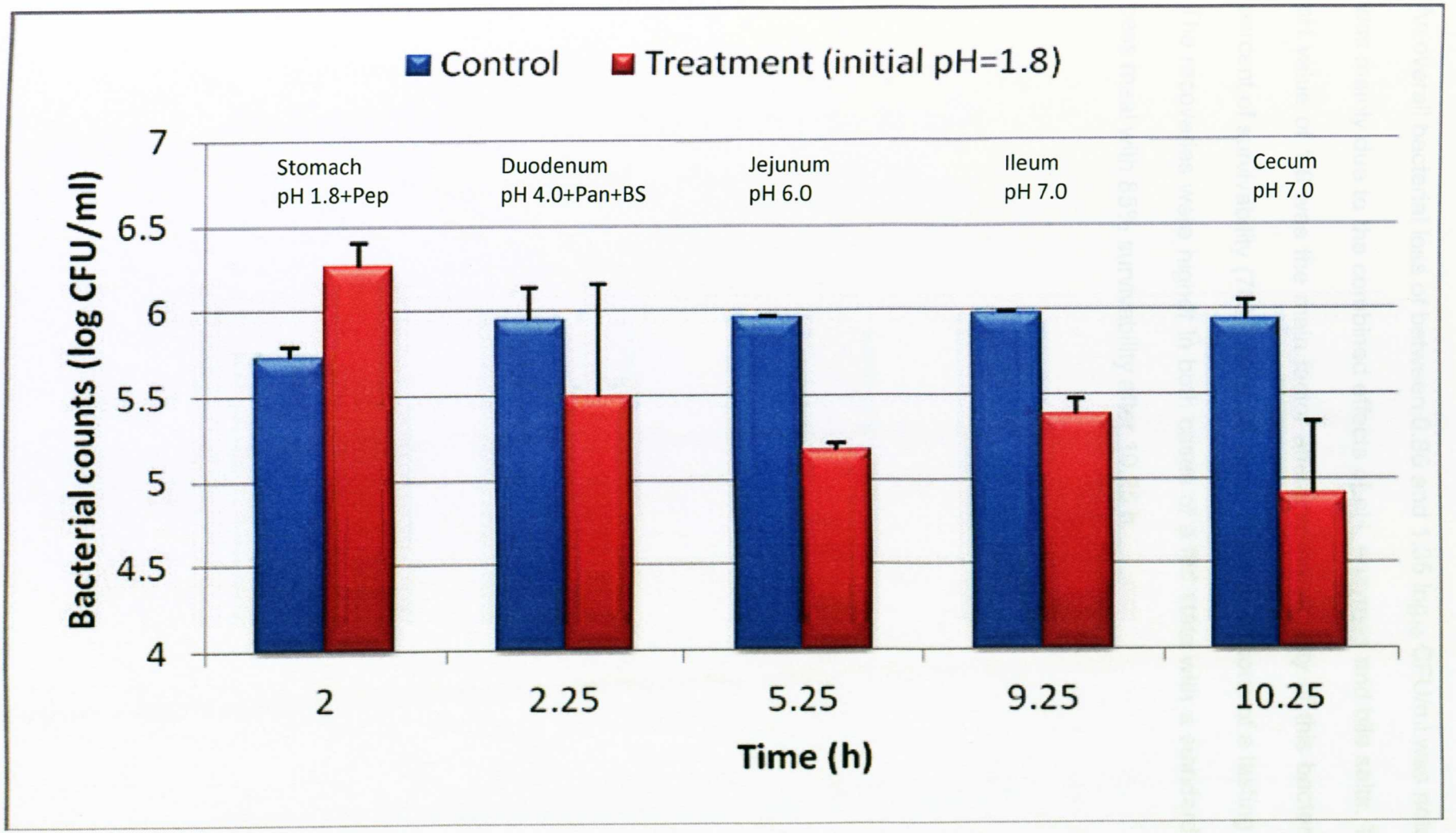

Figure 3.10 The survival of *B.animalis* subsp. *lactis* (B6) (log CFU/ml) under "fasting state (no food)" condition of stomach and small intestine influenced by the presence of acid (pH), bile salt (BS), pepsin (Pep), pancreatin (Pan) and transit time (h)
Data are means ± SD of two replications

The results demonstrated that B6 was not sensitive to low pH, enzymes and bile salt. An overall bacterial loss of between 0.86 and 1.35 $\log_{10}$ CFU/ml was noted. This loss was mainly due to the combined effects of pH, enzymes and bile salts. The stomach pH value of 1.8 was the main factor affecting the viability of this bacterium. Lowest percent of survivability (78%) were obtained in the conditions of a fasting state. The recoveries were higher in both cases of a fed state with a standard meal or excess meal with 85% survivability after 10.25 h.

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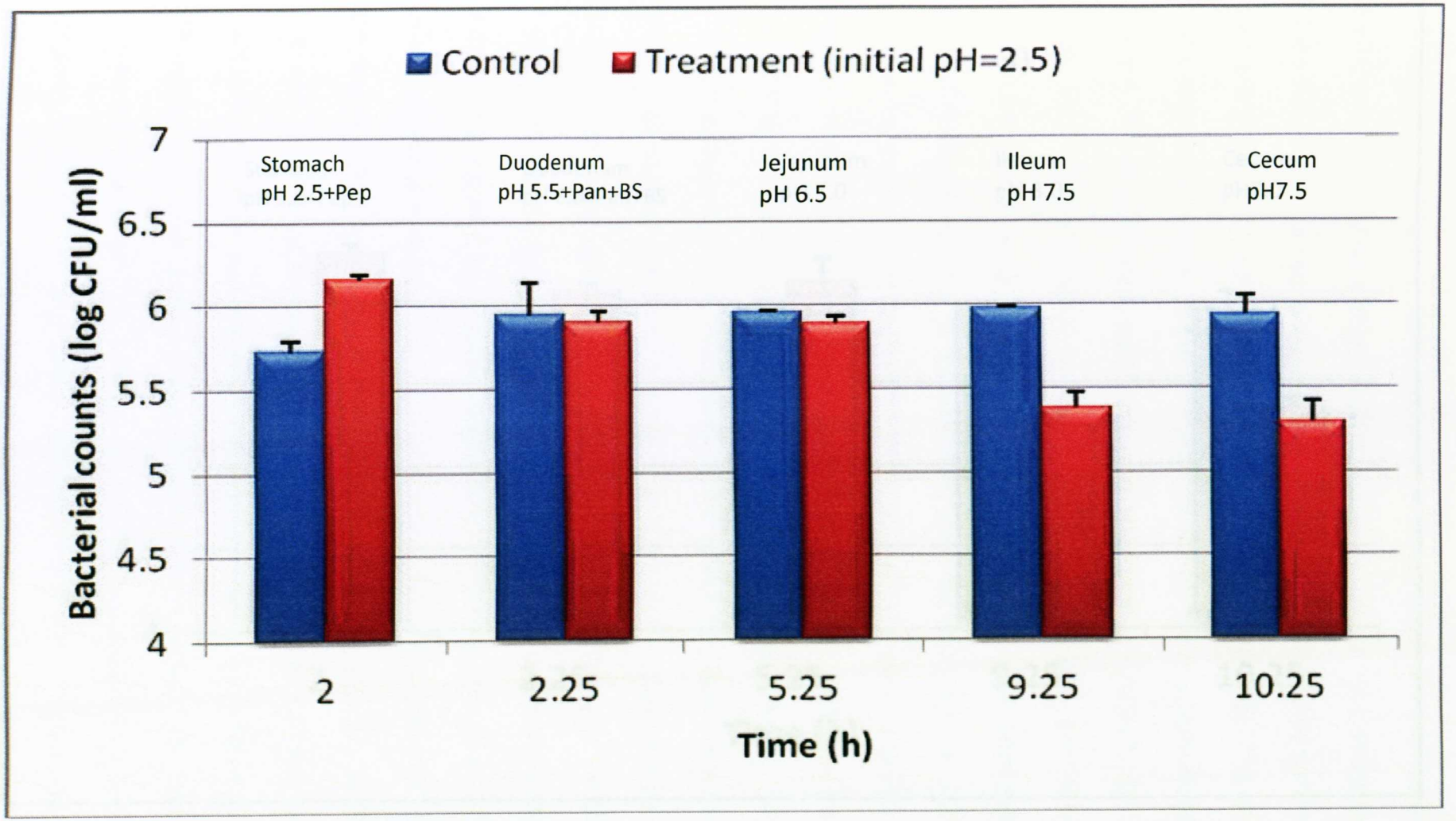


Figure 3.11 The survival of *B.animalis* subsp. *lactis* (B6) (log CFU/ml) under "fed state (standard food)" condition of stomach and small intestine influenced by the presence of acid (pH), bile salt (BS), pepsin (Pep), pancreatin (Pan) and transit time (h)
Data are means ± SD of two replications

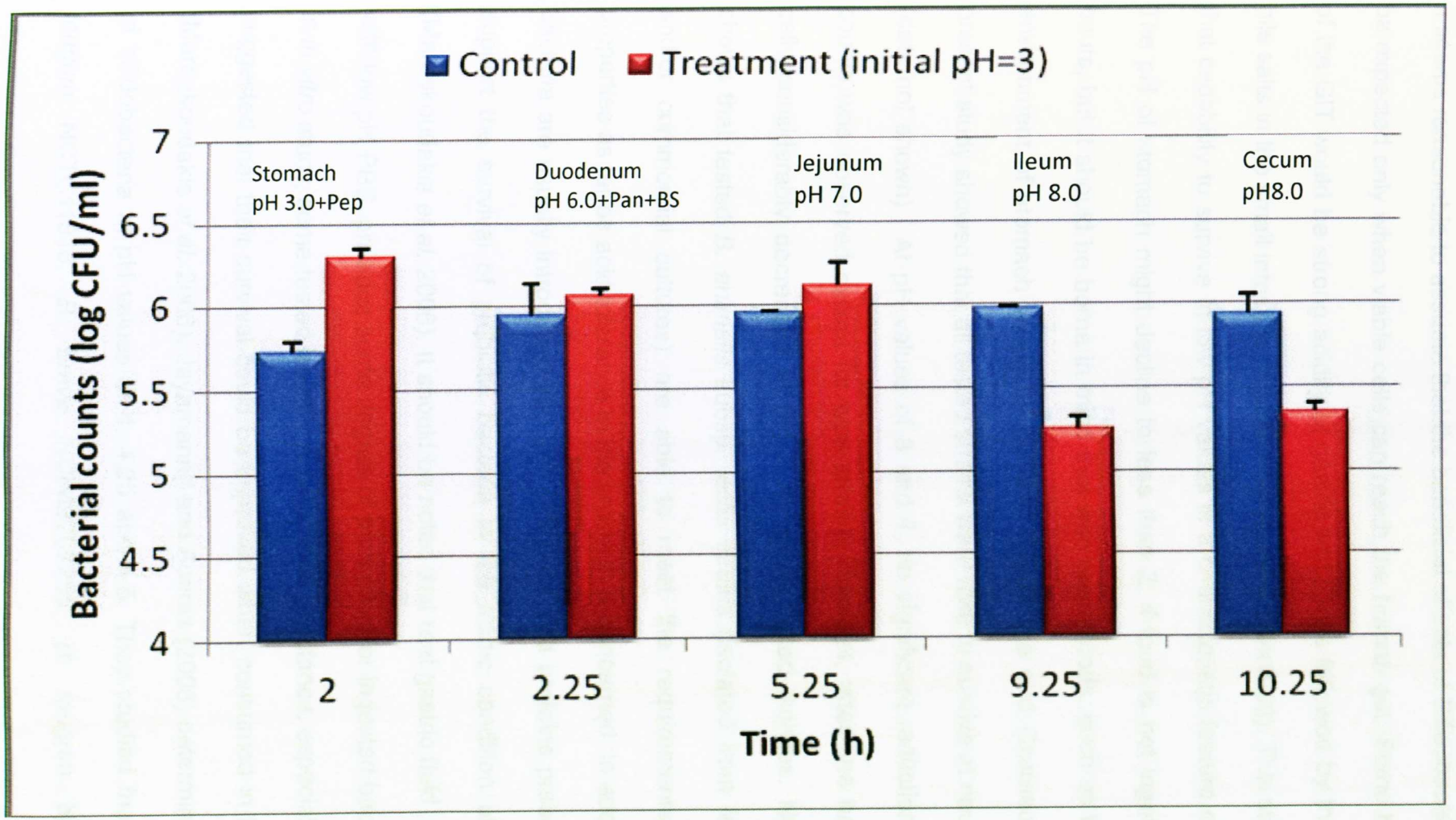


Figure 3.12 The survival of *B.animalis* subsp. *lactis* (B6) (log CFU/ml) under "fed state (excess food)" condition of stomach and small intestine influenced by the presence of acid (pH), bile salt (BS), pepsin (Pep), pancreatin (Pan) and transit time (h)
Data are means ± SD of two replications

3.4 DISCUSSION

It seems reasonable to assume that the beneficial effects of *Bifidobacterium* spp. can be expected only when viable cells can reach the human gut. Some harsh features of the GIT would be strong acidity of gastric secretions followed by the presence of bile salts in the small intestine (Klaenhammer and Kullen1999). This study confirmed that capability to survive in low pH values is a characteristic feature of the isolates. The pH of stomach might decline to less than 2, if food is not ingested for some hours, but it should be borne in mind that with some foods, such as fatty foods the environment of stomach would be buffered (Goderska and Czarnecki 2007). The present study showed that all tested strains were able to survive at neutral pH for 3 h (data not shown). At pH values of 3 and 4, no significant reduction in the viable counts was observed except for type strains. However, after one hour, the viable cells considerably decreased at pH 2 for some tested isolates. Nevertheless, it shows that tested *B. animalis* subsp. *lactis* strains (isolated from fermented food and/or commercial cultures) are able to meet the requirements for probiotic properties as far as acid tolerance in the stomach is concerned. In addition, probiotic bacteria are usually introduced into milk products, and proteins present in the milk support the survival of probiotic bacteria in the acidic condition of the stomach (Maragkoudakis *et al.* 2006). It should be noted that real gastric fluid , in conjunction with low pH PBS, provides some degree of protection for ingested bacteria, although in *in vitro* study, some tested isolates showed less resistance, especially at pH 2. It is suggested that their survival could be improved when consumed in fermented milk (Maragkoudakis *et al.* 2006). Jayamanne and Adams (2006) determined the survival of bifidobacteria at pH values of 4, 4.25 and 4.5. They studied the survival of *B. longum* NCTC11818, *B. breve* NCIMB702258, *B. longum* biotype *infantis*

NCIMB702205, *B. adolescentis* NCIMB702204, *B. bifidum* NCIMB702203 and *B. animalis* subsp. *lactis* which was isolated from fermented milks sold in the UK. *Bifidobacterium animalis* subsp. *lactis* was the only *Bifidobacterium* strain that was resistant to acidity, which is in line with the results of this study.

Bile salt tolerance is also a prerequisite for the live cells to reach the small intestine and colon and to show metabolic activity. In contrast to acid resistance, all tested isolates showed good resistance to different levels of bile salts, even after 6 h and were able to retain their viability. This might be due to presence of bile salt hydrolase (BSH) activities in those isolates which deconjugate bile salts, so the toxic effects of bile salts on bacteria were decreased. The deconjugation of bile salts in human GIT starts at the end of ileum and is completed in the large intestine (Tanaka *et al.* 2000). Noriega *et al.* (2006) reported that lactobacilli and bifidobacteria have a protective system of BSH which enables deconjugation of bile salts.

Bielecka *et al.* (1998) isolated 17 strains of probiotic bacteria from healthy adults and observed that about 30% were completely resistant to pH and bile salts. They also found a considerable variation in their ability to survive at low pH and in the presence of bile salts.

BSH activity in probiotic bacteria might be considered as an advantage because it may lead to more excretion of bile salt, resulting in a significant drop in serum cholesterol, however steatorrhoea could develop as a consequence of substantial deconjugation of bile salts (Tanaka *et al.* 2000).

It is estimated that during intestinal transit only 10-40 % of consumed probiotic bacteria remain alive (Matto *et al.* 2006). Obviously, addition of pepsin to gastric fluid improved the tolerance of studied bacteria to low pH, especially for *B. animalis* subsp. *lactis*. However, the protective effects of pepsin were not as noticeable for *B. breve,*

B. longum and *B. bifidum,* which indicated that at pH 2 and 3, these bacteria are exteremely sensitive to acidic conditions.

The findings herein reported on viability of *B. animalis* subsp. *lactis* in the presence of pepsin are also in agreement with the existing literature (Maragkoudakis *et al.* 2006; Matto *et al.* 2006).

Depending on feeding or fasting states, pH values in different parts of GIT may vary (Vandamme *et al.* 2002). Therefore, in this study, three possibilities were defined for GIT conditions (i.e. fasting condition fed state with a standard food and fed state with receiving excess food).

Bifidobacterium animalis subsp. *lactis* (B6) was selected for this study because it performed well in preliminary screening and showed good resistance to the harsh GIT conditions and numbers remained relatively stable during 10.25 h of experiment. This resistance was also observed with bile salts and pancreatic secretions. The cell counts of B6 decreased to about 78-85% of the initial value, however, this decrease was not considered significant. Ritter *et al.* (2009) used a single reactor system to evaluate the survival of bifidobacteria through the GIT passage. They stated that only *B. animalis* subsp. *lactis* showed acceptable survival for a successful passage in the simulation system.

Bifidobacterium animalis subsp. *lactis*, which is considered the most frequently used *Bifidobacterium* species in fermented milks, presented excellent survival in the *in vitro* GIT. Furthermore, from a technical point of view, the use specifically of *B. animalis* subsp. *lactis* in probiotic food is more reasonable than other bifidobacteria, due to its particular resistance to severe conditions. It is believed that the acid tolerance of *B. animalis* subsp. *lactis* is related to the induction of membrane H^+-ATPase activ-

ity in low pH environments, and also pepsin that possibly induces bacteria to produce more ATP for pumping out protons (Matsumotoa *et al.* 2004; Matto *et al.* 2006)

3.5 CONCLUSION

All tested isolates apart from type strains presented good resistance to low pH (2, 3, and 4) after 3 h and also showed high resistance to bile salts at strength corresponding to the concentrations in the small intestine. During this study, the protective effect of pepsin, enhancing acid tolerance of *Bifidobacterium* species was confirmed.

Chapter 4

In vitro assessment of the isolates for functional properties: antibiotic resistance

4.1 INTRODUCTION

The safety aspects of probiotic bacteria, which are used as adjunct cultures in fermented dairy products, are important and antibiotic resistance genes are a principal concern (Aimmo *et al.* 2007). Antibiotic resistance is a worldwide problem which has originated from extensive and inappropriate use of antibiotics (Ouoba *et al.* 2008), although a significant reduction in infectious diseases has been achieved with antibiotics. Nowadays antibiotics are used in a wide range of applications, such as clinical use against infectious diseases, in veterinary medicine and in agriculture for plant diseases. Over the last 60 years up to 10 million tons of antibiotics have been released to the biosphere and it might be a main reason for the evolution of resistant strains (European Commission 2005).

Examining the antibiotic resistance pattern of probiotic organisms is one of the safety measures recommended by FAO/WHO (2002).

Therefore, although not heavily empahasised in the literature, antibiotic resistance profile of probibiotics should be considered as one of the many selection criteria. This issue is considered as having two cutting edges due to the benefits and risks associated with probiotic strains that are resistant to certain antibiotics. It would be advantageous when a patient is under treatment with drugs for infectious diseases, that the antibiotic would only affect the target pathogens and some of the resident microbiota, but not the probiotics. On the other hand, one result of interaction between the probiotics and the gut flora could be the gene transfer. This means that the probiotics, when ingested in large numbers, could contribute to the potential risk of the genetic materials (i.e. antibiotic resistance genes) being transferred to other organisms in the gut, commensal bacteria or more importantly the pathogens. The acquisition of such genes is undesirable as it may lead to development of antibiotic

resistance (Licht and Wilcks 2005). Therefore, from a safety point of view, the probiotics will become a complex and maybe controversial issue if they possess acquired antibiotic resistance genes.

From a safety perspective, it is necessary to distinguish between intrinsic and acquired resistance genes. Below a description of different types of resistance is presented.

4.1.1 Physiological resistance to antibiotics

Physiological resistance is expressed when bacteria are in a particular physiological condition like when they produce biofilm and the bacteria adopt a sessile biofilm lifestyle. In such conditions, it is hard to eradicate bacteria by antibiotics. The reason for this resistance is not clear. It might be due to slow penetration of antibiotics through the biofilm, which surrounds the bacteria. Other reason for this resistance might be that bacteria in biofilm condition are in a stationary phase and their growth and death are in balanced state (Normark and Normark 2002). Bacteria in the stationary phase tend to be more resistant; the biofilm environment will enhance this.

4.1.2 Intrinsic antibiotic resistance

This resistance may be inherent in some bacteria and is also called "natural" antibiotic resistance. In this case, all members of a bacterial species are resistant to a specific antibiotic and there is no genetic alteration involved. For example some bacteria, such as mycoplasma, which are free of peptidoglycan in their cell wall, are resistant to β-lactams (Ammor *et al.* 2007).

4.1.3 Acquired antibiotic resistance

Horizontal gene transfer and sequential mutations are two main occurrences which lead to acquired resistance to antibiotics. There are a number of mechanisms by which microorganisms can overcome the effects of each group of antibiotics, that can be the reason for resistance (Normark and Normark 2002). Some of these mechanisms, which can cause antibiotic resistance, are as follows:

- Decreased uptake of the antibiotic.
- Increased export of the antibiotic.
- Inactivation or modification of the antibiotic targets within the cell.
- Introduction of a new antibiotic resistant target.
- Breakdown of the antibiotics.
- Modification of the antibiotics, such as production of an aminoglycoside-modifying enzyme.

However, the risk of transfer would be less in intrinsic resistance and also in acquired resistance which happens through mutation, but acquired resistance as a consequence of added genes is presumed to present a high risk of horizontal spread.

4.1.4 Bifidobacteria and antibiotic resistance

LAB and bifidobacteria are the two main inhabitant bacteria in the GIT. In addition, they are distributed in different food sources and always there is a concern for dissemination of antibiotic resistance genes to pathogenic bacteria (Sato and Lino 2010).

The International Dairy Federation (IDF) reported the consumption of 22 kg fermented milks per capita in Europe, which is equivalent to 8.5 billion kg of

fermented milk per year (Hummel *et al.* 2007). Thus, fermented foods are considered as important vehicles of living bacteria. Bacterial used in fermented milks whether starter cultures or adjunct cultures may carry antibiotic resistance genes with the potential of transfer to pathogen or commensal bacteria.

These bacteria acquire resistance from other bacteria which live in the same environment and also when a bacterium achieves antibiotic resistance, this gene could be amplified and transferred to other bacteria. Therefore, it is critical that probiotic bacteria to be checked for transferable antibiotic resistance genes, and also a major criterion for selection of probiotic bacteria is their potential to transfer antibiotic resistance genes to the host (Ammor *et al.* 2007). Conjugation is the main mechanism by which bacteria can spread antibiotic resistance genes to other bacteria. Aimmo *et al.* (2007) stated that one of the safety criteria for use of LAB and bifidobacteria as probiotic cultures is that, they should not have the potential to transfer antibiotic resistance genes.

Probiotic bacteria are generally recognized as safe (GRAS), and have shown a safe use throughout history as food-associated bacteria. However, some characteristics of these bacteria, such as antibiotic resistance, have not been well studied. Due to increasing bacterial drug resistance and also the expansion of probiotic usage in the market, there is particular attention to this emerging issue (Aimmo *et al.* 2007). A significant portion of the intestinal flora in the healthy human is bifidobacteria which are third highest in the GIT population after the genera *Bacteroides* and *Eubacterium* (Gomes and Malcata 1999). Bifidobacteria are rarely associated with infection except *B. dentium,* which causes dental cavities (Turroni *et al.* 2011). They are GRAS, but in connection with their use in probiotic products, the concern for transferable antibiotic resistance gene has been raised (Charteris *et al.* 1998).

Bifidobacterium animalis subsp. *lactis* is the most common species among bifidobacteria, which are widely included in fermented dairy products and, some beneficial effects such as reduction of the risk of diarrhoea and reduction of allergy, have been attributed to these bacteria (Gueimonde *et al.* 2010). In the early part of this research, all strains, which were isolated from fermented dairy products, have been identified as *B. animalis* subsp. *lactis* and the presence of antibiotic resistance determinants in these strains is of high priority for food safety.

European Food Safety Authority (EFSA) launched a new concept which is similar to GRAS system in the US. This system has been named Qualified Presumption of Safety (QPS) and allows only the strains with safety status and documented history to enter the market (Hummel *et al.* 2007).

So far, there have been limited reports on transferability of antibiotic resistant genes in probiotics. A study by Rosander *et al* (2008) on *Lactobacillus reuteri* ATCC 55730 found that *tet*(W) and *lnu*(A) to be potentially transferable, while the presence of transferable *erm*(B) plasmid in *L. reuteri* L4:12002 was reported by Ouoba *et al* (2008).

The aim of this part of the research was to study the antibiotic resistance profiles of bifidobacteria, the detection of possible antibiotic resistance gene in these isolates and also the ability of bifidobacteria to transfer resistances genes to other bacteria.

4.2 MATERIALS AND METHODS

4.2.1 Microorganisms

A- Strains tested for antibiotic resistance

The bacteria which were tested in this experiment were as follows (Table 4.1):

a) Ten *Bifidobacterium* spp. which were isolated from yogurt and other fermented milk products and were identified as *B. animalis* subsp. *lactis* (B1-B10)

b) Two commercial cultures of *B. animalis* subsp. *lactis* Bb-12 and vital probiotic yogurt, kindly provided by two starter culture suppliers, Chr. Hansen and Danisco (B11 and B12).

c) Three different type strains purchased from National Collection of Type Cultures (Salisbury, UK) *B. longum* NCTC11818, *B. breve* NCTC11815, *B. bifidum* NCTC13001 (B13, B14 and B15, respectively).

Table 4.1 Organisms used for the detection of antibiotic resistance and their origin

Code	Bacteria	Origin
B1	*Bifidobacterium animalis* subsp. *lactis*	Plain yoghurt
B2	*Bifidobacterium animalis* subsp. *lactis*	Goat's yogurt
B3	*Bifidobacterium animalis* subsp. *lactis*	Chocolate milk drink
B4	*Bifidobacterium animalis* subsp. *lactis*	Yogurt drink
B5	*Bifidobacterium animalis* subsp. *lactis*	Plain low fat yogurt
B6	*Bifidobacterium animalis* subsp. *lactis*	Fruit yogurt
B7	*Bifidobacterium animalis* subsp. *lactis*	Whole fat yogurt
B8	*Bifidobacterium animalis* subsp. *lactis*	Greek style yogurt
B9	*Bifidobacterium animalis* subsp. *lactis*	Fruit yogurt
B10	*Bifidobacterium animalis* subsp. *lactis*	Yogurt drink
B11	*Bifidobacterium animalis* subsp. *lactis*	Pure culture from Chr-hansen (Bb12)
B12	*Bifidobacterium animalis* subsp. *lactis*	Culture from Danisco
B13	*Bifidobacterium longum* NCTC 11818	National Collection of Type Cultures
B14	*Bifidobacterium breve* NCTC 11815	National Collection of Type Cultures
B15	*Bifidobacterium bifidum* NCTC 13001	National Collection of Type Cultures

B) Positive control organisms for gene transfer study shown in Table 4.2

The following microorganisms were applied as positive controls for determination of the resistance gene. They were kindly provided by Technical University of Denmark, (National Food Institute, Antimicrobial Resistance Centre, Denmark).

Table 4.2 The organisms used as positive controls for the detection of antibiotic resistance

Bacteria	Gene related
Staphylococcus rissem 7522486-1	*aph*(3")-I
Enterococcus faecalis	*aadE*
Salmonella typhimurium	*aadA*
Staphylococcus aureus RN422	*erm*(C)
Enterococcus Faecalis JH2-2	*erm*(B)
Staphylococcus aureus Tn554	*erm*(A)
Staphylococcus aureus PSTS 9-like	*tet*(L)
Staphylococcus aureus PT181-like	*tet*(K)
Staphylococcus intermedius 2567	*tet*(M)
Escherichia coli	*tet*(Q)
Listeria monocytogenes BM4210/PIP811	*tet*(S)
Escherichia coli K2	*ant*(2")-I
Enterococcus JH2-1-5	*aph*(3")-III
Escherichia coli	*tet*(W)

4.2.2 Determination of minimum inhibitory concentration (MIC)

Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. Minimal Inhibitory Concentration (MIC) for 24 antibiotics was determined using a sensititre plate of 96 wells containing variable amounts of

antibiotics (TREK Diagnostic Systems Ltd, UK). After two consecutive subcultures, a selection of single colonies on Reinforced Clostridial Agar (RCA) plates was suspended in 1 ml of sterile MRD (this was used as stock solution for preparing the final inocula). The working inoculum suspension was prepared by diluting stock inoculum in 5 ml MRD in glass boiling tube and using a calibrated sensititre nephelometer (TREK - Diagnostic Systems Ltd, UK) to measure the turbidity.The final concentration of cells per tube was adjusted to 0.5 McFarland standard (10^7-10^8 CFU/ml). The liquid medium used was Reinforced Clostridial Medium (RCM) (CM0149; RCM, Oxoid, Basingstoke, UK) because it supports the growth of all bifidobacteria used in this study. The working inoculum suspension was then diluted 100-fold in fresh RCM and 50µl of inoculated RCM was dispensed in each well. Incubation was carried out at 37°C for 48 h in an anaerobic cabinet (Don Whitley, Skipton, UK) in an atmosphere of 80% N_2, 10%H_2 and 10% CO_2. The antibiotic plates were observed with a magnifier mirror after the incubation period and visible growth (precipitated cells at the bottom of wells) or no visible growth was reported. For determination of degree of resistance/susceptibiliy a breakpoint for each antibiotic was established based on published literature (Table 4.3).

The resulting MIC of each isolate for individual antibiotics were compared with that of proposed breakpoints and the isolate was considered resistant to any particular antibiotic if the MIC was greater than the breakpoint. A further investigation was carried out to study the presence or absence of some selected genes in the isolates that showed resistance to some antibiotics.

Table 4.3 The concentration of each antibiotic tested and the suggested breakpoints

Antibiotics	Range of concentration studied (µg/ml)	Proposed breakpoints (µg/ml)	Reference
Amikacin	0.5-64	>16	EUCAST
Amoxicillin/clavulanic acid	1/0.5-32/16	>2	Ouoba *et al.* (2008)
Ampicillin	1-32	2	EFSA
Cefoxitin	0.5-32	>32	Moubareck *et al.* (2005)
Ceftiofur	0.12-8	8	Ouoba *et al.* (2008)
Ceftriaxone	0.25-64	>4	Karlowsky and Jones (2003)
Ciprofloxacin	0.015-4	4	Ouoba *et al.* (2008)
Chloramphenicol	2-32	4	EFSA
Daptomycin	0.25-16	≥ 8	King and Phillips (2001)
Erythromycin	0.25-8	0.5	EFSA
Gentamycin	0.25-1024	64	EFSA
Kanamycin	8-1024	>256	Ouoba *et al.* (2008)
Lincomycin	1-8	≥ 1	UKPAR
Linezolid	0.5-8	≥ 8	Ouoba *et al.* (2008)
Nalidixic acid	0.5-32	32	Ouoba *et al.* (2008)
Nitrofurantoin	2-64	≥ 128	Ouoba *et al.* (2008)
Penicillin	0.25-16	4	Ouoba *et al.* (2008)
Quinupristin/dalfopristin	0.5-32	1	EFSA
Streptomycin	512-2048	128	EFSA
Tetracycline	4-32	8	EFSA
Tigecycline	0.015-0.5	≥ 8	Nord *et al.* (2006)
Trimethoprim/sulfamethoxazole	0.12/2.38-4/76	>32/512	Ouoba *et al.* (2008)
Tylosin tartrate	0.25-32	≥ 32	Ruzauskas *et al.* (2010)
Vancomycin	0.25-8	2	EFSA

EUCAST: European Committee on Antimicrobial Susceptibility Testing
EFSA: European food safety authority

4.2.3 Identification of the resistance genes

The genetic background of antibiotic resistance genes was studied by PCR reactions and specific primers for antibiotic resistance genes. For an antibiotic to which a tested isolate had resistance, PCR was carried out for most frequent genes for the following four antibiotics. The genes associated with resistance are as follows:

- Tetracycline: *tet*(M), *tet*(L), *tet*(S), *tet*(Q), *tet*(K), *tet*(O), *tet*(w)
- Kanamycin: *aph*(3") *-I, ant*(2") *-I, aph*(3") *-III*
- Streptomycin: *strA, strB, aadA, aadE*
- Erythromycin: *erm*(A), *erm*(B), *erm*(C)

The PCR reagents which were used for tetracycline, kanamycin and erythromycin were as follows: (it should be stated that for each investigated gene the corresponding set of its primers were applied)

High purity water 41.4 µl, 10×PCR buffer (with 15 mM $MgCl_2$) 5 µl, dNTP (1.25 mM) 0.5 µl, Primer 1 (21 pmol/µl) 0.5 µl, Primer 2 (21 pmol/µl) 0.5 µl, Taq DNA Polymerase (5 u/µl) 0.1 µl, DNA 2 µl and volume in total was 50 µl.

The PCR reagents for investigation of two corresponding genes (*str A and str B*) for streptomycin, were as follows,

High purity water 39.4 µl, 10×PCR buffer (with 15 mM $MgCl_2$) 5 µl, dNTP (1.25 mM) 0.5 µl, $MgCl_2$ (25 mM) 2 µl, Primer 1 (21 pmol/µl) 0.5 µl, Primer 2 (21 pmol/µl) 0.5 µl, Taq DNA Polymerase (5 u/µl) 0.1 µl, DNA 2 µl and volume in total was 50 µl.

The PCR reagents for investigation of the resistance gene *aadA* for streptomycin were as follows,

High purity water 40.9 µl, 10×PCR buffer (with 15 mM $MgCl_2$) 5 µl, dNTP (1.25 mM) 0.5 µl, $MgCl_2$ (25 mM) 0.5 µl, Primer 1 (21 pmol/µl) 0.5 µl, Primer 2 (21 pmol/µl) 0.5 µl, Taq DNA Polymerase (5 u/µl) 0.1 µl, DNA 2 µl and volume in total was 50 µl.

The PCR reagents for investigation of resistance gene *aadA* for streptomycin were as follows:

High purity water 38.4 µl, 10×PCR buffer (with 15 mM $MgCl_2$) 5 µl, dNTP (1.25 mM) 0.5 µl, $MgCl_2$ (25 mM) 3 µl, Primer 1 (21 pmol/µl) 0.5 µl, Primer 2 (21 pmol/µl) 0.5 µl, Taq DNA Polymerase (5 u/µl) 0.1 µl, DNA 2 µl and volume in total was 50 µl.

All PCR amplifications were performed in a thermocycler (GeneAmp PCR 2700 system) using the following temperature program:

Initial denaturation temperature was 94°C for 3 min, 25 or 35 cycles of 94°C for 1 min, 45-68°C according to annealing temperature for the individual primers (Table 4.4) and 72 °C for 1 min and a final extension step at 72 °C for 10 min. 10 µl of PCR products were separated by electrophoresis on 1.5% w/v agarose gel and visualised by ethidium bromide staining.

Table 4.4 The studied genes and their specific primers and annealing temperatures

Resistance genes	Primers	Annealing temperature(°C)
tet(M)	5'-GTT AAA TAG TGT TCT TGG AG-3' 5'-CTA AGA TAT GGC TCT AAC AA-3'	45°C
tet(L)	5'-GTT GCG CGC TAT ATT CCA AA-3' 5'-TTA AGC AAA CTC ATT CCA GC-3'	54°C
tet(S)	5'-TGG AAC GCC AGA GAG GTA TT-3' 5'-ACA TAG ACA AGC CGT TGA CC-3'	55°C
tet(Q)	5'-ATG TTC AAT ATC GGT ATC AAT GA-3' 5'-GCG GAT ATC ACC TTG CTT C-3'	55°C
tet(K)	5'-TTA GGT GAA GGG TTA GGT CC-3' 5'-GCA AAC TCA TTC CAG AAG CA-3'	55°C
tet(O)	5'-GAT GGC ATA CAG GCA CAG AC-3' 5'-CAA TAT CAC CAG AGC AGG CT-3'	55°C
aph(3")-I	5'-AAC GTC TTG CTC GAG GCC GCG-3' 5'-GGC AAG ATC CTG GTA TCG GTC TGC G-3'	68°C
ant(2")-I	5'-GGG CGC GTC ATG GAG GAG TT-3' 5'-TAT CGC GAC CTG AAA GCG GC-3'	67°C
aph(3")-III	5'-GCC GAT GTG GAT TGC GAA AA-3' 5'-GCT TGA TCC CCA GTA AGT CA-3'	52°C
strA	5'-CTT GGT GAT AAC GGC AAT TC-3' 5'-CCAATCGCAGATAGAAGGC-3'	55°C
strB	5'-ATC GTC AAG GGA TTG AAA CC-3' 5'-GGA TCG TAG AAC ATA TTG GC-3'	56°C
aadA	5'-ATC CTT CGG CGC GAT TTT G-3' 5'-GCA GCG CAA TGA CAT TCT TG-3'	56°C
aadE	5'-ATG GAA TTA TTC CCA CCT GA-3' 5'-TCA AAA CCC CTA TTA AAG CC-3'	50°C
erm(A)	5'-AAG CGG TAA AAC CCC TCT GAG-3' 5'-TCA AAG CCT GTC GGA ATT GG-3'	55°C
erm(B)	5'-CAT TTA ACG ACG AAA CTG GC-3' 5'-GGA ACA TCT GTG GTA TGG CG-3'	52°C
erm(C)	5'-CAA ACC CGT ATT CCA CGA TT-3' 5'-ATC TTT GAA ATC GGC TCA GG-3'	48°C
tet(w)	5'-GCCATCTTGGTGATCTCC-3' 5'-TGGTCCCCTAATACATCGTT-3'	55°C

4.2.4 Sequencing of the resistance genes

The identity of a positive amplicon *(tet* (W), in this case) was confirmed by PCR followed by sequencing.

The amplification was carried out with the DNA of *B. animalis* subsp. *lactis* strains. The PCR products of detected resistance gene, *tet*(W), were purified using a QIA quick PCR purification kit (Qiagen Ltd, UK), and sent for sequencing (Gene servicing, cambridge, UK) in order to verify and confirm their sequences.

4.2.5 Transferability of the resistance genes (*in vitro* conjugation)

The ability of *B. animalis* subsp. *lactis* to transfer *tet*(W) gene to other bacteria was investigated by conjugation experiments according to Ouoba *et al.* (2008). Isolate B10 was selected as a reliable representative of tested *B. animalis* subsp. *lactis* and its potential for donation of *tet*(W) to other Gram positive bacteria, such as *Enterococcus faecalis* JH2-2 and *Enterococcus faecium* BM 4105 (recipients) was studied. Donor and recipients were subcultured twice at 37 °C for 24 h in, RCA (donor) and non-selective BHI (CM1135, Oxoid)(recipient) in anaerobic and aerobic conditions respectively. An inoculum suspension was prepared for each and final concentration of cells was adjusted to 0.5 McFarland standards (10^7-10^8 CFU/g) by using of sensititre nephelometer (TREK Diagnostic Systems Ltd, UK) to measure optical density.

At the ratio of 10:1, 10 ml of donor suspension was mixed with 1 ml of recipient suspension at equal concentrations of cells and was filtered through a sterile membran filter (0.45 µm) (Whatman Laboratory Division, Maidstone, England) using a filter holder (Whatman Laboratory Division, Maidstone, England) and a vacuum pump (Welch Vaccum Thomas, Model No. 2522C-02 USA) (Figure 4.1).

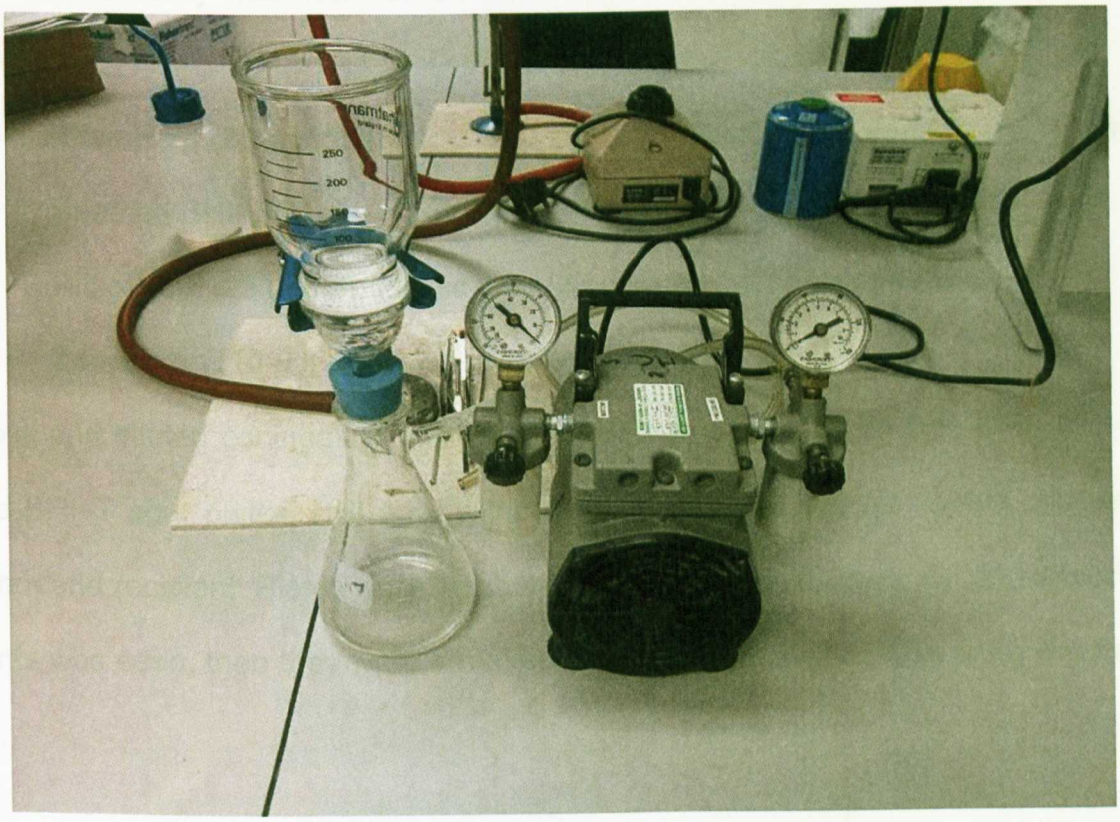


Figure 4.1 Arrangement of vacuum pump and membrane filter support system used for filtration of conjugation mixture of donor and recipient cells

For increased transfer frequency, 10 ml of sterile MRD (Maximum Recovery Dilution) was passed through the membran after filtering the mixed donor and recipient suspensions to trap the cells more tightly in the membrane (Sasaki *et al.* 1988; Gevers *et al.* 2003).

The filters were immediately laid on BHI agar (Oxoid) and incubated aerobically at 37 °C which is the optimum condition for recipients and a replicate was also set up to incubate anaerobically at the same temperature (best condition for donor).

After 48 h incubation, filters containing grown bacteria were inserted in 10 ml sterile MRD and shaked gently. Serial dilutions up to 10^{-5} were prepared of the conjugation mixture.

The transferability was detected on three different selective BHI agar plate (Oxoid) containing:

- Rifampicin (25 µg/ml), fusidic acid (25 µg/ml) and tetracyclin (10 µg/ml) which isnamed BHI–RFT.
- Rifampicin (25 µg/ml), fusidic acid (25 µg/ml) which is named BHI–RF.
- Tetracyclin (10 µg/ml) which is named BHI–T.

Stock and diluted conjugation mixture, 100 µl, was spread on to BHI–RFT, BHI–RF and BHI–T agar plates. BHI agar without antibiotic was used for control cultures of donor and recipient. Plates were incubated at 37 °C for 7-10 days. When transconjugants was seen, then they were streaked once more on BHI-RFT to verify the transfer.

4.2.6. Confirmation of identity of the transconjugants

The transconjugant cells were verified to be true transconjugants using rep-PCR and their fingerprints were compared with the fingerprints of recipients and donor.

4.3 RESULTS

Fifteen strains of *Bifidobacterium* species were selected from a variety of sources to study their antibiotic resistance profile by determination of MIC. Their genetic background of antibiotic resistance genes was studied by PCR reactions followed by gene transferability, which was carried out by filter conjugation.

4.3.1 Susceptibiliy to antimicrobials (including antibiotics)

Antibiotics can be categorised depending on their functions and also their specific targets. A summary of the results for each category is shown in Table 4.5.

Table 4.5 Antibiotics and their mechanisms of action (target)

Mechanism of inhibition	Antibiotic
Cell wall directed	β-lactam antibiotics, vancomycin
Protein synthesis	Aminoglycosides (such as streptomycin, kanamycin, gentamycin, amikacin), tetracycline, chloramphenicol, lincomycin and macrolides (such as erythromycin).
Nucleic acid synthesis	Quinolones, such as nalidixic acid and ciprofloxacin
Antimetabolite	Sulfonamides

4.3.1.1 Cell wall directed antibiotics

Table 4.6 shows all results obtained for MIC on tested isolates. The β-lactam antibiotics appeared to be the most effective antibiotics and all tested bacteria showed susceptibility to them. Penicillin was the most active and had a MIC value < 0.25 μg/ml for the all tested bacteria. With regard to the breakpoint, the isolates were considered susceptible to penicillin. The MIC value determined for ampicillin was < 1 μg/ml. Results demonstrated that all tested bacteria showed susceptibility to beta lactam, which inhibit the cell wall synthesis. Also all isolates showed susceptibility to vancomycin, which is not a β-lactam, but acts on cell wall synthesis. The MIC for vancomycin ranged from 0.25 to 1μg/ml.

4.3.1.2 Protein synthesis inhibitory antibiotics

Antibiotics that bind to the 30S ribosomal subunit act as inhibitors of protein synthesis. They are aminoglycosides, such as streptomycin, kanamycin, gentamycin, amikacin. The aminoglycosides irreversibly bind to the 30S ribosome and freeze the 30S initiation complex and no further initiation can occur. The aminoglycosides also slow down protein synthesis that has already initiated and induce misreading of the mRNA. It should be stated that tetracycline is also able to bind to the 30S ribosomal subunit, but it is not included in the group of aminoglycoside. Other antibiotics, which act as inhibitors of protein synthesis, are able to bind to the 50S ribosomal subunit. Chloramphenicol, lincomycin and macrolides, such as erythromycin are included in this group. Chloramphenicol and lincomycin bind to the 50S ribosome and inhibit peptidyl transferase activity. With the exception of B13, B14 and B15. strains, all other bacteria were resistant to kanamycin and streptomycin. The MIC value for

kanamycin ranged from 512 to < 1024 µg/ml and for streptomycin was more than 64 µg/ml.

All 15 isolates showed sensitivity to chloramphenicol; however, their reaction to other 50S ribosomal subunit directed antibiotics, such as tetracycline and erythromycin was variable. It should be pointed out that tolerance to erythromycin was lower than to chloramphenicol and tetracycline (Table 4.6). Three tested type strains (B13, B14, and B15) showed sensitivity to tetracycline, with MIC < 4 µg/ml; however, commercial strains showed resistance to tetracycline with MIC ranging from 16 to 32 µg/ml. Thirteen out of 15 isolates were susceptible to erythromycin and their MIC was < 0.25 µg/ml, but two commercial isolates (B2 and B7) showed reduced susceptibility and their MIC was 0.5 µg/ml.

4.3.1.3 Nucleic acid synthesis-inhibiting antimicrobials

Quinolones, such as nalidixic acid and ciprofloxacin, are able to inhibit the DNA synthesis. These antibiotics bind to the A subunit of DNA gyrase (topoisomerase) and prevent super coiling of DNA, thereby inhibiting DNA synthesis. Both commercial bifidobacteria and also three type strains tested in this study showed low susceptibility to nalidixic acid and ciprofloxacin. The MIC for nalidixic acid (32 µg/ml) was much more than for ciprofloxacin (4 µg/ml).

4.3.1.4 Antimetabolite antibiotics

They mainly inhibit folic acid synthesis. They bind to dihydrofolate reductase and inhibit formation of tetrahydrofolic acid.

There was no resistance to a combination of trimethoprim and sulfamethoxazole and all strains showed susceptibility with MIC ranging from < 0.12/2.38 to 4/76 µg/ml.

Table 4.6 Minimal Inhibitory Concentrations (µg/ml) of tested bifidobacteria

Strains / Antibiotic	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15
Amikacin	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)
Amoxicillin/ Clavulanic acid	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)	<1/0.5 (S)
Ampicillin	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)
Cefoxitin	1 (S)	<0.5 (S)	1 (S)	1 (S)	2 (S)	2 (S)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	<0.5 (S)	16 (S)	8 (S)	0.5 (S)
Ceftiofur	0.25 (S)	0.25 (S)	0.12 (S)	0.12 (S)	0.25 (S)	0.5 (S)	0.25 (S)	0.25 (S)	0.12 (S)	0.12 (S)	0.12 (S)	0.12 (S)	0.5 (S)	0.5 (S)	0.12 (S)
Ceftriaxone	1 (S)	0.5 (S)	0.5 (S)	0.5 (S)	1 (S)	1 (S)	0.5 (S)	1 (S)	1 (S)	0.5 (S)	1 (S)	0.5 (S)	2 (S)	2 (S)	0.5 (S)
Ciprofloxacin	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)
Chloramphenicol	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)	<2 (S)
Daptomycin	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	8 (R)	>16 (R)	1 (S)
Erythromycin	<0.25 (S)	0.5 (R)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	0.5 (R)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)

Table 4.6 *continued*: Minimal Inhibitory Concentrations (µg/ml) of tested bifidobacteria

Antibiotic \ Strains	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15
Gentamycin	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	256 (R)	256 (R)	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	256 (R)	<128 (R)	<128 (R)	<128 (R)
Kanamycin	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	512 (R)	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	512 (R)	256 (S)	256 (S)	256 (S)
Lincomycin	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)	<1 (S)
Linezolid	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	1 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)
Nalidixic acid	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)
Nitrofurantoin	<2 (S)	<2 (S)	<2 (S)	4 (S)	4 (S)	4 (S)	4 (S)	4 (S)	<2 (S)	4 (S)	4 (S)	4 (S)	<2 (S)	<2 (S)	<2 (S)
Penicillin	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)
Quinupristin/dalfopristin	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)
Streptomycin	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	<32 (S)	<32 (S)	<32 (S)
Tetracycline	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	16 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	<4 (S)	<4 (S)	<4 (S)

Table 4.6 *continued*: Minimal Inhibitory Concentrations (µg/ml) of tested bifidobacteria

Strains / Antibiotic	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15
Tigecycline	>0.5 (S)	0.25 (S)	0.25 (S)	0.5 (S)	0.25 (S)	0.12 (S)	0.5 (S)	0.25 (S)	0.25 (S)	0.25 (S)	0.25 (S)	0.25 (S)	0.12 (S)	0.12 (S)	0.12 (S)
Trimethoprim/ sulfamethoxazole	0.5/9.5 (S)	0.25/4.75 (S)	0.12/2.38 (S)	0.12/2.38 (S)	0.12/2.38 (S)	0.12/2.38 (S)	0.12/2.38 (S)	0.5/9.5 (S)	0.12/2.38 (S)	0.12/2.38 (S)	0.12/2.38 (S)	0.12/2.38 (S)	4/76 (S)	2/38 (S)	1/19 (S)
Tylosin tartrate	0.5 (S)	0.5 (S)	2 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	<0.25 (S)	<0.25 (S)	0.5 (S)	0.5 (S)	1 (S)	<0.25 (S)	<0.25 (S)	<0.25 (S)
Vancomycin	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	1 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	1 (S)	0.5 (S)	0.5 (S)	<0.25 (S)

(R) Indicated as Resistance, (S) Indicated as Sensetive.
B1-10 are *B. animalis* subsp. *lactis* strains isolated from probiotic dairy products
B11 and **B12** are commercial cultures of *Bifidobacterium animalis* subsp. *lactis,* Bb-12 and vital probiotic yogurt
B13, B14 and **B15** are type strains of *B. longum* NCTC11818, *B. breve* NCTC11815, *B. bifidum* NCTC13001, respectively.

4.3.2 Detection and characterization of *tet* (W) resistance gene

Based on the results obtained for bacterial resistance to antibiotics (Table 4.6), four out of 24 tested antibiotics were selected to be assessed for resistance determinants. As seen on Table 4.6, there is not uniformity among the tested isolates based on resistance or susceptibility; therefore, these 4 antibiotics along with their most common known genes were examined.

- Tetracycline: *tet*(M), *tet*(L), *tet*(S), *tet*(Q), *tat(*K), *tet*(O), *tet*(W)
- Kanamycin: *aph*(3") *-I, ant*(2") *-I, aph*(3") *–III*
- Streptomycin: *strA, strB, aadA, aadE*
- Erythromycin: *erm*(A), *erm*(B), *erm*(C)

The DNA of 15 tested isolates was subjected to genetic screening for all 17 different resistance genes using PCR, for the 4 selected antibiotics, mentioned above. The DNA was amplified by PCR with primers specific for the respective antibiotic resistance genes. Except *tet*(W), no positive amplicon was observed for tested strains. It should be pointed out that positive control was used for accuracy of experiment, and in all related experiments, they produced a clear positive band.

B. animalis subsp. *lactis* isolates showed positive PCR for resistance genes. Positive amplicons was obtained for resistance genes encoding tetracycline from all isolates of *B. animalis* subsp. *lactis*. Detected amplicon had a similar size compared to the positive control. However when the amplicon was sequenced, it was similar to published *tet*(w) sequences in the GenBank database.

Figure 4.2 shows the result of all 14 isolates for *tet*(W). A clear positive amplicon is observed for all *B. animalis* subsp. *lactis* strains (B1, B2,..., B12) and the positive control.

Due to absence of a positive PCR, the results of other tested genes were not shown.

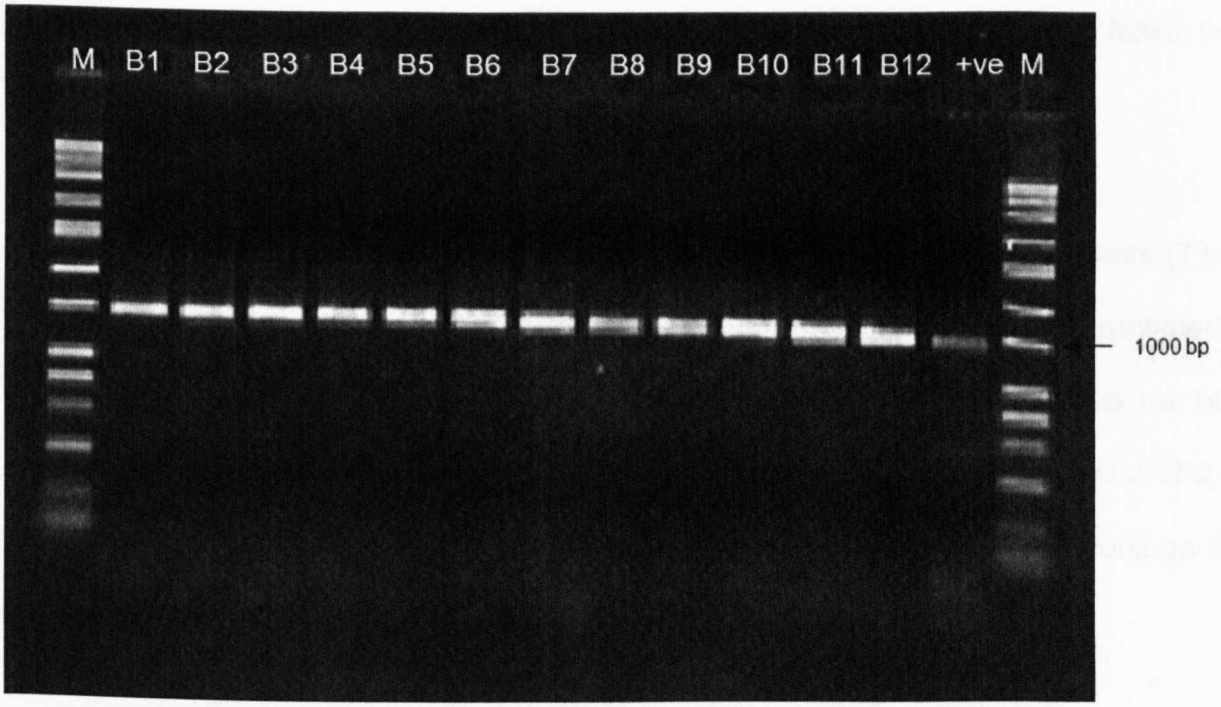


Figure 4.2 The presence of *tet*(W) in all tested isolates of *B. animalis* subsp. *lactis* (B1-B12) and positive control (+ve), M: DNA marker

4.3.3 *In vitro* conjugation for transfer of *tet*(W)

This transfer was chosen because *tet*(W) gene was detected in all isolates of *B. animalis* subsp. *lactis* and confirmed by sequencing and identified by the BLAST in GenBank database.

Due to presence of rifampicin (25 µg/ml) and fusidic acid (25 µg/ml), donor is not able to grow in BHI-RFT. Moreover, tetracyclin (10 µg/ml) suppress the growth of recipients in BHI-RFT.Only transconjugant cells form colonies on BHI–RFE agar plates.

After 2 days incubation of conjugation mixtures, growth was observed on BHI–RF and BHI-T which was related to the growth of recipient and donor respectively; however, transconjugant cells required at least a week to be visible on BHI–RFT agar and their growth on BHI–RFT agar was sparse.

Seven possible transconjugant cells were isolated from the double selective medium (BHI-RFT agar) with conjugation experiments with *E. faecalis* JH2-2. It should be

stated that conjugation experiment with *E. faecium* BM 4105 as recipient failed to show any transconjugants.

4.3.4 Typing of transconjugants

Rep-PCR was used for confirmation of transconjugants. Seven transconjugants (T1-T7) and *E. faecalis* JH2-2 (E) along with B10 as reference strains were differentiated by rep-PCR using GTG5 primer. As Figures 4.3 shows the fingerprints for six out of seven transconjugants exhibited the same profile as the recipient *E. faecalis* JH2-2. However, T6 showed a different pattern and was not confirmed as transconjugant and was omitted.

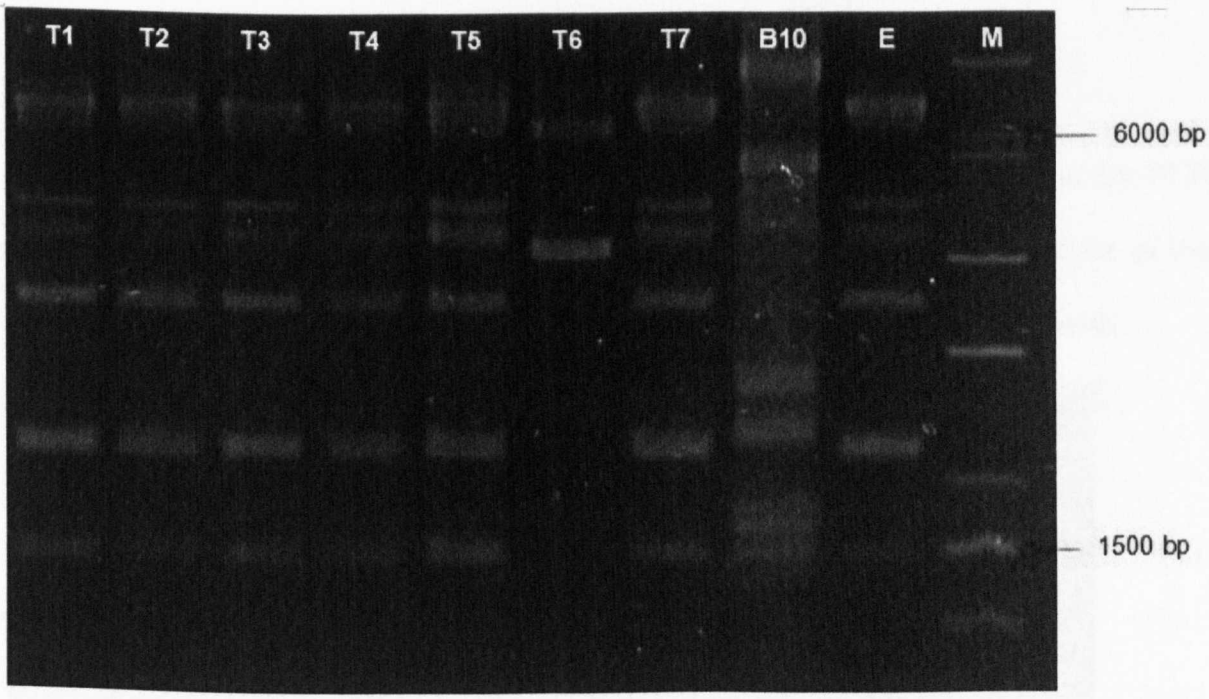


Figure 4.3 Rep- PCR profiles of transconjugants (T1- T7), donor (B10/*B. animalis* subsp. *lactis*) and recipient (E/ *E. faecalis* JH2-2), M: DNA marker

4.3.5 Antibiotic susceptibility testing and MIC determination for transconjugants

MIC was determined for all six identified transconjugants using a sensititre plate 96 well. The original recipient *E. faecalis* JH2-2 showed an MIC≤1 µg/ml and was sensitive to tetracycline. However, five out of six transconjugants showed MIC ranging from 8 to16 µg/ml and one presented 32 µg/ml which were very close to determined MIC for donor (16-32 µg/ml). It should be clearly stated that increased MIC in transconjugants was only due to conjugation with *B. animalis* subsp. *lactis*. According to EFSA report (2005) MIC breakpoint for entrococci is 16 µg/ml.

4.3.6 Detection of *tet*(W) gene in transconjugants by PCR

Finally, the presence of *tet*(W) in six transconjugant cells was determined by PCR amplification and specific primers as described previously (See 4.2.3). None of the six transconjugants showed positive PCR for the investigated resistance gene.

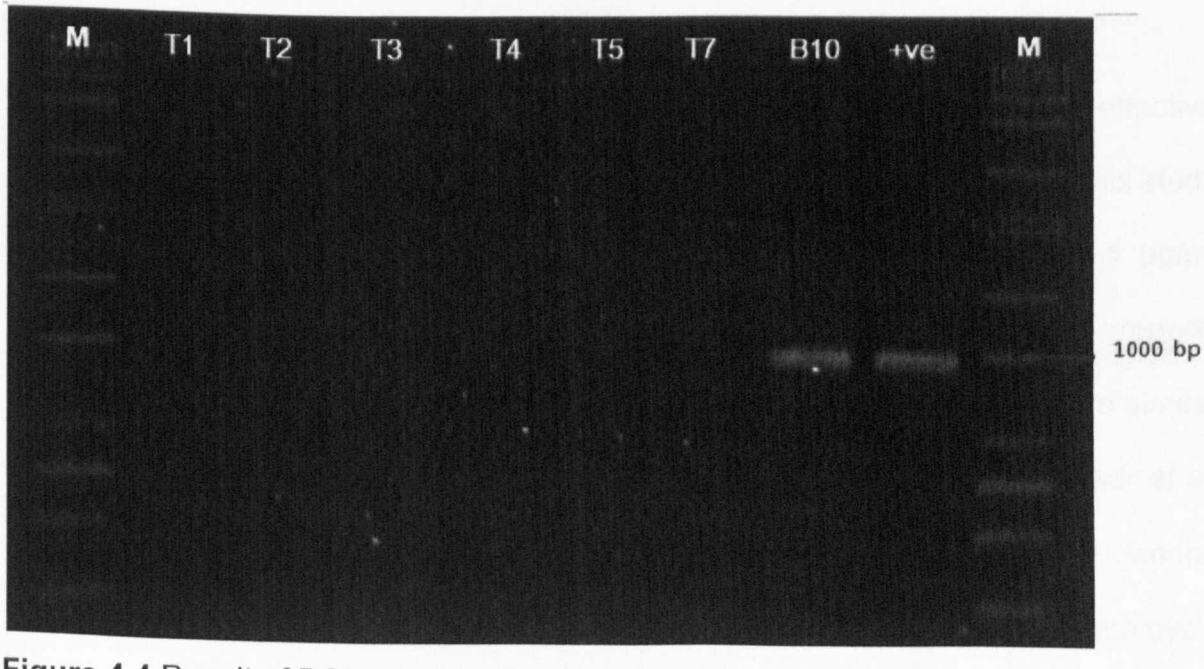


Figure 4.4 Result of PCR amplification for detection of *tet*(W) in transconjugants (T1-T7), donor (B10/*B. animalis* subsp. *lactis*) and positive control (+ve), M: DNA marker

4.4 DISCUSSION

Kheadr *et al.* (2004) stated two main reasons for study of antibiotic susceptibility: first evaluation of strains which may carry transferable antibiotic resistance genes and, secound also identification of some antibiotics for formulation of selective media.

Lim *et al.* (1993) determined the susceptibility of 37 strains of bifidobacteria from commercial yogurts and starter cultures to 18 antibiotic agents and reported that erythromycin was the most active agent with MIC of 0.19 µg/ml. They also reported a wide range of MIC for β-lactam antibiotics and susceptibility to tetracycline was variable with MIC ranging from 0.39 to 50 µg/ml. However, chloramphenicol had a narrow range from 1.56 to 6.25 µg/ml and the test organisms were most resistant to kanamycin and nalidixic acid.

Kheadr *et al.* (2004) stated that due to lack of cytochrome mediated drug transport in anaerobic bacteria, they show resistance to aminoglycosides. Also, Charteris *et al.* (1997) reported resistance of bifidobacteria to aminoglycosides

Vancomycin resistance is considered a major concern because it is widely effective against clinical infections (Zhou *et al.* 2005). However, all tested isolates in this study showed susceptibility towards vancomycin with MIC ranging from 0.5 to 1 µg/ml (under 0.25 only for one strain). The literature shows contradictory reports concerning the susceptibility of bifidobacteria to vancomycin. Our results are similar to those of Zhou *et al.* (2005) and Lim *et al.* (1993), but disagree with Kheadr *et al.* (2004) and Charteris *et al.* (1998) who stated vancomycin resistance is a general characteristic of bifidobacteria. Lim *et al.* (1993) determined the MIC for vancomycin which ranged from 0.39 to 3.12 µg/ml for 37 bifidobacteria strains and, as a result, the tested bifidobacteria were considered sensitive to vancomycin. However, Kheadr

et al. (2004) observed MIC above 250 µg/ml for bifidobacteria, and they reported that bifidobacteria were remarkably resistant to vancomycin. It should be stated that these researches were carried out using different methods. Accordingly, the contradictions in literature might be because of variations in assay techniques, methodologies and media used, because vancomycin diffuses poorly in agar media (Zhou *et al.* 2005).Therefore, it is necessary to standardize the applied methods.

The results from this study showed that all *B. animalis* subsp. *lactis* were resistant to kanamycin, gentamycin, nalidixic acid, amikacin, tetracyclin, ciprofloxacin, streptomycin and daptomycin. Also it was demonstrated that they are susceptible to cefoxitin, chloramphenicol, ceftriaxone, amoxicillin/clavulanic acid, ceftiofur, trimethoprim/ sulfamethoxazole, ampicillin, tigecycline, erythromycin, penicillin, vancomycin, nitrofurantoin, tylosin tartrate, quinupristin/dalfopristin, lincomycin and linezolid. This is also in agreement with the results of previous studies (Temmerman *et al.* 2003; Moubareck *et al.* 2005). Also, bifidobacteria show resistance to mupirocin and this antibiotic was recommended in the selective media for their isolation (Thitaram *et al.* 2005). Mupirocin is now the selective agent in the selective medium TOS-MUP for enumeration of bifidobacteria in fermented milks (IDF/ISO 2010).

Resistance to certain antibiotics is not an unusual feature of starter and probiotic cultures. Much of this resistance is due to complex intrinsic features, such as cell wall structure or metabolic properties. However, *tet*(W) was found in this research as gene associated to tetracycline resistance

The *tet* genes are most abundant antibiotic resistance determinants which were described for bifidobacteria and are involved in resistance to tetracycline and coding for ribosomal protective proteins (Gueimonde *et al.* 2010). Aires *et al.* (2007) stated that *tet*(W) is the most commonly found gene in bifidobacteria and also the G+C content

in *tet*(W) (50-55%) is very close to that in the bifidobacteria chromosome (58%); therefore, it might be the reason for widespread occurrence of this gene among bifidobacteria.

Eighty nine strains of bifidobacteria were studied by Aires et *al.* (2007) of which 33% of tested bifidobacteria was reported as tetracycline resistant. They could not find *tet* (O) in any of tested bifidobacteria, but *tet*(W) was found in 83% of tetracycline resistant strains and also prevalence of *tet*(M) was 21% (Aires *et al.* 2007). It should be stated that they did not report any *tet*(M) in four strains of *B. animalis* subsp. *lactis* which were included in their study and these findings agree with the research herein reported.

Gueimonde *et al.* (2010) also reported the presence of *tet*(W) gene in 26 strains of *B. animalis* subsp. *lactis* from a variety of sources, such as probiotic supplements and fermented dairy products.

To our knowledge, there is little research about transferability of *tet*(W) in *B. animalis* subsp. *lactis*. Gueimonde *et al.* (2010) reported no transfer of *tet*(W) to any of tested recipients.

In this research, the possibilty of horizontal transfer of *tet*(W) from *B. animalis* subsp. *lactis* to *E. faecalis* by *in vitro* filter conjugation approach was studied. The transfer frequency might be affected by several parameters. Size and type of membran filter, age and ratio of donor and recipient, the volume of fluid passing through the filter (Sasaki *et al.* 1988; Gevers *et al.* 2003). Sponge like membrane with 0.45μm pore size was suggested by Sasaki *et al.* (1988) for improvement of transfer frequencies. Ouoba *et al.* (2008) stated that incubation time is an important factor for recovering of transconjugant cells. Current research confirms this statement as transconjugants were recovered after 7-10 days incubation at 37°C rather than 2 days.

However, no transconjugants could be obtained from conjugation with *E. faecium* BM 4105 and it seems the detected *tet*(W) gene could not be transferred from B10 to *E. faecium* BM 4105.

Some useful information, which has been derived from this research are as follows:

- The best result of conjugation was obtained when filter conjugation experiment was carried out under optimum growth condition of the recipient. Therefore, the conjugation mixture incubated in anaerobic condition (donor maximum growth condition) did not present any transconjugants. These results support Sasaki *et al.* (1988) who stated that for a best result of transconjugation, optimum growth of recipient cells is necessary.
- Most transconjugants were recovered from stock solution (contained filter) and no growth was seen for diluted samples.
- Transconjugants were only obtained when experiment was carried out under vaccum filtration.

4.5 CONCLUSION

In conclusion, out of the 17 studied resistance genes, *tet*(W) was discovered in *B. animalis* subsp. *lactis* which demonstrated resistance to tetracycline with MIC above 32 µg/ml. While some of the strains seem to be intrinsically resistant to amikacin, ciprofloxacin, gentamicin, nalidixic acid, kanamycin and streptomycin, all tested strains were sensitive to a number of clinically effective antibiotics. In conclusion, *B. animalis* subsp. *lactis* may be considered a reservoir of organisms of antibiotic resistance gene which contain *tet*(W) gene for tetracycline and is transferable to other bacteria.

Chapter 5

In vitro assessment of the isolates for functional properties: biofilm formation

5.1 INTRODUCTION

The performance of microorganisms (e.g. bacteria) that grow on a surface could be different from free-floating bacteria. Biofilm can be defined as a population of attached microorganisms which grow on a surface and are in the form of multicellular aggregates enclosed by an exopolysaccharide matrix (Donlan 2002).

It has been well documented that in natural environment, bacterial cells are most often discovered in close association with surfaces and interfaces as surface attached biofilm. They are surrounded with extracellular matrix that are produced by themselves and protect them against harsh conditions (Branda *et al.* 2005). Biofilms also play an essential role in the close relationship between the human body and its inhabitant bacteria (Lebeer *et al.* 2007). There are enormous changes during conversion of planktonic microorganisms to sessile cell communities. Four stages of conversion are observed during biofilm formation: initiation, maturation, maintenance and dissolution (O`Toole *et al.* 2000). Usually, these transformations are a response to specific environmental stresses. Microorganisms are able to apply a variety of resistance mechanisms when some environmental factors worsen. In nature, biofilm networks play a role as a protective shelter for microorganisms against environmental stresses, such as antibiotics, bacteriocins, disinfection materials, pH and nutrient shortage/availability (Xu *et al.* 2000). Usually, two types of microorganisms are observed in biofilm networks: (a) single microbial species that are present in infections and on the surface of medical implants, and (b) multiple microbial species, usually mixed microbial species predominant in most environments (O`Toole *et al.* 2000).

As mentioned above, biofilm formation might be affected strictly by nutrient availability or shortage in several environmental conditions. In most Gram negative

microorganisms, transition from planktonic cells to attached cells occur when they are located in nutrient rich medium and if fresh medium is not provided they will detach from the biofilm state and revert to free living mode to search for a new source of nutrients (Kolter *et al.* 1993). It should be noted that environmental stresses vary among microorganisms. Some organisms, such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens,* are able to produce biofilm in any growth condition. Some strains, such as *Escherichia coli* K-12 and *Vibrio cholerae,* form biofilms only in amino acid fortified medium, but *Escherichia coli* O157:H7 can form biofilms in minimal medium (Dewanti and Wong 1995). Many other environmental stresses have been reported to affect biofilm production, such as temperature, osmolarity, pH, oxygen and bile salts (Lebeer *et al.* 2007).

The potential of probiotic bacteria for adherence and also colonisation have drawn attention as main contributing aspects for immune modulation, enhanced contact with the mucosa and pathogen inhibition. Ideally, probiotics are expected to remain in the body for a long-lasting period of time after ingestion. However, that is not always the case and most probiotics are considered as transient flora, resulting in a sharp decline of probiotic organisms after discontinued consumption. It is suggested that production of biofilm by these organisms could ensure or at least increase the chance of their colonization in the intestines. In a study by Lebeer *et al.* (2007), they demonstrated that *Lactobacillus rhamnosus* GG is able to form biofilms on abiotic surfaces *in vitro*, in contrast to other related lactobacilli tested.

Therefore, probiotic bacteria would be able to improve the inhabitant bacteria, which build up an essential part of the mucosal hurdle and colonisation resistance against pathogens (Tuomola and Salminen 1998; Tuomola *et al.* 1999).

Biofilm formation by *Bifidobacterium* spp. has not been investigated so far; although a few studies have been carried out for determination of LAB potential for biofilm formation (Lebeer *et al.* 2007, Kubota *et al.* 2008). In this study, the potential for biofilm formation was investigated in 10 isolates of *B. animalis* subsp. *lactis* from fermented milks, two commercial cultures of *B. animalis* subsp. *lactis* kindly provided by Chr. Hansen and Danisco and two type strains of *B. longum* and *B. breve*. Furthermore, the biofilm formation capacity of the above mentioned bacteria in different conditions of nutritional stress was compared.

5.2 MATERIAL AND METHODS

5.2.1 Media

The media used in this study were Reinforced Clostridial Medium (CM0149; RCM, Oxoid, Basingstoke, UK), Tryptone Soya Broth (CM0129; TSB, Oxoid, Basingstoke, UK), de Man Rogosa Sharpe broth (MRS) (CM0359, Oxoid, Basingstoke, UK) and MRS broth supplemented with 0.5% w/v cysteine (MRSC). Also, the four media mentioned above were diluted to 1/2 and 1/20 strength; hence, 12 variants of the four media (MRS broth, MRSC broth, RCM, TSB and their 1/2 and 1/20 dilutions) were prepared and autoclaved at 121°C for 25 min. Anaerobic conditions were achieved by the use of anaerobic incubator (80% N_2, 10% CO_2, and 10% H_2; Don Whitley, Skipton, UK).

5.2.2 Quantitative biofilm assay

Quantification of biofilm formation was performed according to the method of Stepanovic *et al.* (2007) with some modifications.

The inuculum was prepared from overnight culture of isolates (grown colonies on RCA). The harvested colonies were added to MRD solution and adjusted to to make a standard suspension (final concentration of ca 10^7-10^8 CFU/ml) using a calibrated sensititre nephelometer (TREK, Diagnostic Systems Ltd., UK).

Each medium (180 µl) was poured into the wells of a sterile 96 well flat bottomed polystyrene micro plate (Sero-Well, UK). The inoculum (20 µl) was added to each well containing 180 µl of the medium (a ratio of 1:10). The negative control wells contained just 200 µl broth (MRSC, MRS, RCM, TSB and their 1/2 and 1/20 dilutions). The micro plates were incubated anaerobically for 48 h at 37 °C. At this stage the OD was read as indicator of planktonic growth at 595nm. Then the

contents of the micro plates were poured out. Each well was washed three times with 200 µl of sterile phosphate buffered saline (PBS, pH 7.2). After washing, the remaining attached bacteria were fixed by keeping the plates for an hour at 60 °C. The adherent biofilm layer in micro plates were stained with 200 µl per well of 0.1% v/v crystal violet solution (Sigma-Aldrich, UK) for 15-30 min at room temperature. Excess stain was rinsed off by placing the micro plate under running tap water. The micro plates were then air driedand the dye bound to the adherent cells was re-solubilised with 200 µl of bleaching solution (ethanol/acetone 80/20). The optical density of each well containing the bleaching solution was measured at 595 nm. Based on the OD obtained, the following categories were suggested: (a) no biofilm producer, (b) weak biofilm producer, (c) medium biofilm producer and (d) strong biofilm producer. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control (un-inoculated medium) (Stepanovic *et al.* 2007).

ODC: (Mean negative control OD_{595} + 3 SD)

OD: Mean sample OD_{595}

Strains were therefore classified as follows:

- OD ≤ ODc = no biofilm producer
- ODc < OD ≤ (2 x ODc) = weak biofilm producer
- (2 x ODc) < OD ≤ (4 x ODc) = moderate biofilm producer and
- (4 x ODc) < OD = strong biofilm producer

All tests were carried out in triplicate and the results were averaged.

5.3 RESULTS

This study aimed to determine the potential biofilm formation among bifidobacteria strains and possible differences within the tested strains and species.

The results of the evaluation of cell growth of bifidobacteria cultivated in four different media revealed that all tested strains were able to grow well in MRSC broth and RCM. However, their growth in other tested broth media (MRS and TSB) was not as much as MRSC broth and RCM (data are presented in appendix 3). Diluting the medium reduces the available nutrients, which significantly influenced the cell growth of tested microorganisms.

5.3.1 Influence of growth media on biofilm formation

The results of biofilm formation on plastic surfaces by bifidobacteria cultivated in different media and dilutions thereof revealed that all tested cultures except *B. longum* and *B. breve* produced biofilm in original medium and also in dilutions of the medium. However, the quantities of biofilm produced by strains were greater in MRSC broth (Figure 5.1) and RCM (Figure 5.3) than those produced in MRS broth (Figure 5.2) and TSB (Figure 5.4). The nutrient content of diluted medium influenced the quantity of biofilm produced by tested isolate. MRSC broth was the most effective medium in promoting biofilm production as 10 out of 14 tested strains were considered moderate biofilm producer in this medium (Table 5.1), followed by RCM, in which half of tested strains were considered as medium biofilm producer. However, TSB and MRS broth were the least effective media with no significant difference between them for all tested strains (weak biofilm formation) (Figure 5.2 and 5.4). The results also illustrated that diluting all media to 1/2 and 1/20 strength resulted in much less biofilm formation. This could indicate that nutrients are effective

factor in promoting biofilm production by tested strains and as conditions turn worse at 1/20 dilution, the quantity of biofilm was much less than in 1/2 dilution.

Bifidobacteria are sensitive to oxygen and their growth in presence of oxygen is negligible. MRS broth is not a suitable medium for bifidobacteria perhaps due to lack of reducing agents, such as L-cystein and they can not grow well in this medium. L-cystein as an oxido-reductant agent can provide strict anaerobic conditions in growth media. Figure 5.2 shows that biofilm formation in MRS broth is much less than MRSC broth and it might be because of presence of dissolved oxygen. All tested isolates except type strains were able to produce some biofilm in RCM medium, but not as much as in MRSC broth. However, the potential of biofilm formation for all isolates was reduced as the medium became nutrient limited by dilution (Table 5.1).

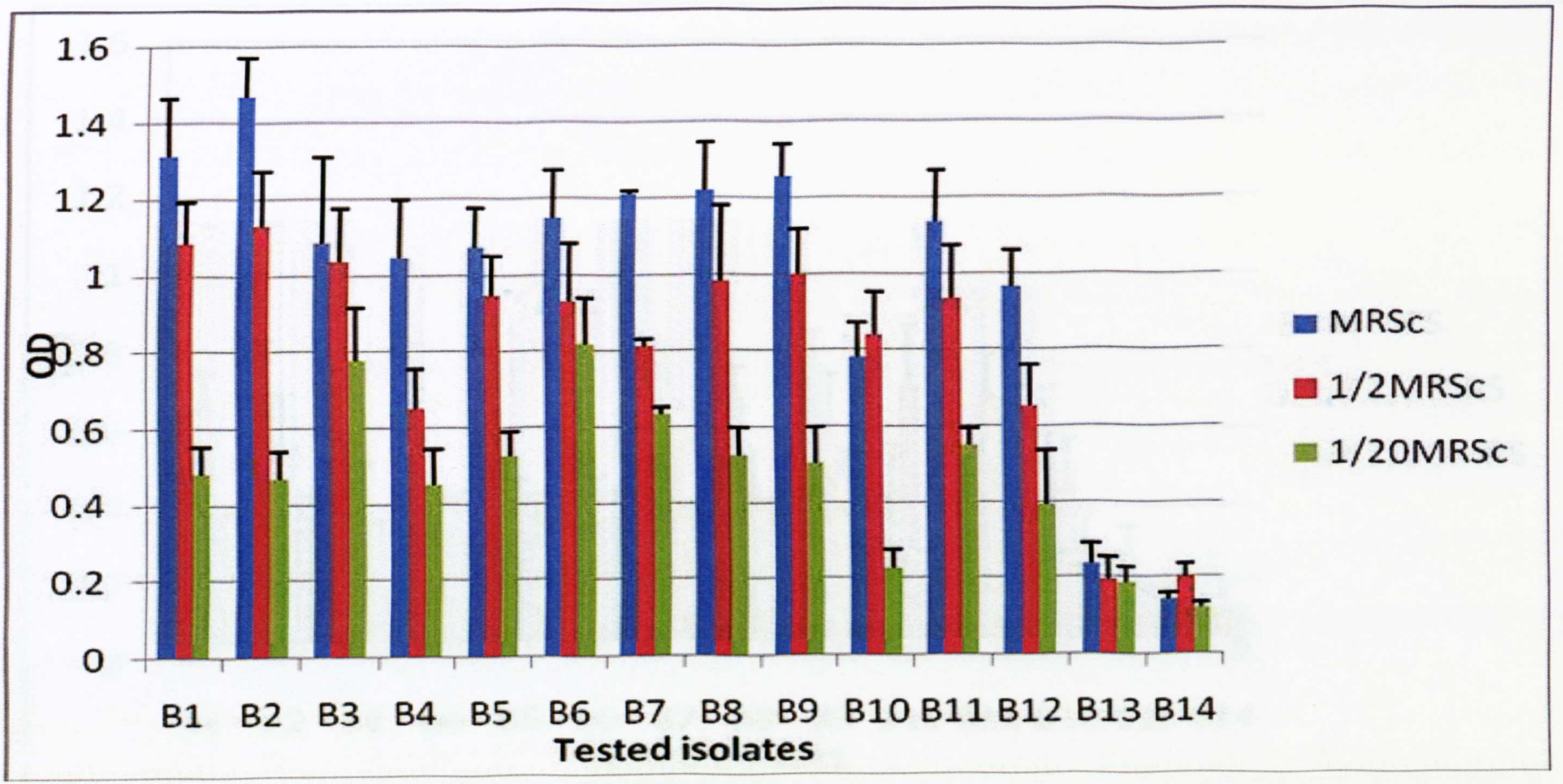


Figure 5.1 Biofilm formations of isolates (expressed as OD) in original and diluted MRSC broth after 48 h incubation at 37°C
B1-B12: *B. animalis* subsp. *Lactis,* B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815, OD: optical density,
Data are means ± SD of three replications

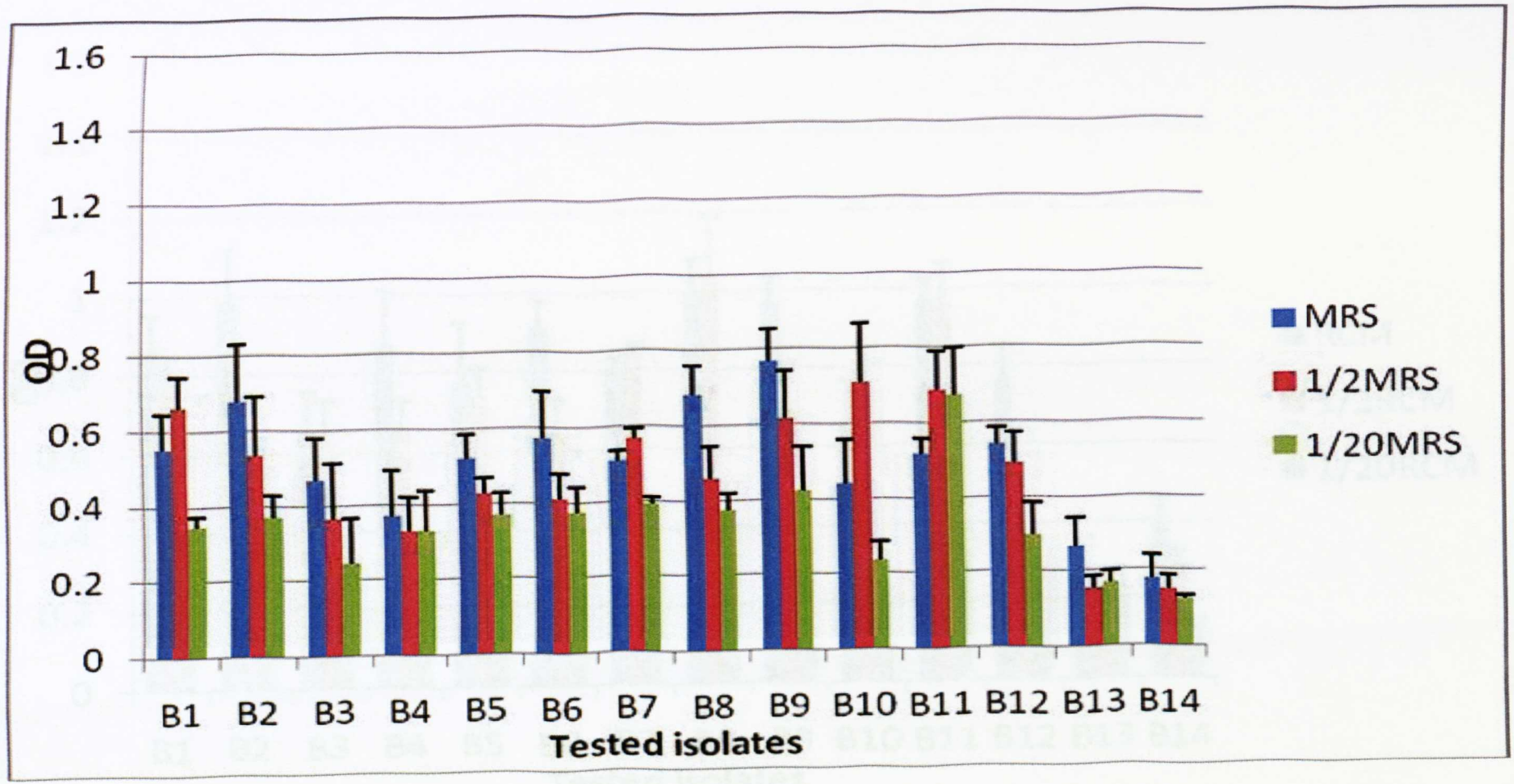


Figure 5.2 Biofilm formations of isolates (expressed as OD) in original and diluted MRS broth after 48 h incubation at 37°C B1-B12: *B. animalis* subsp. *Lactis,* B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815, OD: optical density, Data are means ± SD of three replications

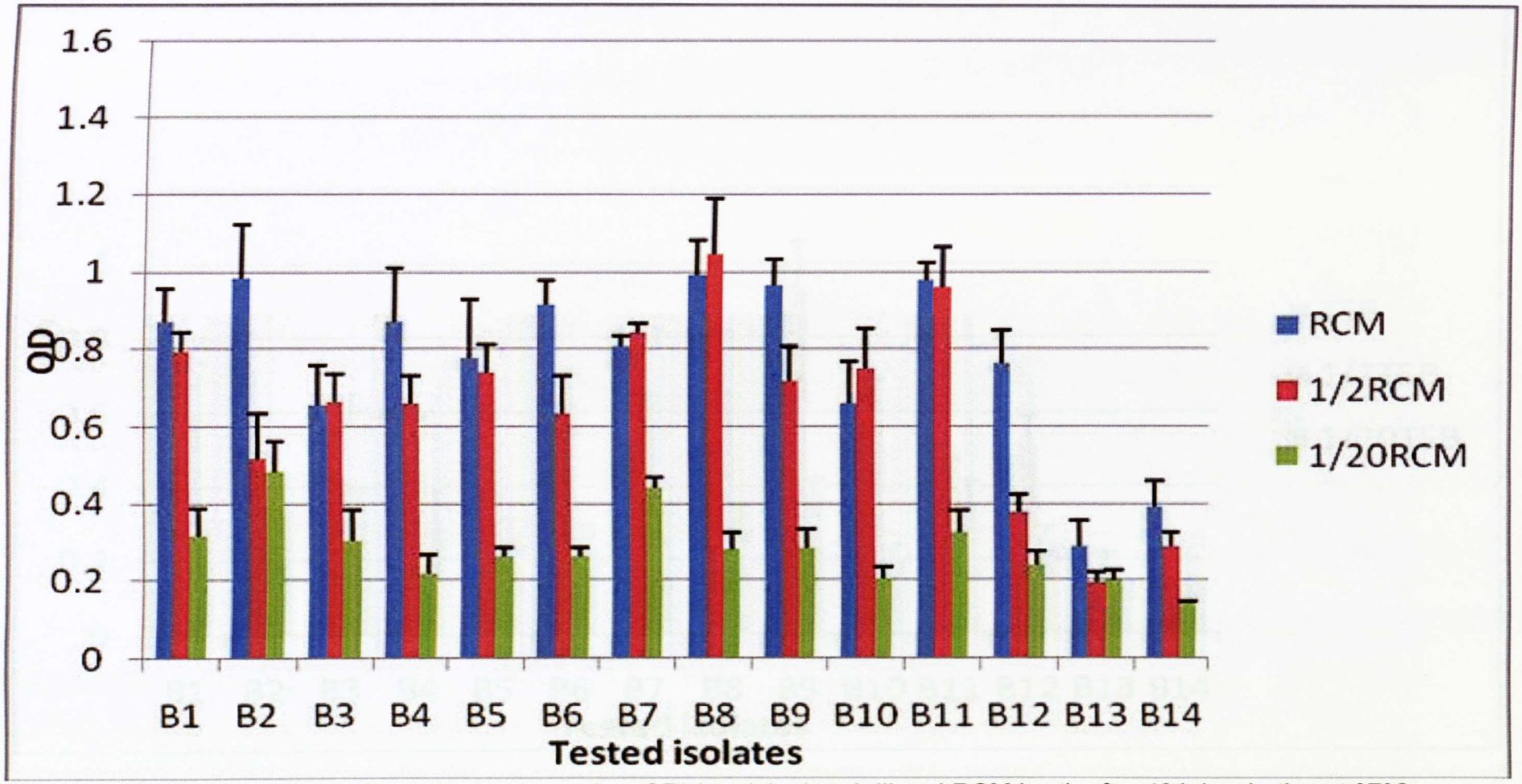


Figure 5.3 Biofilm formations of isolates (expressed as OD) in original and diluted RCM broth after 48 h incubation at 37°C
B1-B12: *B. animalis* subsp. *Lactis,* B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815, OD: optical density,
Data are means ± SD of three replications

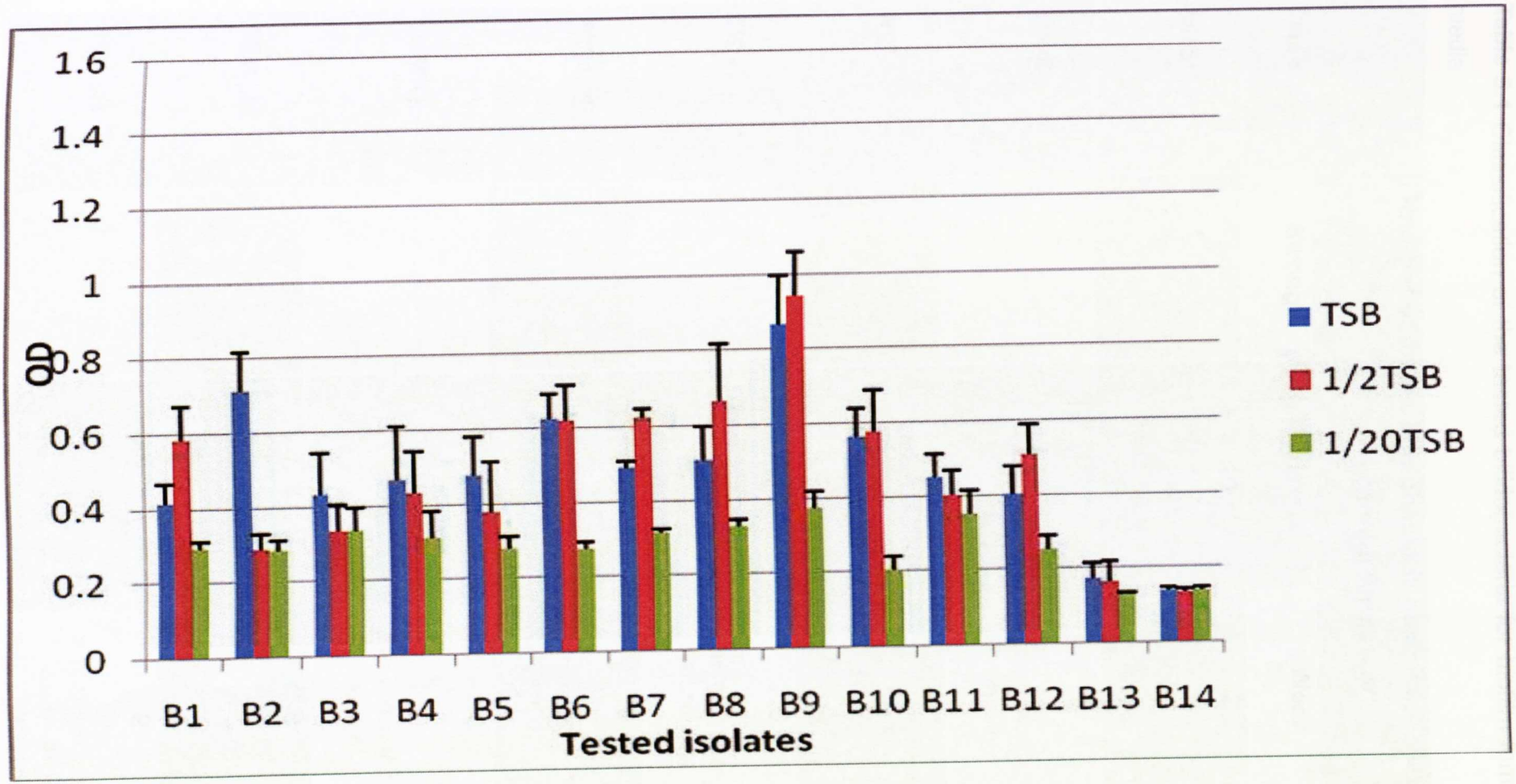


Figure 5.4 Biofilm formations of isolates (expressed as OD) in original and diluted TSB broth after 48 h incubation at 37°C B1-B12: *B. animalis* subsp. *Lactis*, B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815, OD: optical density, Data are means ± SD of three replications

Table 5.1 Classification of the tested bifidobacteria for biofilm formation in different media

	Number of tested bifidobacteria classified in each category for biofilm formation			
Media	**Strong**	**Moderate**	**Weak**	**No biofilm**
MRSC		10 (B1,B2,B3,B4,B5,B6,B9, B10,B11,B12)	2 (B7,B8)	2 (B13,B14)
MRS		3 (B4,B5,B9)	7 (B1,B2,B3,B6,B7,B10, B11,B12)	4 (B8,B13,B14)
RCM		7 (B1,B3,B4,B5,B6 ,B10,B11)	6 (B2,B7,B8,B9,B12,B14)	1 (B13)
TSB		0	10 (B1,B2,B3,B4,B5,B6,B9 ,B10,B11,B12)	4 (B7,B8,B13,B14)
1/2MRSC		6 (B2,B5,B7,B8,B9,B11)	7 (B1,B3,B4,B6,B10,B12, B14)	1 (B13)
1/2MRS		0	10 (B1 ,B3,B4,B5,B6,B7,B9, B10,B11,B12)	4 (B2,B8,B13,B14)
1/2RCM		4 (B1,B7,B8,B11)	9 (B2,B3,B4,B5,B6,B9, B10,B12,B14)	1 (B13)
1/2TSB		1 (B6)	9 (B1,B4,B5,B7,B8,B9, B10,B11,B12)	4 (B2,B3,B13,B14)

Table 5.1 *continued*: Classification of the tested bifidobacteria for biofilm formation in different media

	Number of tested bifidobacteria classified in each category for biofilm formation			
Media	**Strong**	**Moderate**	**Weak**	**No biofilm**
1/20MRSC		1 (B1)	9 (B2,B3,B5,B6,B7,B8,B9,B10,B11)	4 (B4,B12,B13,B14)
1/20MRS		0	7 (B2,B3,B6,B8,B9,B10,B11)	7 (B1,B4,B5,B7,B9,B12,B13,B14)
1/20RCM		1 (B6)	7 (B1,B2,B3,B4,B5,B9,B10)	6 (B7,B8,B11,B12,B13,B14)
TSB		2 (B6,B11)	8 (B1,B2,B3,B4,B5,B7,B9,B10)	4 (B8,B12,B13,B14)

B1-B12: *B. animalis* subsp. *Lactis*, B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815

5.4 DISCUSSION

The experiments performed in this study enabled quantification of biofilm formation of tested bacteria. Evaluation of biofilm formation by *B. animalis* subsp. *lactis* in this study revealed that these isolates possess a high capacity for biofilm formation on plastic surfaces. However, the significance of this study originates from the fact that no study has been reported for bifidobacteria so far.

In general, stainless steel and glass are hydrophilic materials while plastic and rubber are hydrophobic materials. Sinde and Carballo (2000) have shown that microorganisms adhere in higher numbers to more hydrophobic materials. It was for this reason that micro plates were used for biofilm formation in this study.

It is well known that many factors influence biofilm formation and composition of media could be one of them (Djordjevic *et al.* 2002). Therefore, four broth media at different dilutions were selected for this study: MRSC (cystein helps to create anaerobic condition) and RCM were chosen as nutritious laboratory media that are frequently used for cultivation and enumeration of anaerobes; MRS that lacks L-cystein and TSB, which is a relatively less rich culture medium but frequently used in biofilm investigation. Diluted media (1/2 and 1/20) were chosen to mimic some food that are less nourishing, since these diluted media may not supply as much nutrients as rich laboratory media (Leriche and Carpentier 2000).

The four culture media used (undiluted and diluted) to investigate the biofilm production, led to different levels of biofilm formation by tested bacteria. Hood and Zottola (1997), who investigated biofilm formation by five different bacterial species, found that the medium, which induced the production of the highest quantities of biofilm, was different for each tested microorganism. They showed that *Listeria monocytogenes*, in general, produced more biofilm in nutrient rich media than

Salmonella typhimurium which produced more biofilm in nutrient limited medium. However, the composition of the medium did not have the same extent of influence on all tested strains.

The majority of tested *B. animalis* subsp. *lactis*, but not all tested strains, produced moderate quantities of biofilm in MRSC broth (71.4%). Furthermore, irrespective of the composition of the medium, quantities of produced biofilm by tested strains classified them only as moderate or weak biofilm producers.

Biofilm formation is a complex process regulated by diverse factors, including the growth medium (Donlan 2002), but is still poorly understood. One possible explanation for different response of microorganism to environmental conditions could be the results of mutations in genes that control biofilm formation (Romling *et al.* 1998).

This study demonstrated that *B. animalis* subsp. *lactis* readily form biofilm on plastic surfaces. Biofilm formation by these bacteria was significantly affected by the growth medium composition. In general, *B. animalis* subsp. *lactis* produced more biofilm in nutrient rich medium. It seems that there is a direct relationship between biofilm formation and growth of free living cells in media. Nutrient shortages and low availability of fermentable carbon sources (in diluted media) led to limited or no growth. However, growth limitation was not enough to induce biofilm formation and sometimes this reaction might be different among several strains of one microorganism. Stepanovic *et al.* (2004) investigated the biofilm formation by *Salmonella* spp. and *L. monocytogenes* strains on a plastic surface. They found that composition of the medium did not have the same influence on all tested *Salmonella* spp. and *L. monocytogenes* strains, at least, not to the same extent.

Much has been said about the formation of microbial biofilms on a wide range of natural environments for example sediments, soils and animal body especially in the oral cavity, skin as well as the gastrointestinal tracts (Macfarlane and Macfarlane 2006).

Attachment to epithelial cells, potential colonization power and better contact with the mucosa are regarded as causative factors for some beneficial effects of probiotics including modulating the immune system and combating the pathogens by their prohibition (Tuomola and Salminen 1998).

The colonization capacity of probiotic strains has been the subject of many *in vitro* studies where mucus or intestinal epithelial cell lines like Caco-2 and HT-29 cells have been used (Tuomola *et al.* 1999). Although such investigations could signify the primary connection of ingested probiotics with the intestinal wall, they may not necessarily be considered as a guaranteed indication of colonization.

Earlier researches have proven that biofilms are not just stacks of microorganisms and a slime matrix that bonds and safeguards them, but they are highly programmed and distinguished population with a greater capability to defy environmental stresses. It is also suggested that mucosal biofilm in the human colon could play a role in diseases process. Although many acute contagious diseases are caused by the so called free-floating (also known as planktonic) bacteria, it is estimated that more than 80% of infectious diseases in the developed world are caused by biofilms. Examples include infections of the urogenital and gastrointestinal tract (National Institutes of Health "NIH" 2002). The importance of biofilm formation by probiotics lies in their relationship with antimicrobials.

When grown under planktonic conditions many bacteria are sensitive to antimicrobials; however, biofilms are extremely hard to treat with antimicrobials. The

mechanisms are not clear, but it is suggested that they may inactivate or prevent penetration of antimicrobials. Such capacity could lead to resistance of probiotics to antibiotics which is considered encouraging when probiotics are given along with antibiotics for treatments of GIT infections. On the other hand biofilms could increase the opportunity for gene transfer between/among bacteria. Hence, from this point of view, production of biofilm by probiotics will not be welcomed, since such gene resistance transfer to neighbouring organisms could turn them into a virulent pathogen (NIH 2002).

5.5 CONCLUSION

Bifidobacterium animalis subsp. *lactis* was able to produce relatively good level of biofilm in two of the tested media. These data show some differences among the 10 isolates from fermented milk, which in turn could be interpreted as being different strains or more likely be a result of going through different processing conditions and exposed to various stress condition. This, however, needs further investigations.

Chapter 6

In vitro assessment of the isolates for functional properties: antimicrobial activities

6.1 INTRODUCTION

Several criteria have been considered for a strain to be recognised as probiotic, of which inhibition of pathogenic bacteria is one of the most important ones (Gopal *et al.* 2001). Bifidobacteria exert an essential role in the management of gastrointestinal infections (Cheikhyoussef *et al.* 2008). Their potential role in prevention and treatment of intestinal disturbance and diseases has been documented elsewhere (Collado *et al.* 2005, wildt *et al.* 2011).

Most probiotics, such as bifidobacteria, have the ability to produce organic acids like acetic and lactic acids, so they are able to lower the pH in the intestine which, as a result the growth of pathogenic bacteria, may be restricted. It has also been reported that some bifidobacteria produce bacteriocins which have antagonistic effects against pathogenic bacteria (Yusof *et al.* 2000).

The first report of antimicrobial activity by bifidobacteria was published by Tissier; where he stated several antagonistic effects of *Bifidobacterium bifidum* over *Escherichia coli* (Tissier 1900). The antagonistic effect of bifidobacteria could be exclusively due to the production of lactic and acetic acids with their pH lowering effect (Ibrahim and Bezkorovainy 1993). However, others believe that in addition to the pH effects, the secretion of proteinaceous compounds also contributes to antimicrobial activities of bifidobacteria (Lee *et al.* 2003; Collado *et al.* 2005).

Probiotic bacteria are able to adhere to the host colon cells and locate in the mucin layer and make a defence layer against pathogenic bacteria. The adhesion of bifidobacteria in healthy adults' intestinal mucosa is less in elderly people. Bifidobacteria have the ability to block the specific receptors by steric obstacles and hence the adhesion of pathogenic bacteria to host mucosa would be restricted (Bevilacqua *et al.* 2003).

Generally, bacteriocins are antimicrobial proteins, which are produced by some Gram positive bacteria. It seems that they exert more bactericidal than bacteriostatic effects. Bacteriocins are resistant to variable pH and temperature, but they are sensitive to proteolytic enzymes. They are mainly secreted in the early stage of the stationary phase and their synthesis is associated with a plasmid encoded gene, hence it could be considered as a strain specific characteristic (Yildirim *et al.* 1999).

In this part of the project, the potential antagonistic activity of 14 *Bifidoacterium* spp. against four pathogen organisms was studied. Due to growth difficulties, *B. bifidum* was excluded from this experiment.

6.2 MATERIALS AND METHODS

6.2.1 Media

De Man, Rogosa, Sharp agar (MRS), Reinforced clostridial agar (RCA), Reinforced clostridial medium (RCM), Maximum Recovery Diluent (MRD) Tryptone soy broth (TSB) and Nutrient agar (NA) all from Oxoid, UK were prepared as recommended by manufacturer.

6.2.2 Microorganisms

The antimicrobial activities of 10 isolates of *B. animalis* subsp. *lactis* from fermented milks, two commercial cultures of *B. animalis* subsp. *lactis* kindly provided by Chr. Hansen and Danisco and two type strains of *B. longum* NCTC11818 and *B. breve* NCTC11815 was assessed.

The inhibitory properties of the isolates were investigated against the following four bacteria (indicator organism), from Microbiology Research Unit (MRU) culture collection:

- *Salmonella typhimurium* DT124
- *Salmonella enteritidis* P125582 (PT4)
- *Escherichia coli* NCIMB 555
- *Escherichia coli* NCTC 12900

6.2.3 Screening bifidobacteria isolates for antimicrobial activities

6.2.3.1 Spot test

I) Preparation of *Bifidbacterium* spp. inoculum:

A suspension in MRD (final concentration of ca 10^7 CFU/ml) was made from overnight culture of isolates grown on RCA. For this purpose, after two consecutive subcultures of Bifidobacteria on RCA, a stock solution was prepared by suspending colonies in 1 ml of sterile MRD, then a few drops of this stock solution was added to 5 ml MRD until final concentration of cells reach to 0.5 MacFarland standard (10^7-10^8 CFU/ml) using a calibrated sensititre nephelometer (Trek Dignostic Systems Ltd, UK). This suspension was used as inoculum for further experiments.

II) Preparation of indicator organisms:

The active overnight culture of the indicator bacteria was prepared by growing each organism on nutrient agar. The harvested colonies were added to MRD solution and adjusted to to make a standard suspension (final concentration of ca 10^7-10^8 CFU/ml) using a calibrated sensititre nephelometer (TREK, Diagnostic Systems Ltd., UK).

III) The test:

The spot test recommended by Bernet *et al.* (1993) was used. An aliquot (2 µl) of active suspension of test bacteria was spotted on RCA, and plates were left for half an hour to dry at room temperature and then incubated at 37 °C for 18 h under anaerobic condition. The grown colonies were overlaid with 10 ml of soft TSB (containing 0.8% w/v agar) at 45°C which was seeded with 100 µl (final concentration of ca 10^7 CFU/ml) of the indicator organisms. The overlaid plates were incubated at 37°C for another 18 h aerobically. It should be stated that for

assessment of competion for nutrient between isolates and indicator bacteria, overlaying of isolates with indicator bacteria was carried out simultaneously. For this purpose, 2 µl of active microbial suspension was spotted on RCA, and then were immediately overlaid with 10 ml of soft TSB which already was seeded with indicator bacteria and incubated for 24h at 37°C.

6.2.3.2 Blank disk assay

The spot test assay was slightly modified by replacing the direct addition of test isolates on the agar with placing sterile disk papers (5 mm diameter). Inoculated disks (with 2 µl of bacteria suspension of the test organism) were placed onto RCA and incubated at 37 oC for 18 h under anaerobic condition. The aim was to obtain a uniform colony of test isolates without any displacement.

6.2.3.3 Well diffusion assay

The well diffusion method described by Toure *et al.* (2003) was used for examination of production of inhibitory substances in the culture medium. An overnight culture of isolates in RCM was centrifuged at 4°C and 3287 × *g* for 10 minutes and the supernatant immediately was used for well diffusion assay. In this method, 20 ml of TSB containing 0.8% w/v agar was inoculated with 200 µl of indicator bacteria at a final concentration of around 10^7 CFU/ml and poured into a sterile plate to solidify. Afterwards, three wells were excavated in the solidified agar using sterilized pipet tip and the wells were filled with 100 µl of filtered (0.2 µm) supernatant of the test organisms. All plates were kept 3-5 hours in a fridge to allow diffusion of the supernatant (test organism) and then incubated at 37°C for 18 h aerobically.

It should be noted that inhibition activities by test colonies or supernatant were considered to be represented by observation of a clear zone around wells. This was measured in mm.

6.3 RESULTS

6.3.1 Spot test

All 14 isolates were studied for their antibacterial potential against four indicator bacteria using the spot test and well diffusion assay. In the spot test, all isolates were first spotted on the appropriate medium and then either straightaway (concurrent overlaying), or after 18 h, overlaid with soft TSA agar seeded with indicator microorganisms. The reason for doing so was to examine whether the potential inhibition was the result of competition of probiotics with indicator organisms for nutrients or space (concurrent over laying) or because of the production of inhibitory metabolites by probiotic organism. The spot test with concurrent overlaying did not result in inhibition zone for any of the tested isolates which was interpreted as lack of nutrient competition between the isolates and indicator organism. As Table 6.1 illustrates all isolates showed clear inhibition zone with overlaying after 18 hours which indicated these isolates were able to inhibit all four used indicator bacteria. Interestingly, B13 and B14 (*B. longum* and *B. breve, respectively*) illustrated great inhibitory activity which was significantly higher than that of *B. animalis* subsp. *lactis* isolates (Figure 6.1).

Table 6.1 Antibacterial activity of *Bifidobacterium* isolates (B1-B10), commercial cultures (B11and B12) and type strains (B13 and B14) against indicator bacteria using spot test.

Microorganism	Indicator microorganism			
	Salmonella typhimurium	*Salmonella enteritidis*	*Escherichia coli* NCIMB 555	*Escherichia coli* NCTC 12900
B1	+	+	+	+
B2	+	+	+	+
B3	+	+	+	+
B4	+	+	+	+
B5	+	+	+	+
B6	+	+	+	+
B7	+	+	+	+
B8	+	+	+	+
B9	+	+	+	+
B10	+	+	+	+
B11	+	+	+	+
B12	+	+	+	+
B13	+++	+++	++	+++
B14	+++	++	++	++

+: 1<diameter of inhibition zone ≤3, ++: 3<diameter of inhibition zone ≤6, +++: diameter of inhibition zone >6
B1-B12: *B. animalis* subsp. *Lactis,* B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815

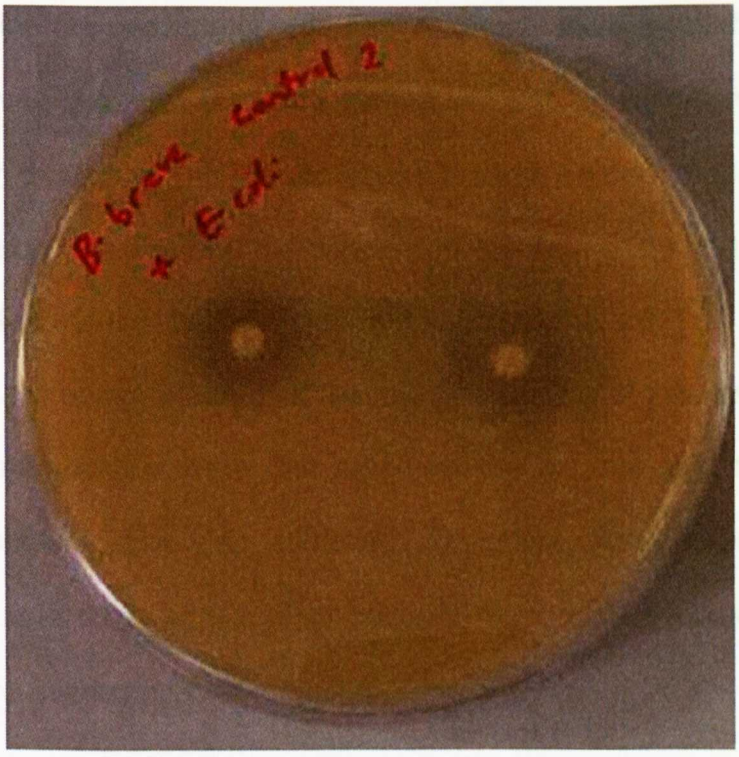


Figure 6.1 Inhibition zone produced by *Bifidobacterium breve* against *Escherichia coli* NCIMB 555 using spot test

The antibacterial activity of all isolates against four indicator bacteria was further examined to elucidate the mechanism (s) involved.

In order to exclude the effect of acid on indicator organisms a buffer was added to the medium to stabilize pH. RCA containing 2 g/l sodium bicarbonate was prepared for this purpose and spot test was carried out again using overlaying technique after 18 h incubation of isolates. As Table 6.2 shows when medium was buffered by 0.2% w/v sodium bicarbonate, with the exception of the two type strains (B13 and B14) antagonistic property disappeared and no inhibition zone was seen. B13 and B14 displayed a clear inhibition zone of 1-2 mm, which was far less than that of the un-buffered medium (i.e. >6 mm). However, it could be concluded that the production of some inhibitory compounds (e.g. bacteriocin like) other than organic acids is possible.

In order to examine the possible contribution of growth medium to the mechanism of antibacterial activity, the spot test was carried out using two different media (MRS agar and RCA) in duplicate. The results were the same on both media.

One problem encountered during the concurrent overlaying method of spot test was the displacement of the test bacteria during addition of the soft TSA agar. Hence, the test was carried out using "blank disk" assay. The results of this test did not lead to generation of any inhibition zone (the same as the original spot test); however, a more stable inoculum spot was achieved. Therefore, for further studies it was decided to inoculate RCA with 2 µl of bacterial suspension followed by overnight incubation, before overlaying with indicator organism.

Table 6.2 Antibacterial activity of *Bifidobacterium* isolates (B1-B10), commercial cultures (B11 and B12) and type strains (B13 and B14) against indicator bacteria using the medium buffered with sodium bicarbonate in spot test.

	Indicator microorganism			
Microorganism	*Salmonella typhimurium*	*Salmonella enteritidis*	*Escherichia coli* NCIMB 555	*Escherichia coli* NCTC 12900
B1	–	–	–	–
B2	–	–	–	–
B3	–	–	–	–
B4	–	–	–	–
B5	–	–	–	–
B6	–	–	–	–
B7	–	–	–	–
B8	–	–	–	–
B9	–	–	–	–
B10	–	–	–	–
B11	–	–	–	–
B12	–	–	–	–
B13	+	+	+	+
B14	+	+	+	+

-: no inhibition, +: 1<diameter of inhibition zone ≤3
B1-B12: *B. animalis* subsp. *Lactis,* B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815

6.3.2 Well diffusion test

Further studies of antimicrobial activity were accomplished by examining the mechanism (s) involved in antimicrobial activity. The isolates were screened by the well diffusion method for monitoring the likely production of antimicrobial compound(s) in broth media. In this method, when the filtered supernatants (cell free) of isolates were delivered into the wells, no detectable inhibition zone was seen for any of the isolates (data not presneted).

Since none of the cell free supernatants was able to show any antagonistic activity against indicator bacteria, it was thought that it could be because of low concentration of antibacterial compounds in the filtered supernatant. Therefore, the cell free supernatant with original pH of 4.4 was concentrated to 1/10 of initial volumes by freeze drying. For this purpose, first all samples were frozen at -80°C and then lyophilized in a freeze drier (Heto Drywinner, UK). The dried supernatant was reconstituted in 1/10 of its original volume sterile deionised water. These concentrated supernatants were utilized into well diffusion assay, as explained above, and the results are summarised in Table 6.3.

As can be seen in Table 6.3, unbuffered concentrated supernatant showed inhibition zone against indicator bacteria, however, the inhibition strength of the isolates were not consistant amongst tested isolates. For example, *B. breve* and *B. longum* presented a clear inhibition zone which was at least 3 times more than the inhibition zone produced by other tested isolates (Figure 6.2). Opaque (not clear) inhibition zone was interpreted as partial inhibition.

Table 6.3 Antibacterial activity of cell free concentrated supernatant (1/10) against indicator bacteria using the well diffusion method

Microorganism	Indicator microorganism			
	Salmonella typhimurium	*Salmonella enteritidis*	*Escherichia coli* NCIMB 555	*Escherichia coli* NCTC 12900
B1	+	+	+ (P)	+
B2	+	+	+	+
B3	+	+	+	+
B4	++	+	+	+
B5	+	+	+	++
B6	+	+	+ (P)	+ (P)
B7	+	+	–	–
B8	+	+	+ (P)	–
B9	+ (P)	+ (P)	+ (P)	+ (P)
B10	+	+	+	+
B11	+	+	+ (P)	+ (P)
B12	+	+	+	+
B13	+++	+++	++	+++
B14	+++	+++	+++	+++

-: no inhibition, +: 1<diameter of inhibition zone ≤3, ++: 3<diameter of inhibition zone ≤6, +++: diameter of inhibition zone >6, P: Partial inhibition zone (not clear)
B1-B12: *B. animalis* subsp. *Lactis,* B13: *B. longum* NCTC11818, B14: *B. breve* NCTC11815

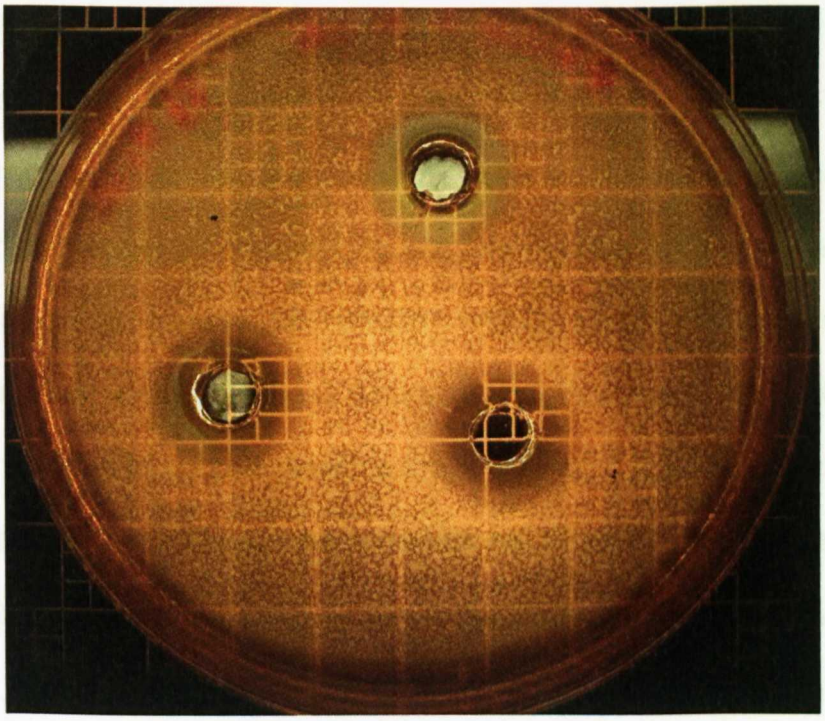


Figure 6.2 Inhibition zone produced by *Bifidobacterium breve* against *Salmonella typhimurium* DT124 using well diffusion test

Also, in order to elucidate the mechanism of inhibition in the concentrated filtered supernatant the experiment was repeated by neutralising the supernatant to pH 7 using 2M NaOH. The aim was to exclude any inhibitory effect from organic acid. Here again no inhibition zone was detected around neither the test isolates from fermented milk nor type strains.

6.4 DISCUSSION

Some LAB are able to compete with each other by producing antimicrobial compounds, which may put their viability at risk. Niku-Paavola *et al.* (1999) categorised these biological compounds into two groups: (a) non proteinaceous low molecular mass compounds (<1000 Dalton), and (b) proteinaceous compounds with high molecular mass (>1000 Dalton). There is little information about the capacity of bifidobacteria isolates to secrete proteinaceous antimicrobial compounds.

The antagonistic property of bifidobacteria has not been described in as much detail as for LAB. Some researchers have shown the inhibition of pathogenic bacteria by bifidobacteria. Ibrahim and Bezkoroaing (1993) stated that the antagonistic activity of bifidobacteria is related to their pH lowering effect, but Toure *et al.* (2003) attributed it to the secretion of proteinaceous compounds in addition to the pH lowering effect.

Gibson and Wang (1994) showed that *B. Infantis* is able to inhibit growth of *Clostridium perfringens* and *E. coli* by some metabolites other than organic acid, which were called "antibacterial substances". The existence of such compounds was confirmed when the filtered and neutralised supernatant of *Bifidobacterium Infantis* was able to inhibit the growth of several pathogenic bacteria (Toure *et al.* 2003).

Lievin *et al.* (2000) showed that some infant *Bifidobacterium* spp. are able to secrete a lipophilic molecule with molecular weight of < 3500 Dalton. They suggested that bifidobacteria, as part of resident human gastrointestinal microflora, could participate in the defence effect of indigenous microflora.

Yildirim and Johnson (1998) stated that bifidocin B secreted by *B. bifidum* NCFB 1454 is the sole bacteriocin excreted by bifidobacteria.

In this study it was demonstrated that all tested bifidobacteria show good degree of antimicrobial activity against 4 different indicator organisms using spot test. Such activity was more noticeable by *B. longum* and *B. breve*. Neutralisation of RCA with sodium bicarbonate, in spot test, eliminated any antimicrobial activity in all isolates except for B13 and B14, where inhibition zone was observed around the isolates but to a much lesser degree compared to contol (RCA with original pH). Such observation, however, was not repeated during well diffusion test as all isolated failed to produce any inhibition zone following neutralisation of the concentrated cell free supernatant.

These results are similar to those of Harris *et al.* (1989) and Toure *et al.* (2003). This might be due to low concentration of antibacterial compounds in the supernatant or as Toure *et al.* (2003) concluded, the inability of supernatants to suppress the indicator bacteria might be due to the lack of cell to cell contact between the isolates and indicator bacteria in the well diffusion method. In addition, it is postulated that the presence of isolates together with indicator bacteria in the same medium might induce the secretion of suppression compounds by the test organisms.

The results also indicated that while concentrated neutralized supernatant did not show antimicrobial activities against indicator bacteria, the original (non-neutralised) supernatant showed an inhibition zone with more than 3mm diameter with all four tested indicator organisms. Based on these observations, it was concluded that the antimicrobial effect (inhibition) of the cell free supernatant relies on organic acids produced by the isolates. Since the concentration of these acids in the supernatant is not enough to prevent the growth of indicator bacteria or more likely the acids are diluted as a result of diffusion into agar, concentrating the cell free supenatant by

freeze drying could increase the concentration of organic acids in the supernatant sufficiently to inhibit the growth of indicator bacteria.

In addition, in the spot method, isolates remained at least 48 h at the optimum growth conditions before being overlaid by indicator bacteria, but in well diffusion test, isolates were allowed to produce antimicrobial compounds only for 18 h. The potential effect of prolonged incubation up to 48 h should be studied in more detail in future.

It is interesting to notice that B13 and B14 showed some inhibitory activity against the indicator organisms, when spot test was applied on neturilised RCA agar. However, this was not the case when the well difusion technique was used with neutralised concentrated supernatant. It seems that neutralisation of RCA agar in spot test provides a localised and ongoing neutralisation. In other words, the acids produced by *Bifidobacterium* isolates are neutralised as they are produced and as a result no redution in pH occurs, whereas in well diffusion test the acid is produced and neutralisation occurs at the end of incubation. It is believed that the activity of some bacteriocin-like inhibitory substances (BLIS) is pH dependent and that some BLIS could maintain their activities at very low pH (e.g. 2-6), but not at pH higher than 8 (Zouhir *et al* 2011). Hence, the low pH of the supernatant (i.e. 4.4) in this experiment should not adversely affect their activity, however, more studies needed to confirm this different behaviour. Therefore, it is more likely that the inhibition of indicator organisms by type strain bifidobacteria (*B. breve* and *B. longum*) is the result of the combined effect of organic acids and BLIS. Nevertheless, such presumption needs further investigation.

6.5 CONCLUSION

Overall, the isolates from fermented milk expressed limited antimicrobial activity compared to tested type strains. It is also seems very unlikely that the isolates from fermented milks have the potential to secreting antimicrobial compounds other than that of organic acids. This, however, needs to be substantiated by conducting *in vivo* studies. Moreover, the potential of the two type strains screened in this study deserve additional examination.

Chapter 7

In vitro assessment of the isolates for functional properties: production of conjugated linoleic acid

7.1 INTRODUCTION

Conjugated linoleic acid (CLA) was discovered in 1933. It was realised that the UV absorbency was increased when polyunsaturated fatty acids reacted with alkaline compounds. Booth *et al.* (1935) found the bovine milk produced in the summer showed more UV absorbency at 230 nm than winter milk and stated that the variation in absorption at 230 nm is genuinely seasonal character. Reiser (1951) attributed this to conjugation of the double bonds of the polyunsaturated fatty acids (Kapoor *et al.* 2005). In recent years, conjugated linoleic acid (CLA) has attracted great attention for its health promoting effects. Usually, linoleic acid (LA) is converted to CLA in presence of isomerase enzyme of rumen bacteria (Sieber *et al.* 2004).

7.1.1 Conjugated linoleic acid (CLA)

CLA comprises a mixture of positional and geometric conjugated isomers of linoleic acid (LA), with conjugated double bonds at carbon positions 7 and 9, 8 and 10, 9 and 11, 10 and 12, 11 and 13 or 12 and 14 (Lin 2000; Sieber *et al.* 2004; Rodriguez-Alcala 2011). To date 28 positional and geometrical CLA isomers are known which naturally occur during biohydrogenation (BH) of LA. However, *cis-9* (*c9*), *trans-11* (*t11*) CLA is considered as the major bioactive isomer which is also named rumenic acid, while *t10-c12* CLA is the second major isomer of CLA. As shown in Figure 7.1, CLA, the same as other fatty acids, contains a hydrocarbon chain linked to a carboxyl group. However, due to presence of conjugated double bonds along the eighteen carbon chain molecule, it is a unique fatty acid.

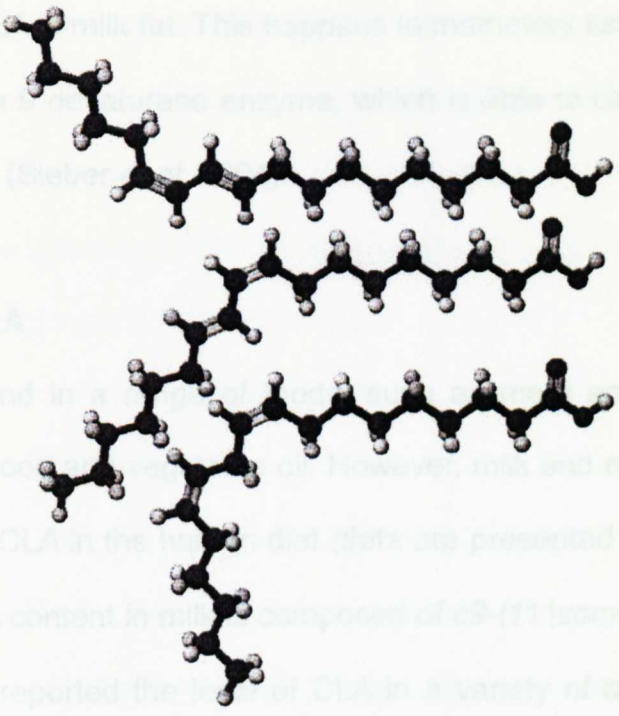


Figure 7.1 Structures of *t10-c12* CLA (top), *c9-t11* CLA (middle), and linoleic acid (bottom) (adapted from Pariza *et al.* 2001)

7.1.2 CLA synthesis

CLA is formed through conversion of vaccenic acid in the mammary gland and also by the conversion of dietary LA in the rumen. *Butyrivibrio fibrisolvens* is one of the most widely known anaerobic bacteria in rumen fluid, which is capable of generating CLA due to presence of LA isomerase (Lin 2006; Nieuwenhove *et al.* 2007). This bacterium was also found in human faeces. Usually, *c9-t11* CLA is formed during biohydrogenation of LA to stearic acid by *B. fibrisolvens* in strictly anaerobic conditions via the action of LA isomerase. This process takes place in three steps. At the beginning of bioconversion process, LA is isomerated to *c9-t11* CLA. In second step *c9-t11* is hydrogenated to *t11* C18:1 which is named trans-vaccenic acid and this is reduced to stearic acid at a later stage. It is worth mentioning that there is an alternative way for CLA production in milk which is estimated to be the route for 78%

of the total *c9- t11* CLA in milk fat. This happens in mammary tissue of lactating cows by the action of delta 9 desaturase enzyme, which is able to convert trans-vaccenic acid into *c9,-t11* CLA (Sieber *et al.* 2004).

7.1.3 Sources of CLA

CLA is naturally found in a range of foods, such as meat and milk of ruminants, poultry (turkey), seafood and vegetable oil. However, milk and meat of ruminants are the main sources of CLA in the human diet (data are presented in appendix 4). More than 90% of the CLA content in milk is composed of *c9-t11* isomer.

Akalin *et al.* (2007) reported the level of CLA in a variety of dairy products ranges from 0.55 to 9.12 mg CLA/g fat. In another study, the analysis of dairy products suggested a wide range of concentrations of CLA. For example, raw milk contained 0.83 - 5.5, Brick cheese as high as 7.1 and fat-free yogurt 1.7 - 5.3 mg CLA/g fat (Lin *et al.* 1999). Usually, full-fat fermented dairy products comprise more CLA than non-fermented products. An increase in CLA content was reported from 4.4 mg CLA of fresh milk to 5.3 mg CLA/g fat of yogurt. Also, an elevated level of CLA was described in dahi (Indian yogurt) (26.5 mg CLA/g fat) compared to its raw material (5.5 mg CLA/g fat) (Lin *et al.* 1999). Among dairy products, cheese is considered the main source of CLA. The content of CLA in cheese depends on the initial amount of CLA in milk and bacterial fermentation (Nieuwenhove *et al.* 2007). For instance the amount of CLA in Cheese Whiz, a processed cheese sauce, (8.81 mg CLA/g fat) was much higher than raw milk (0.83 mg CLA/g fat) (Lin *et al.* 1999).

Some of the most important parameters that affect the CLA content in milk are: (a) cows fed on fresh pasture, (b) addition of some oil to cow's diet, (c) breed of cow, and (d) season. On fresh pasture, the CLA content of raw milk reaches 10.9 mg/g fat

compared to 4.6 mg/g with regular corn diet. It is interesting that the CLA content in cow's milk, pastured on high Alpine grazing lands is 23.6 compared to milk from low lands with 8.7mg/g fat, and it is due to different plants consumed. Also, the CLA content of milk is considerably higher in May, June and July (Northern hemisphere) (Sieber *et al.* 2004; Nieuwenhove *et al.* 2007).

7.1.4 Health benefits of CLA

Researchers have identified a number of beneficial biological effects of CLA which include anti-inflammatory activities, antidiabetogenic, increased body protein, and decrease of colon cancer risk, low density lipoprotein (LDL) concentration and whole body fat. Different isomers of CLA may present different biological benefits. *C9-t11* CLA is expected to be active as anti-carcinogenic and antioxidant agent and body fat reduction is ascribed to *t10-c12* CLA (Lin *et al.* 2003; Sieber *et al.* 2004; Nieuwenhove *et al.* 2007; O'Shea *et al.* 2012).

7.1.5 CLA intake

As CLA is produced during organic synthesis, the conversion of LA to CLA does not occur in considerable amounts, thus the quantities of CLA isomers produced in the human body are far less than the physiologically effective level (Park *et al.* 2011). Conjugated linoleic acid quantity in human body predominantly depends on diet and could be increased by consumption of dairy and meat products which are considered as the main sources of CLA. In recent years, and as an alternative, the regular consumption of adequate probiotic bacteria with the potential for LA conversion has been suggested (Jiang *et al.* 1999; Coakley *et al.* 2003).

To exhibit a cancer protective effect, a daily intake of 620 mg *c9-t11* CLA for men and

441 mg for women, has been recommended (Sieber *et al.* 2004). However, some animal studies have concluded that an estimated daily intake of CLA for cancer prevention should be at least 3 g, which is much greater than the normal human daily intake of 0.1-1 g of CLA. Mean daily intake of CLA was estimated to be 360 mg for women and 440 mg for men in Germany, with nearly two thirds coming from dairy products and one-fourth from meat products. Also, the total CLA intake for North America was reported as 212mg/d for men and 151 mg/d for women (O'Shea *et al.* 2012).

7.1.6 Conjugated linoleic acid formation by bifidobacteria

Due to higher level of CLA in fermented milks, it was hypothesised that the formation of CLA is through the activities of starter cultures. It is now obvious that the addition of some starter cultures to dairy products contributes to the production of CLA (Yadav *et al.* 2007). The fact that CLA is formed through the metabolism of rumen bacteria, such as *B. fibrisolvens* and *Megasphaera elsdenii,* guides us to a theory that this vital metabolite might be synthesised by other microorganisms as well. Bifidobacteria are normal inhabitants of the human digestive tract and are now being used as probiotics in the production of fermented dairy products. Coakley *et al.* (2003) found that *Bifidobacterium* spp. are able to produce CLA and their sensitivity to linoleic acid is considerably different among species.

In this study, previously isolated bifidobacteria from fermented milk and two commercial probiotic cultures along with three bifidobacteria purchased from NCTC culture collection (*Bifidobacterium breve*, *B. longum* and *B. bifidum*) were used to examine their potential, as a probiotic, to increase CLA production *in vitro*.

7.2 MATERIALS AND METHODS

Lipid standards, LA and CLA, were obtained from Sigma-Aldrich (Poole, Dorset, UK). All other chemicals used in the fatty acids analysis were of analytical grade and purchased from Sigma unless otherwise stated.

7.2.1 Substrate preparation

A stock solution of LA (30 mg/ml) was prepared in 2% v/v Tween 80 (polyoxyethylene sorbitan monooleate) solution to improve its solubility, filter sterilized through a 0.45 µm syringe filter (Nalgene, Fisher Scientific, UK) and stored in the dark at - 20°C until use.

7.2.2 Bacterial growth and sampling

This study employed 10 strains of *B. animalis* subsp. *lactis* which were isolated from yogurt and other fermented milk products, two commercial cultures of *B. animalis* subsp. *lactis* kindly provided by Chr. Hansen (BB12) and Danisco (vital probiotic yogurt) as well as three different type strains purchased from National Collection of Type Cultures (NCTC, Salisbury, UK); *B. breve* NCTC11815, *B. longum* NCTC11818 and *B. bifidum* NTCT 13001.

All strains were subcultured twice anaerobically at 37 °C for 24 h on RCA (CM0151, Oxoid) before use. An inoculum suspension was prepared in sterile MRD and final concentration of cells was adjusted to 0.5 MacFarland solutions (~10^7-10^8 CFU/g) using sensititre nephelometer (TREK - Diagnostic Systems Ltd, UK).

Inoculum of 1% v/v of each strain was added to the 10 ml RCM (CM0149; RCM, Oxoid, Basingstoke, UK) containing 500 µg/ml of LA, and CLA production was determined after incubation at 37 °C for each strain after 24 and 48 h as described

by Coakley *et al.* (2003). The CLA content in the cultures was expressed as percentage of LA added.

Viable cell number, pH and CLA production were determined at time 0 and after 24 and 48h incubation in the above conditions. The CLA percentages were determined using the following equation: % CLA = CLA/(CLA + LA)×100.

7.2.3Analysis of CLA by gas chromatography (GC)

The inoculated RCM was centrifuged at 4 °C at 7000 x *g* for 10 min. Cells and supernatant were separated and extraction of fatty acids was carried out on 500 µl of both fractions (i.e. cell and supernatant).

7.2.4 Analysis of fatty acids

Free Fatty Acids (FFA) profiles of samples were determined by gas chromatography after esterification as described below.

7.2.4.1 Extraction

Lipids were extracted by homogenising 500 µl of sample (cell pellet or supernatant) in 15 ml hexhane/isopropanol (HIP) (3:2 v/v) and 0.01% v/v butylated hydroxytoluene (BHT) was added to the solvent to prevent potential oxidation. All samples were flushed for about 1 min by bubbling nitrogen gas through the liquid and stored overnight at 4°C.

7.2.4.2 Partitioning

The HIP mixture was completely evaporated under nitrogen, and 3 ml of chloroform/methanol (2:1 v/v), BHT (0.01% v/v) and 1 ml of distilled water was added

to each tube. The tubes were vortexed and centrifuged at 2504 x *g* for 5 min. After centrifugation, the organic phase (bottom layer) was clearly separated from the aqueous phase (top layer). The lipid enriched organic phase was collected and transferred to new tubes and the solvent was completely evaporated under nitrogen flush.

7.2.4.3 Extraction of methyl ester

Four ml of 15% v/v acetyl chloride in methanol was added to tubes containing dried organic phase. The tubes were flushed with nitrogen, sealed and left in the oven at 70°C for 3 h. After oven treatment, 4 ml of 5% w/v NaCl and 2 ml of petroleum ether were added to each tube. The tubes were shaken for few seconds. The upper petroleum ether layer was transferred into a new tube containing 2 ml of 2% w/v potassium bicarbonate. Another 2 ml of petroleum ether was added to each of the original tubes, the tubes were shaken and the upper petroleum ether was transferred to the tube containing 2% w/v potassium bicarbonate. The tubes were vortexed and the upper layer was transferred to a new tube containing 100-200 mg of dried granular sodium sulphate. The solution containing fatty acid methyl esters (FAME) in petroleum ether was transferred to a 3ml glass vial. The petroleum ether was removed under nitrogen and the samples were dissolved in 1ml heptane and BHT (0.01% v/v). The samples were flushed under nitrogen and stored at -20 °C until use.

7.2.4.4 Gas chromatography

FAME was analysed by a capillary gas liquid chromatograph (HRGC MEGA 2 Series, Fison's Instruments, Italy). Injection (1 µl) was performed automatically with an inlet temperature of 250 °C and a split ratio of 100:1. Hydrogen was used as a

carrier through a BPX-70 capillary column (60 m.320 μm). The oven operated at an initial temperature of 140 °C for 1 min, with four ramps to attain 180, 184 and 240 °C at a rate of 1.2, 0.5 and 30 °C/min respectively. Detection was carried out by flame ionization at 270 °C.

The relevant peaks were identified by comparison of retention times with authentic standards and calculation of equivalent chain length values. The peak areas were quantified by a computer chromatography data system (Agilent EZ Chrom Elite chromatography Data System version 3.2, Scientific Software, Pleasanton, CA).

7.3 RESULTS

In this research, a number of bifidobacteria were studied for their ability to convert free LA to CLA. The origin of tested bifidobacteria varied from different dairy products, commercial cultures and type strains. *B. breve,* previously reported to synthesize CLA from free LA; therefore, it was used as positive control in the CLA biosynthesis assay (Coakley *et al.* 2003).

7.3.1 Screening isolated bifidobacteria from fermented milks for CLA-production

Screening 10 isolates of *B. animalis* subsp. *lactis* and two commercial cultures for CLA production in treated RCM with 500 µg/ml free LA was undertaken after anaerobic incubation at 37°C for 24 h (Table 7.1). It was found that seven out of 12 tested isolates were capable of synthesising CLA in the supernatant and the amount of CLA produced by the others was not detectable.

All tested isolates/strains grew well in presence of LA, but isolates B2, B4, B7, B8 and B10 (from fermented milks) did not convert LA to CLA at a detectable level.

Table 7.1 Formation of CLA by isolates and commercial *B. animalis* subsp. *lactis* in RCM with 500 µg/ml of LA (all values are average of triplicate measurements)

Isolates	Source	%CLA converted from 500µg/ml of LA
B1	Isolated from plain yoghurt	2.22
B2	Isolated from goat's yogurt	ND
B3	Isolated from chocolate milk drink	5.85
B4	Isolated from yogurt drink	ND
B5	Isolated from plain low fat yogurt	4.36
B6	Isolated from fruit yogurt	2.23
B7	Isolated from whole fat yogurt	ND
B8	Isolated from Greek style yogurt	ND
B9	Isolated from fruit yogurt	1.25
B10	Isolated from yogurt drink	ND
Commercial B11	From Chr. Hansen Co. (BB12)	4.41
Commercial B12	From Danisco Co. (vital probiotic yogurt)	2.05

B1-10 are *B. animalis* subsp. *lactis* strains *isolated* from probiotic dairy products
ND: not detected

7.3.2 Effect of incubation time and growth medium on the production of CLA by bifidobacteria

In this part of the research, the effect of two different media and also incubation time on the production of CLA by certain bifidobacteria was investigated. BB12 was selected as an authentic representative of tested *B. animalis* subsp. *lactis* and its capability for production of CLA was compared with three different type strains from National Collection of Type Cultures (NCTC). All bacteria were cultivated in modified MRS broth (MRS with 0.05% w/v L-cysteine; MRSC; Sigma) and RCM containing 500 µg/ml free LA. It should be mentioned that without Tween 80, LA does not dissolve in the medium.

Conjugated linoleic acid production was monitored after 24 and 48 h of incubation. The effect of free LA on cell counts of tested bacteria was determined by enumeration of viable cells after 24 and 48 h of incubation. As shown in Figure 7.2, the growth of BB12 and *B. breve* in RCM was not greatly affected by presence of LA. The growth of *B. longum* and *B. bifidum* was rather inhibited after 24 h incubation, declining by 1.5 and >2 log CFU/ml, respectively, but after 48 h incubation, the counts of *B. longum* increased by one log CFU/ml; however, *B. bifidum* declined even further.

In MRSC broth , only BB12 showed good growth in presence of LA where the growth of other tested bacteria commenced after 24 h (Figure 7.3). When enumerated after 48 h, the counts of *B. breve* and *B. longum* increased by less than one log CFU/ml, but *B. bifidum* continued to decline.

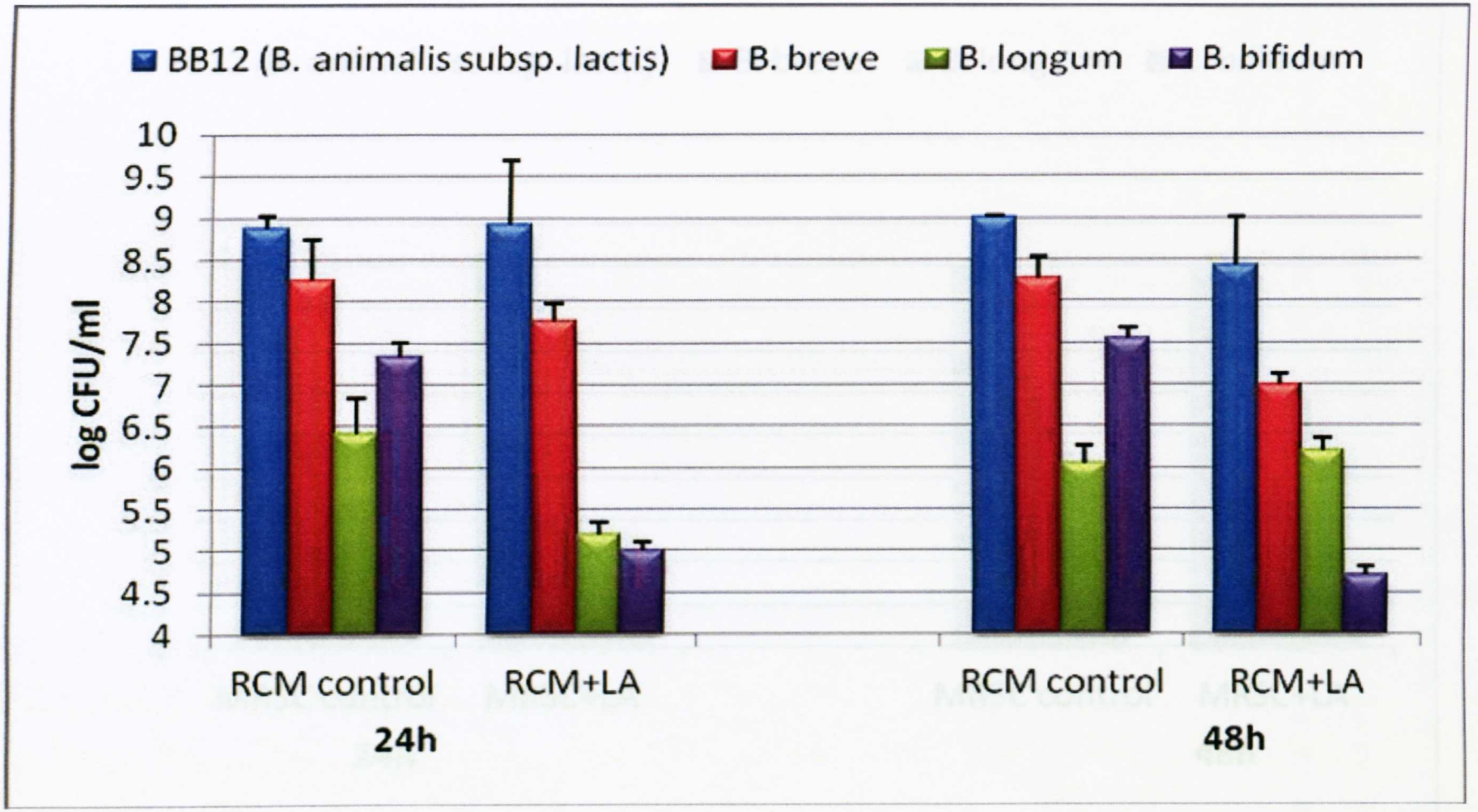


Figure 7.2 Changes in total plate counts of four bifidobacteria in RCM without LA (control) and with LA (500 µg/ml) after 24 and 48h anaerobic incubation at 37 °C
RCM control : Reinforced Clostridial Medium without linoleic acid
RCM+LA: Reinforced Clostridial Medium with 500 µg/ml linoleic acis
Data are means ± SD of three replications

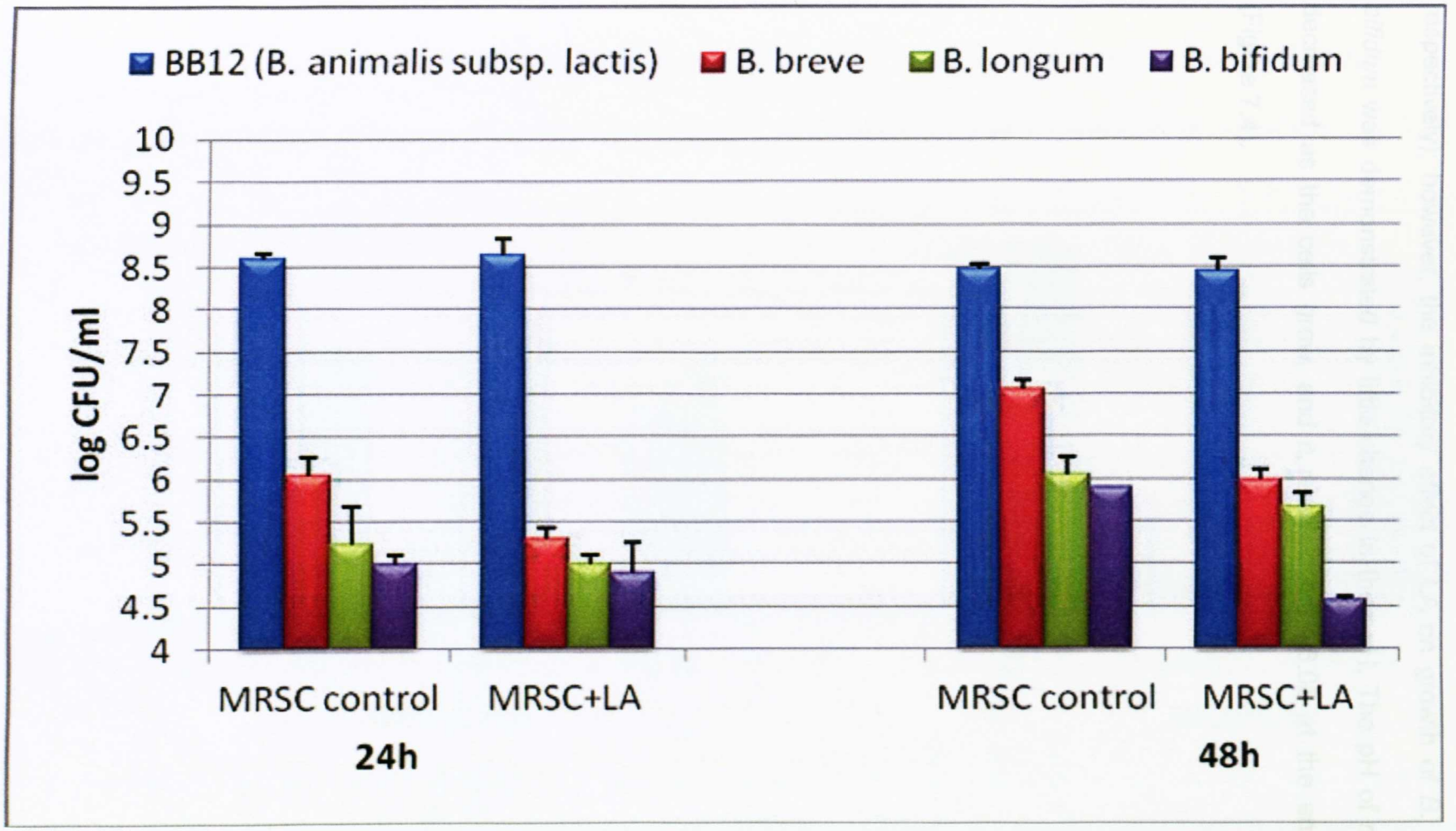


Figure 7.3 Changes in total plate counts of four bifidobacteria in MRSC broth without LA (control) and with LA (500 µg/ml) after 24 and 48h anaerobic incubation at 37 °C
MRSC control : De Man Rogosa Sharpe plus cystein without linoleic acid
MRSC+LA: De Man Rogosa Sharpe plus cystein with 500 µg/ml linoleic acis
Data are means ± SD of three replications

When pH changes was measured during cultivation, BB12 showed a similar pH profile in RCM and MRSC broth after 24 and 48 h incubation (i.e. around 4.5 and 4, respectively); however, the inhibitory effect of LA on growth of *B. longum* and *B. bifidum* was demonstrated by little change in their pH. The pH of media gradually decreased as the cells grew, and it reached 4.01–6.06 at the end of cultivation (Figure 7.4).

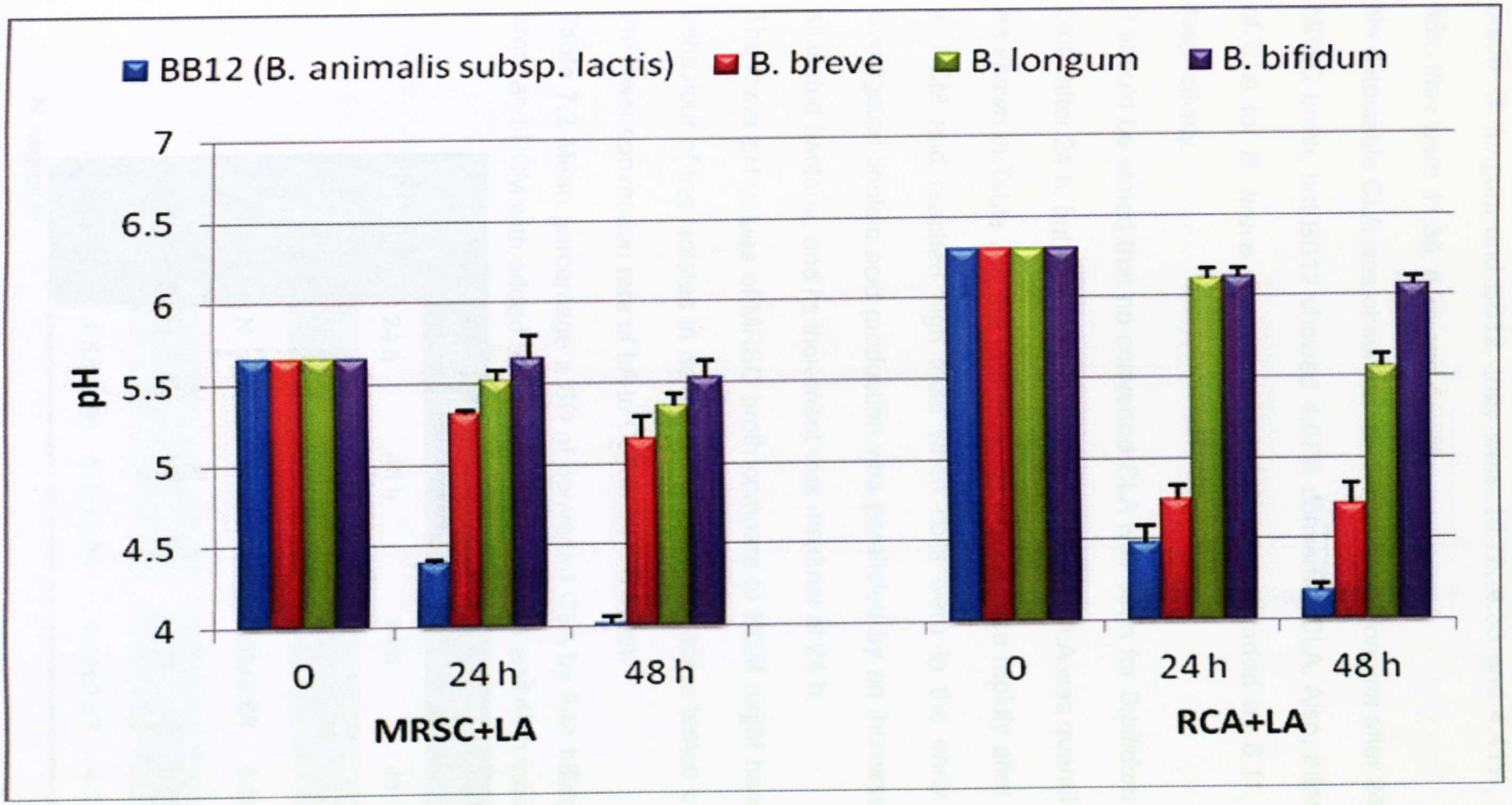


Figure 7.4 Effect of LA on pH changes by four bifidobacteria in RCM and MRSC broth without LA (control) and with 500 µg LA/ml after 24 and 48h anaerobic incubation at 37 °C
MRSC+LA: De Man Rogosa Sharpe plus cystein with 500 µg/ml linoleic acis
RCM+LA: Reinforced Clostridial Medium with 500 µg/ml linoleic acis
Data are means ± SD of three replications

Table 7.2 shows the percentage of converted CLA in RCM after 24 h and for *B. breve, B. longum* and BB12, they were 10.78, 4.33 and 4.41%, respectively. After 48h, they were 11.36, 5.26 and 4.23%.

No detectable CLA was observed for *B. breve* and *longum* after 24 h of incubation in MRSC broth, but BB12 showed 4.57% converted CLA. Also, after 48h, the quantity of CLA for *B. breve, longum* and BB12 was recorded as 6.11, 1.14 and 5.45%, respectively.

It should be stated that no converted CLA was seen for *B.bifidum* in RCM or MRSC broth after 24 h, but after 48 h incubation in RCM, CLA was quantified as 0.29%.

As shown in Table 7.2, the total quantity of CLA rose rapidly after 24 h of incubation in RCM and reached high level when cells were in the early stationary phase. Conjugated linoleic acid production was paralleled by an increase in cell count with all tested bacteria, and its increment was maximal at 24 h.

The lower pH values of MRSC broth compare to RCM might have had an effect on behaviour of the isolates in delaying the growth of some tested cultures resulting in the lower conversion rate of LA to CLA in MRSC broth.

Table 7.2 Mean percentage ± SD of converted CLA by four bifidobacteria in MRSC broth and RCM with added 500 of µg/ml LA, after 24 and 48 h incubation

	%CLA in MRSC broth		%CLA in RCM	
Organism	24 h	48 h	24 h	48 h
B. breve	N	6.11±0.79	10.78±2.79	11.36±3.1
B. longum	N	1.14±0.19	4.33±0.68	5.26±1.03
B. bifidum	N	N	N	0.29±0.06
BB12	4.57±0.58	5.45±0.86	4.41±0.52	4.23±0.95

N: negligible

7.3.3 GC analysis of CLA isomers produced by fermentation of bifidobacteria

The percentage of two major isomers of CLA, *c9-t11* and *t10-c12*, was determined in RCM for four tested bifidobacteria (Table 7.3). By comparing the retention times of the products produced in this study with standards, it was concluded that *c9-t11* CLA was the major CLA isomer which was formed by the fermentation process (Figure 7.5). It was determined that *B. breve, longum, bifidum* and BB12 produced the *c9-t11* CLA isomer, which accounted for 62 to 88% of the total CLA isomer products (calculated based on the results presented in Table 7.3).

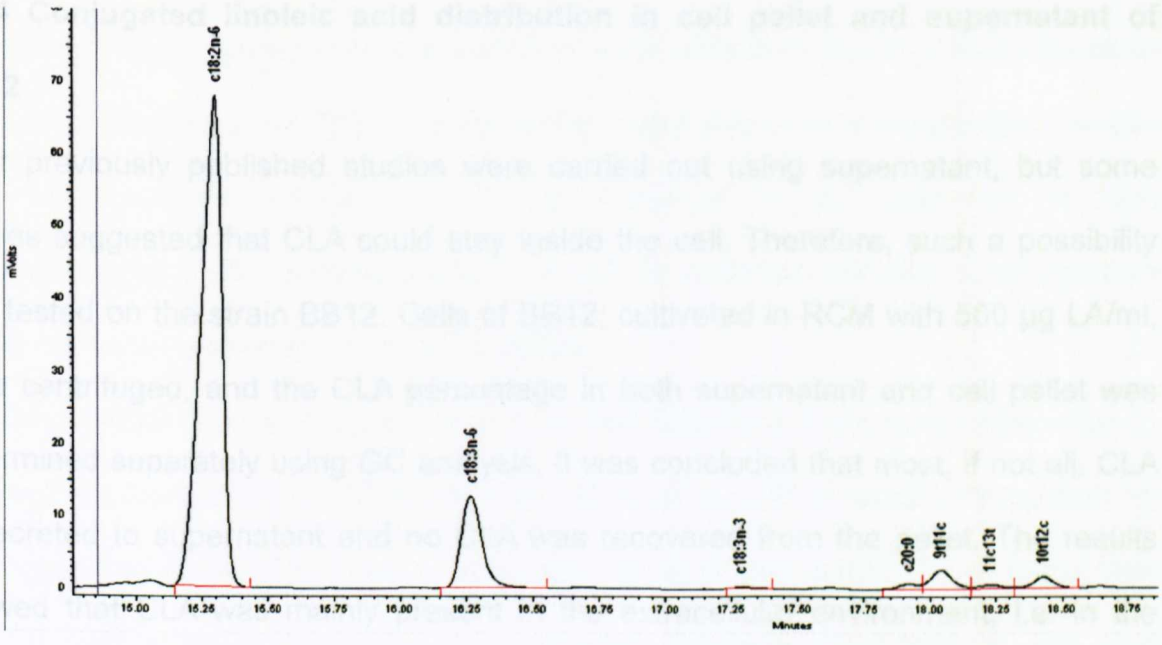


Figure 7.5 Representative GC chromatogram of the fatty acid composition of the fermented RCM supplemented with 500 µg/ml of LA.

Table 7.3 Percentages of CLA isomers produced by four bifidobacteria in RCM

	24 h		48 h	
Organism	*c9-t11*	*t10-c12*	*c9-t11*	*t10-c12*
B. breve	9.23	0.07	10.02	0.08
B. longum	3.39	0.58	4.03	0.95
B. bifidum	N	N	0.18	N
BB12	3.37	0.44	3.7	0.31

N: negligible

7.3.4 Conjugated linoleic acid distribution in cell pellet and supernatant of BB12

Most previously published studies were carried out using supernatant, but some studies suggested that CLA could stay inside the cell. Therefore, such a possibility was tested on the strain BB12. Cells of BB12, cultivated in RCM with 500 µg LA/ml, were centrifuged, and the CLA percentage in both supernatant and cell pellet was determined separately using GC analysis. It was concluded that most, if not all, CLA is secreted to supernatant and no CLA was recovered from the pellet. The results showed that CLA was mainly present in the extracellular environment, i.e. in the growth medium.

7.4 DISCUSSION

The production of health-promoting fatty acids, such as CLA, could be an example of pharmabiotic production by intestinal bacteria (O'Shea *et al.* 2012).

LA isomerase is considered an essential enzyme for conversion of LA to CLA and is found in many bacteria. Several studies on *Lactobacillus* and *Bifidobacterium* cultures, either from culture collections or from human faeces, have revealed their potential for production of CLA (Coakley *et al.* 2003; Barrett *et al.* 2007).

Various parameters like pH, media and growth stage can affect on CLA production (Van Nieuwenhove *et al.* 2007). It has been demonstrated that the toxicity of saturated fatty acids to rumen bacteria is less than polyunsaturated fatty acids; therefore, biohydrogenation is considered as a detoxification mechanism by bacterial cells. Also, the toxicity of CLA, as a derivative metabolite of biohydrogenation, is less than LA (Jenkins and Courtney 2003). However, in the presence of high concentrations of LA, biohydrogenation would be inhibited and the microorganism would not be alive anymore (Kim *et al.* 2000).

Due to potential toxicity of LA to bacteria, as free fatty acid, it is not recommended to be used as food additive at high dosage. Choi *et al.* (2008) used monolinolein, a monoglyceride form of LA, as a substrate for CLA production. They found that monolinolein is more easily used by probiotic bacteria than free LA, for CLA production in yogurt.

It should be stated that toxic effects of LA for bacteria depends on its concentration and the study reported herein showed that tested bifidobacteria could grow well in presence of 500 µg LA/ml in RCM.

The potential of some strains of probiotic bacteria to produce CLA from LA was studied by Coakley *et al.* (2003). Among fifteen bifidobacteria isolates, *B. breve* was

the most capable CLA producer which showed conversion rate of 27-65% and *B. bifidum* was the least capable with 0.18 % conversion.

Park *et al.* (2011) introduced *B. breve* LMC520 as a functional probiotic which could be applied for human consumption to increase CLA production. According to their research, *B. breve* LMC520 was able to convert 15% of LA to CLA under conditions pertaining to the rumen, which was two times higher than of *B. fibrisolvens*. As mentioned earlier, *B. fibrisolvens* is a key CLA producer in the rumen.

In Rosberg-Cody's study (2004), which was designed to isolate different strains of *Bifidobacterium* from the faeces of neonates and to assess their potential for production of CLA, the most efficient CLA producers belonged to the species *B. breve*, exhibiting between 7.7 and 29% conversion.

The percentage of conversion to CLA reported by Barrett *et al.* (2007) was from 4.12% to 76.65% for *B. breve* and from 3.68% to 60.12% for *B. longum*.

However, the present study showed that the growth of type strain bifidobacteria in MRSC broth treated with LA was not the same as in RCM and consequently the quantity of CLA was not considerable in MRSC broth . This might be explained by the differences in the composition of these two media or the pH value or maybe the conversion pathway functions more optimally at the higher pH of RCM. Chung *et al.* (2008) stated that proteins in the growth media may have facilitated the production of CLA by enhancing the interaction of substrate with the bacteria. Macouzet *et al.* (2009) reported that MRS broth is not a suitable medium for conversion of LA to CLA. According to their research *L. acidophilus* was not able to produce CLA in MRS broth.

Jiang *et al.* (1998) screened 19 dairy starter cultures for their ability to generate CLA. The results showed that none of them was able to produce CLA in MRS broth, but all

of them generated CLA in skim milk. These findings point out that skim milk might be a better and perhaps more realistic medium for production of CLA. It is more likely that casein and whey protein are able to neutralize the toxic effect of LA in milk.

Ogawa *et al.* (2001) suggested that CLA produced by *L. acidophilus* La5 is associated with the cells and that this is advantageous to consumer. However, probiotic bacteria are not digested in human gut, hence, accumulated CLA in the cell might not be available for the consumer. On the other hand, however, this study showed no CLA accumulated in cell pellets of BB12 and CLA appeared only in the supernatant. Therefore, this is more valuable to the consumer, since the cells export it to the environment, i.e. the GIT.

Up to now, the number of studies, which assessed the ability of *B. animalis* subsp. *lactis* to produce CLA is few. However, Coakley *et al.* (2003) stated that *B. animalis* subsp. *lactis* are able to convert LA to CLA at the rate of 27.98%.

The effect of two different growth media on bifidobacterial growth in presence of 500 µg LA/ml was examined. All tested strains were able to survive and grow better to higher numbers in RCM supplemented with LA and showed the potential for production of CLA, but only BB12 presented good growth in both RCM and MRSC broth supplemented with LA. These results indicated that the capability of growth in presence of LA and also CLA production is species and perhaps strain dependent. However, from the results of the current study it is hard to speculate on the effect of strain on CLA production, yet isolates of *B. animalis* subsp. *lactis* showed considerable ability to produce CLA. The rep-PCR data illustrated a very similar DNA fingerprint for all these isolates. Therefore, such a difference could be explained only by the effect of environmental stresses that might have resulted in differences in metabolic activities of the isolates. This, however, needs further investigation.

Moreover, *B. breve* could produce more CLA than other tested bacteria and as expected, most was *c9-t11* the major CLA isomer, which was produced by all isolates, while less *t10-c12* was produced.

The results obtained in this study may vary to some extent from other research in this area. This could be partly explained by,

- Different concentration of LA in the medium.
- Time of incubation.
- Intrinsic characteristics of tested bacteria.
- Isomeration of the CLA produced to other saturated fatty acids due to detoxification process.

7.5 CONCLUSION

Bifidobacterium animalis subsp. *lactis* presented the potential for production of CLA in the presence of 500µg LA/ml in two different media. The largest percentage of CLA was produced during the first 24h of incubation. *C9-t11* CLA was the main isomer detected, which constituted nearly 80% of total CLA produced. As a secondary metabolite, CLA was secreted to the fluid medium and no CLA was discovered in the cell pellet.

Chapter 8

General discussion

Characterisation of probiotic organisms is important from various points of view including legislation, costumer protection, health claims, safety and technical perspective. Characteristics of the organisms are scrutinised during the selection process both *in vitro* and *in vivo*, however, independent investigations are needed to examine whether the probiotic organisms used in probiotic food products could actually demonstrate the expected qualities. Features of such assessment could be grouped into a number of categories, such as: (a) safety (e.g. presence of antibiotic resistance gene and their transferability), (b) dosage (e.g. enumeration at certain intervals before the end of shelf-life), and (c) selection criteria (e.g. resistance to acid, bile salts and digestive enzymes) as well as functional properties (e.g. antimicrobial activity, production of valuable metabolites, adhesion and/or colonisation ability in the gut).

The first aim of the current project was to assess the presence of bifidobacteria in 24 probiotic fermented dairy products from the UK market at the time of purchase and at the end of shelf-life. The isolated bifidobacteria were further studied to indentify them by means of phenotypic and genotypic methods followed by a further discriminatory examination at strain level using Rep-PCR. Using *in vitro* studies, the isolates were then characterised for the following qualities:

- Resistance to acid, bile and digestion enzymes
- Antimicrobial activity
- Production of conjugated linoleic acids (CLA)
- Biofilm formation

The used strains as probiotic culture in dairy products should be GRAS (generally recognised as safe) organism and also contain some of the beneficial properties mentioned for good probiotic strains (Ronka *et al.* 2003).

Clinical studies always are not feasible because of high costs, ethical considerations and safety regulations and it would be easier to use *in vitro* experiments. Having said that and despite their advantages, *in vitro* studies can not fully mimic the biological processes in the living organism. Therefore, a thorough examination should include *in vivo* (animal and clinical human) studies on behaviour of ingested probiotic bacteria. This, however, is not an easy task and as a result not enough studies have been carried out and limited publications are available.

8.1 Isolation and identification of bifidobacteria from fermented milks

To achieve the first aim of this study, isolation and identification of bifidobacteria from fermented milks and subsequently investigation of their probiotic characteristics, it was considered essential to use a suitable medium for isolation of bifidobacteria. BIM-25 as a known selective medium was compared to TOS-MUP (recently developed by IDF).

The selectivity of these two media and their reliability was verified by the results of molecular biology based techniques used for the identification of the isolates (Chapter 2). Overall, it was concluded that although the selectivity of both BIM-25 and TOS-MUP were good enough, the ease of preparation and shorter incubation time for TOS-MUP were advantageous; however, some Gram positive bacteria are able to form small red or pink colonies on BIM-25 (Munoa and Pares 1988). Therefore, successful enumeration is possible if special attention is paid to the appearance of colonies on BIM-25. The results obtained in this research are in line with those of Ghoddusi and Hassan (2011).

The results of enumeration of bifidobacteria in 24 samples of probiotic fermented milks showed that apart from one sample (D), all products contained 10^6 CFU/g at

the time of purchase. At the end of shelf-life, the viable counts decreased to some extent, but except product B which showed 1.37 log reductions in viable cells, all other products were in the range of minimum value required for probiotic efficiency. Losses of viable bifidobacteria of between 0 and 3 logs during shelf-life of fermented milk have been reported in the past (Schillinger 1999; Shin *et al.* 2000; Gilliland *et al.* 2002). Hence, our findings are good indication of the improvement that has been made over the years in selecting and applying more resilient strains of bifidobacteria in fermented dairy products.

Susceptibility to pH, oxygen and additives in the carrier food might have been one of the many reasons for low survival of *Bifidobacterium* spp. in fermented milk products in the past (Jayamanne and Adams 2006; Dave and Shah 1997; Shah 2000), but the isolated bifidobacteria were demonstrated to be technologically viable.

Based on the results of this research, there is a greater chance for achieving higher numbers of probiotic when these products consumed earlier than their expiry date.

A correct identification of the employed probiotic strains is certainly of fundamental importance. In this research the combination of the results obtained by phenotypic and genotypic techniques, made it possible to achieve a reliable identification.

Identification of a selection of bifidobacteria isolates with phenotypic methods was shown not to be reliable in most cases. API 50 CHL alone was useful only to determine the carbohydrate fermentation profiles. However, API Rapid ID 32 A, alongside API 50 CHL might be a useful method for identification of bifidobacteria at genus level. Collado *et al.* (2006) also failed to differentiate bifidobacteria with API 50 CHL. However, Vlkova *et al.* (2004) characterised bifidobacteria by using of API 50 CHL and API Rapid ID 32 A. Genotypic techniques are important tools for the identification of bacteria. Although phenotypic methods identified the bacteria at their

genus and species level, but different results were achieved when 16s rRNA was sequenced. These results showed that bifidobacteria cultures isolated from the fermented milks were belonged to *B. animalis* subsp. *lactis*. The presence of these organisms in fermented milks, as an adjuct culture, is in agreement with results of other studies (Biavati *et al.*1992; Iwana *et al.* 1993; Roy *et al.* 1996; Yaeshima *et al.* 1996; Klein *et al.* 1998; Reuter *et al.* 2002)

Moreover, rep-PCR genomic fingerprinting is a useful tool for differentiation of bacteria at species, subspecies and even strain level (Rademaker *et al.* 2005).

Zavaglia *et al.* (2000) reported successful fingerprinting using rep-PCR with BOX sequences which differentiated *Bifidobacterium* strains isolated from faeces of newborn babies. However, in current research, rep-PCR fingerprinting profile with both BOXA1R and GTG5 primers did not reveal relative genetic differences between the tested isolates.

Reports highlighting the presence of mislabelling in market products raise concerns about the behaviour of these bacteria as probiotic culture. In probiotic products, proper labelling regarding the safety and functionality of probiotic cultures is very important (Gueimonde *et al.* 2004). Temmerman *et al.* (2003) reported that of 30 food supplements and 25 dairy products tested, mislabelling was noted in 47% of the food supplements and 40% of the dairy products.

In current research, apart from two samples U and W, which mislabelling was noted, all tested products matched their labelling i.e. the probiotics indicated on their labels were present in the products. Samples U and W ought to be named *B. animalis* subsp. *lactis* rather than *B. longum,* which was claimed on their labels.

8.2 Assessment of probiotic characteristics of the isolates

Ten out of 24 *B. animalis* subsp. *lactis* isolated from fermented milks and two commercial cultures along with three bifidobacteria purchased from the National Collection of Type Cultures (*B. breve*, *B. longum* and *B. bifidum*) were selected throughout the study for characterization of their probiotic properties.

8.2.1 The survival at low pH, bile salts presence

The beneficial effects of *Bifidobacterium* spp. can be expected only when viable cells colonise the human gut. Resistance to harsh condition in the GIT could thus be a critical point in selection of probiotic cultures. Therefore, further characterisation of bifidobacteria isolates based on their ability to tolerate conditions in GIT was carried out (Chapter 3). The value obtained by *in vitro* experiments in simulated gastrointestinal secretions might to some extent indicate the survival rate of consumed strains in a real life conditions. It is important to note that the strains in this study were in non-protected form. However, the behaviour of probiotic bacteria protected by food matrix or other secured form, such as microencapsulation might be different and depending on the desired function of strain, test conditions should be adapted. Such observations (which need to be supported by *in vivo* studies could therefore guide us to:

- Decide on the minimum required intake of probiotics, considering the rate of survival/reduction of the isolates in the stomach
- Decide on the need for protection methods (e.g. embedding in food matrices, microencapsulation) for those isolates that show efficient functionality but some degree of sensitivity to the upper digestive tract condition.

No significant reduction in the viable counts of *B. animalis* subsp. *lactis* was

observed at pH values of 3 and 4. However, pH 2 was decisive only for some of tested isolates for which after 2 h incubation, their viable cells considerably reduced but isolates B1, B2, B5, B6 and B8 showed good resistance in this pH. Three different studied type strains (*B. breve, B. longum* and *B. bifidum*) did not survive at low pH values (2 and 3). This study confirmed that capability to survive in low pH values is a characteristic feature of *B. animalis* subsp. *lactis,* which is in agreement with the results of previous studies (Jayamanne and Adams 2009)

In contrast to acid resistance, bile salt did not show any harmful effects on viability of tested bacteria. It might be due to deconjugation of bile salts by the isolates as a result of the presence of bile salt hydrolase (BSH). Hypothetically, this increases the demand on cholesterol for the synthesis of bile salts, and thus leads to lower blood serum cholesterol levels (De Smet *et al.* 1998; Taranto *et al.* 2000). However, it should be taken into consideration that the presence of BSH activity in probiotic bacteria is controversial. Marteau *et al.* (1995) reported that absence or limited dehydroxilation of bile salt is a priority for probiotic bacteria and stated that excessive BSH activity might be detrimental and undesirable. Tanaka *et al.* (2000) stated that extensive bile salts deconjugation leads to steatorrhoea (excessive discharge of fat in the faeces). However, the survivability of tested bacteria in presence of different concentration of bile salts was examined in this research and BSH activities need to be investigated in future.

Survival of *B. animalis* subsp. *lactis* during incubation in stimulated gastric secretions fortified with pepsin was significantly greater than incubation without pepsin. However, the protective effect of pepsin was not observed to be significant in *B. breve, B. longum and B. bifidum.* When encountering to pH 2 and 3, these bacteria were extremely sensitive to acidic condition. Supporting evidence for protective

effects of pepsin on *B. animalis* subsp. *lactis* in low pH has been accumulating in recent years (Maragkoudakis *et al.* 2006; Matto *et al.* 2006). In addition, in the current market, probiotic bacteria are mainly produced and consumed in the presence of milk proteins (i.e. in fermented dairy products). Milk proteins have shown a protective effect on the starters culture bacteria and thus could have supporting role in bifidobacterial survival in the acidic environment of the stomach (Charteris *et al.* 1998). Also, the gastric fluid itself may offer some degree of protection, when compared with the minimal composition low pH PBS (Maragkoudakis *et al.* 2006).

Although the identification study revealed that the bifidobacteria isolated from different products has the same or very similar origin, their reaction in certain *in vitro* studies were different. This could signify the potential effect of environmental stresses (e.g. acidity, oxygen, low temperature) on probiotics during preparation, production and storage of a probiotic food. Such stresses could be extended to include the effects of GIT secretions, such as acid and bile, which in turn might have a positive or negative effect on functionality of these bacteria in the body. On the other hand, probiotic products are consumed at different states of feeding (empty vs full/half full stomach) which might affect the survivability of probiotic cultures. It is estimated that during intestinal transit only 10-40 % of consumed probiotic bacteria remain alive (Matto *et al.* 2006). However, results from this research showed that about 78-85% of the isolates survived in different states of feeding. These results were supported by the research of Ritter *et al.* (2009).

Bifidobacterium animalis subsp. *lactis*, which was identified the only used *Bifidobacterium* species in tested probiotic fermented milks, presented excellent survival in GIT as indicated in these *in vitro* studies. Furthermore, from a technical point of view, *B. animalis* subsp. *lactis* is more useful than other human isolated

bifidobacteria because of its resistance to harsh conditions. Due to high possibility of colonising, human isolated of bifidobacteria are preferred for human consumption (Gueimonde *et al.* 2004; Sanz 2007). Nevertheless, they are extremely sensitive to low pH and this study confirmed why those strains are replaced by *B. animalis* subsp. *lactis* in fermented milks.

8.2.2 Antibiotic resistance

For the safety evaluation of bacteria, European Food Safety Authority (EFSA) has launched QPS (Qualified Presumption of Safety). According to this system, which is quite similar to GRAS in the US, only strains with safety history and well document background are allowed to enter the market.

The acquired resistance of *B. animalis* subsp. *lactis* to kanamycin, gentamycin, tetracycline and streptomycin was confirmed in this research. This is also in agreement with the results of previous studies (Temmerman *et al.* 2003; Moubareck *et al.* 2005).

For safety reasons, probiotic bacteria should not carry transferable antibiotic resistance. Therefore, some possible genes which were assumed to be associated with acquired antibiotic resistance were studied (Chapter 4).

The role of *tet* genes in tetracyclin resistance has been well documented (Aires *et al.* 2007). Gueimonde *et al.* (2010) stated that *tet* genes are involved in the resistance to tetracycline and coding for ribosomal protection proteins. The most abundant antibiotic resistance gene in this research was found to be associated with *tet*(W), however, it is still not entirely clear how these genes are transfer to other bacteria.

In this research, the possibility of horizontal transfer of *tet*(W) from *B. animalis* subsp. *lactis* to *E. faecalis* and *E. faecium* by *in vitro* filter conjugation approach was

studied. To our knowledge, there is little research on transferability of *tet*(W) in *B. animalis* subsp. *lactis*. Gueimonde *et al.* (2010) reported no transfer of *tet*(W) to any of tested recipients.

In conclusion, six trans-conjugant cells were identified (only with *E. faecalis* conjugation) which presented MIC ranging from 8 to16 μg/ml and in one case 32 μg/ml. The original recipient *E. faecalis* JH2-2 showed an MIC≤1 μg/ml and was sensitive to erythromycin. It should be stated that according to EFSA report (2005) MIC breakpoint for entrococci is 16 μg/ml. However, several attempts to obtain trans-conjugants from conjugation with *E. faecium* BM 4105 were failed and it seems the detected *tet*(W) gene could not be transferred from *B. animalis* subsp. *lactis* to *E. faecium* BM 4105.

PCR was not able to define the genetic background for resistance or to transfer tetracycline resistance. None of six transcojugants showed positive PCR for investigated resistance gene and probably more sophisticated techniques are required for these purposes.

8.2.3 Biofilm formation

Another experiment performed in this study enabled measurement of biofilm formation by *B. animalis* subsp. *lactis*. However, the significance of this study originates from the fact that no such study has been reported for bifidobacteria so far.

The ability to biofilm production is a common property amongst many bacteria from different sources. Such capacity might be considered beneficial to bifidobacteria since it helps to promote the colonisation in different ecosystems. Also after covering of epithelial receptors by bifidobacterial biofilm, undesirable microorganisms will not

be able to colonise. However, this trait has been strongly correlated with the presence of a putative virulence factor, which promotes primary attachment and biofilm formation on abiotic surfaces (Looijesteijn *et al.* 2001; Russell *et al.* 2011). It is well known that many factors influence biofilm formation and composition of growth media might be one of them (Djordjevic *et al.* 2002).

Biofilm formation was studied in four undiluted culture media (MRSC broth, MRS broth, RCM and TSB) and two dilutions thereof (1/2 and 1/20) which led to different levels of biofilm formation by tested bacteria (Chapter 5). *Bifidobacterium animalis* subsp. *lactis* presented good biofilm formation in high nutritious media, such as MRSC broth and RCM. Also environmental stress could have adverse effect on the rate of biofilm formation. The growth of studied bacteria was limited in diluted media due to shortage of nutrients. However, growth limitation could not be enough to induce biofilm formation.

The role of media on biofilm formation was investigated by Hood and Zottola (1997). According to their findings, *L. monocytogenes* was able to form biofilm in nutritious media but *salmonella* presented a contrary reaction with higher biofilm production in nutrient-limited medium and sometimes these reactions vary even among different strains of bacteria (Stephanotis *et al.* 2004).

Based on these results, it is concluded that the *B. animalis* subsp. *lactis* strains have the ability to form a well-structured biofilm, a capacity that could be controversial.

8.2.4 Antimicrobial properties

One principal aspect of bifidobacteria as probiotic cultures used in this study was their antagonistic effects against pathogen bacteria. The potential of bifidobacteria to produce organic acids and other antimicrobial compounds, such as bacteriocins

have been reported (Ibrahim and Bezkorovainy 1993; Gibson and Wang, 1994; Yildirim and Johnson, 1998; Yildirim *et al.* 1999). According to some researches the antagonistic activity of bifidobacteria was related to production of lactic and acetic acid and their pH lowering effect (Ibrahim and Bezkoroaing 1993; Bruno and Shah 2002) but some other studies ascribed this to the secretion of proteinaceous compounds in addition to the pH reducing effects (Meghrous *et al.*1990; Toure *et al.* 2003).

The positive antimicrobial effects of the tested isolates using spot test, even with buffered media, indicated the production of some presumptive antimicrobial compounds (Chapter 6). Regarding the results of well diffusion assay, no antagonistic activity was observed with un-concentrated supernatant which might be due to low concentration of antibacterial compounds or a result of dilution by diffusion into agar. These results are similar to those of Harris *et al.* (1989) and Toure *et al.* (2003). However, concentration of supernatants by freeze drying/vacuum evaporation increased the amount of antibacterial compounds in the supernatant which then showed an inhibition zone of more than 3 mm diameter. When concentrated supernatants were neutralized, then no antimicrobial activities was observed.

Base on these observations, it could be concluded that the inhibition activities of isolates form fermented milks depend on the production of organic acids, such as lactic and acetic, during fermentation. It has been reported that acetic and lactic acid accounted for more than 90% of the organic acids produced by bifidobacteria (Russell *et al.* 2011). *Bifidobacterium longum* and *B. breve* showed more antagonistic property compared to *B. animalis* subsp. *lactis* not only in spot test, but also in well diffusion assay which might be due to the concentration or type of

individual organic acids derived from their metabolisms. There is, however, a possibility of antimicrobial compound production other than organic acids by these organisms.

8.2.5 Production of secondary metabolite (CLA)

In order to complete the study of probiotic properties of bifidobacteria, *in vitro* studies on the capability of conjugated linoleic acid (CLA) formation was also conducted. The production of health promoting fatty acids, such as CLA, could be an example of pharmabiotic production by intestinal bacteria (O'Shea *et al.* 2012).

The potential of bifidobacteria in production of CLA in presence of 500 µg LA/ml in two different growth media (MRSC broth and RCM) was studied (Chapter 7). RCM supplemented with linoleic acid (LA) was found to be the most suitable tested medium for production of CLA by all studied bacteria.

This could be attributed to the differences in the composition of these two media. The role of proteins in the growth media is considerable which may enhance the interaction of substrate with the bacteria and subsequently facilitating the production of CLA (Chung *et al.* 2008). The potential of probiotic bacteria to form CLA from LA has been reported in several studies. Coakley *et al.* (2003) reported *B. breve* was the most capable CLA producers which showed converting rate of 27-65% and *B. bifidum* was the least capable with only 0.18 % conversion. According to this report, *B. animalis* subsp. *lactis* was able to convert LA to CLA at the rate of 27.98%. In similar research, the percentage of converted CLA was reported from 4.12% to 76.65% for *B. breve* and from 3.68% to 60.12% for *B. longum* strains (Barrett *et al.* 2007).

In the current research the highest CLA content was obtained in RCM and in

association with *B. breve* which was quantified at 11.36%. However, based on existing knowledge, intrinsic characteristic of tested bacteria and also experimental conditions could have essential effect on performance of bacteria for CLA production.

Even though probiotic products, such as fermented milks are generally regarded as safe and stable, results of this research emphasises the necessity for continuing studying and publication by researchers which may persuade manufacturers to improve the quality of their products.

Chapter 9

Conclusions and future works

This research initially studied the survival of probiotic bifidobacteria in fermented dairy products marketed in the UK during their shelf-life and also identified the isolated *Bifidobacterium* spp. with biochemical and biomolecular approaches. Furthermore, other main objectives of this project were *in vitro* studies of the potential physiological and functional properties of the isolates, such as resistance to simulated gastric secretions in stomach and bile salts, antibiotic resistance and transfer possibility of antibiotic resistance genes, biofilm formation, antimicrobial activity and production of conjugated linoleic acid (CLA).

Evaluation of two selective media (BIM-25 and TOS-MUP) for the selective enumeration of bifidobacteria demonstrated that both media were effective for enumeration of bifidobacteria in fermented milks. However, TOS-MUP was chosen for its selectivity as well as good recovery of bifidobacteria and ease of preparation.

Twenty-four *Bifidobacterium* spp. were isolated from fermented milk samples, having examined a total of 24 samples and all were identified as *B. animalis* subsp. *lactis* using genotypic analysis. Due to its reliability and degree of differentiation, rep-PCR was used as a valuable technique for rapid and accurate differentiation of probiotic strains in combination with genotyping analysis to identify and discriminate bacteria contained in commercial dairy products.

Using rep-PCR, this study showed that *Bifidobacterium* spp. in fermented milks in the UK lack diversity and all seem to be of the same origin.

With regard to the population of bifidobacteria in the tested products it was found that except in two products, all other samples retained recommended levels of probiotic levels (10^6 CFU/g) at the end of their shelf-life.

Conceivably the decrease in the number of probiotic bacteria in fermented products could be avoided or limited by selection of proper carrier, technological care during

processing and the use of higher initial inoculum levels. It is of great importance to mention that the majority of tested products were inadequately labelled (in terms of number and type of probiotic species) and a few did not correspond with the real identity of the incorporated strains. Other reports commented on mislabelling of the probiotic products; this study indicates that the situation still needs to be improved.

For investigation of probiotic properties, 10 selected strains of *B. animalis* subsp. *lactis* and two commercial cultures, along with three different type strains (*B. breve*, *B. longum* and *B. bifidum*) were selected for further characterization.

Preliminary screening of the above isolates based on survival in low pH conditions demonstrated that *B. animalis* subsp. *lactis* presented greatest resistance in acidic conditions, even at pH 2.0. Subsequent study also indicated that all tested strains had good resistance to various bile salt concentrations.

In this study, an advanced system to simulate the stomach and intestine conditions was also set up for the assessment of survival of a representative of *B. animalis* subsp. *lactis*. This isolate (which was named B6) presented even better resistance to the simulated conditions of upper digestive tract. These studies also showed that other tested bifidobacteria (*B. breve, B. longum* and *B. bifidum*) need a protective system (food matrix or encapsulation) to survive passage through the gut.

The results of acid resistance for *B. animalis* subsp. *lactis* showed that pepsin protected the cells during exposure to low pH. It is suggested that such a protective effect is the result of decreased hyper-polarisation of the cells during exposure to low pH, which in turn implies that it might be linked to the H+-ATPase activity of the cells. This was not examined and is considered worthy of study in future.

There are variants of secretory enzymes produced in the GIT which have different proteolytic activity. For example, in the stomach pepsin is not the only enzyme, and

even so, it is multi-variant (e.g. pepsin A and C) and the gastric antibacterial efficiency could be different for these variants of pepsin.

From a safety point of view, acquired resistance to tetracycline, kanamycin, erythromycin and streptomycin were observed among tested isolates. To the best of our knowledge this is the first study on commercial *B. animalis* subsp. *lactis* strains that has demonstrated the transferability of *tet*(W), as the gene associated to tetracycline resistance, to enterobacteria. It is unfortunate that currently there are no data available to the public or scientists as to whether any of the commercial strains used as probiotic are screened for such risk. Results of the present study suggest that as part of commercialisation procedure for probiotics, such important safety measures need to be put in place. Strains should be tested for the presence of transferable resistance genes before being used as commercial starters and probiotic cultures. However, such data by no means are inclusive and, as a controversial topic, needs further investigations and more evidence.

In vitro assessment of biofilm formation by bifidobacteria concluded that biofilm formation capacity is strongly dependent on the culture medium used. In general, a direct relationship between the extent of biofilm formation and planktonic growth in the medium was observed. Nutrient shortage reduced biofilm growth of bifidobacteria.

Another important aspect of bifidobacteria is their interactions with indigenous organisms of the human. When the culture supernatant of 14 isolates screened for their antimicrobial activities, it was concluded that antagonistic effects against pathogen bacteria were due to production of organic acids and that non-commercial cultures of *B. breve* and *B. longum* were better choices. Although bacteriocin formation by some bifidobacteria species/strains has been reported earlier, the

tested isolates did not show such capacity.

The capability of bifidobacteria to produce CLA was species dependant and also could depend on the growth media. The CLA accumulation ability of bifidobacteria is of interest for the development of probiotic products.

In conclusion, this study revealed a considerable homogeneity in probiotic properties among the different isolates of *B. animalis* subsp. *lactis*. Although *B. animalis* subsp. *lactis* has many technological advantages, their superiority is not necessarily extended to functional properties. The observed differences in some of the functional properties of the isolates could be a result of diverse environmental stresses that the isolates might have been subjected to during preparation.

Suggestions for future works

- Continued search for finding more diverse range of *Bifidobacterium* species/strains for use in fermented dairy products.
- Further research on mislabelling of the probiotic products and suggestions for improvement of current situation.
- Research on possible protective mechanisms of pepsin on probiotic bacteria during exposure to low pH.
- Improve of the quality of *in vitro* studies on resistance to gastric fluid, using actual human gastric fluid, such as secretions which could be obtained from patients undergoing routine gastric drainage.
- Further investigations and more evidences are needed as potential warning that risk of transfer of antibiotic resistance genes, might be associated with commercial probiotic foods.
- Study of the biofilm formation after the probiotic strains are exposed to conditions similar to gastrointestinal tract, such as low pH, presence of bile salts and mucus.
- Further investigations on interactions of bifidobacteria with indigenous organisms of the human.
- Further studies in food systems, such as milk, to maximize the enrichment with CLA and to enhance the health functionality of the product
- Further investigations on the effect of different environmental and physiological stresses on probiotic and functional properties of commercial strains.

REFERENCES

Abe F, Muto M, Yaeshima T, Iwatsuki K, *Aihara H, Ohashi Y and Fujisawa T (2010)* Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability, *Anaerobe* **16** 131–136.

Adams M (1999) Safety of industrial lactic acid bacteria, *Journal of Biotechnology* **68** 171–178.

Aimmo M, Modesto M and Biavati B (2007) Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products, *International Journal of Food Microbiology* **115** 35–42.

Aires J, Doucet-Populaire F and Butel M (2007) tetracycline resistance mediated by *tet*(w), *tet*(m), and *tet*(o) genes of *bifidobacterium* isolates from humans, *Applied and Environmental Microbiology* **73** 2751–2754.

Akalin A, Tokusoglu O, Gonc S and Aycan S (2007) Occurrence of conjugated linoleic acid in probiotic yogurts supplemented with fructooligosaccharide, *International Dairy Journal* **17** 1089–1095.

Ammor M, Florez A and Mayo B (2007) Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria, *Food Microbiology* **24** 559–570.

Angus F, Smart S and Shortt C (2005) Prebiotic ingredients with emphasis on galactooligosaccharides and fructooligosaccharides. In: *Probiotic Dairy Products,* Tamime A (Ed.), pp.120-137, **Oxford,** Blackwell Science Ltd.

Arunachalam K (1999) Role of bifidobacteria in nutrition, medicine and technology, *Nutrition Research* **19** 1559-1597.

Barrett E, Ross R, Fitzgerald G and Stanton C (2007) Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures, *Applied and Environmental Microbiology* **73** 2333–2337.

Bernet MF, Brassart D, Neeser JR and Servin AL (1993) Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions, *Applied and Environmental Microbiology* **59** 4121-4128.

Berrada N, Lemeland JF, Laroch G, Thouvenot P and Piaia M (1991) *Bifidobacterium* from fermented milks: survival during gastric transit, *Journal of Dairy Science* **74** 409–413.

Besselink G, van Santvoort C, Buskens E, Boermeester A, van Goor H, Timmerman H, Nieuwenhuijs V, Bollen T, van Ramshorst B, Witteman B, Rosman C, Ploeg R, Brink M, Schaapherder A, Dejong C, Wahab P, van Laarhoven C, van der Harst E, van ijck C, Cuesta M, Akkermans L and Gooszen H (2008) Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial, *The Lancet* **371** 651-659.

Bevilacqua L, Ovidi M, Mattia E, Trovatelli L and Canganella F (2003) Screening of *Bifidobacterium* strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens, *Microbiology Research* **158** 179-184.

Bezkorovainy A (2001) Probiotics: determinants of survival and growth in the gut. *American Journal of Clinical Nutrition*, **73** 399-405.

Biavati B, Mattarelli B and Crociani P (1992) Identification of bifidobacteria from fermented milk products, *Microbiologica* **15** 7–14.

Bielecka M, Biedrzycka E, Biedrzycka E, Smoragiewicz W and Mieszek M (1998) Interaction of *Bifidobacterium* and *Salmonella* during associated growth, *International Journal of Food Microbiology* **45** 151-155.

Booth G, Kon S, Dann W and Moor T (1935) A study of seasonal variation in butter fat. II. A seasonal spectroscopic variation in the fatty acid fraction, *Biochemistry Journal* **29** 133–137.

Branda S, Vik A, Friedman L and Kolter R (2005) Biofilms: the matrix revisited. *Trends in Microbiology* **13** 20-26.

Bruno A and Shah P (2002) Inhibition of pathogenic and putrefactive microorganisms by *Bifidobacterium* spp., *Milchwissenschaft* **57** 617–621.

Charteris W, Kelly P, Morelli L and Collins K (1998) Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract, *Journal of Applied Microbiology* **84** 759–768.

Charteris W, Kelly P, Morelli L and Collins K (1997) Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial population, *International Journal of Food Microbiology* **35** 1–27.

Charteris W, Kelly P, Morelli L and Collins K (1998) Antibiotic susceptibility of potentially probiotic *Bifidobacterium* isolates from the human GIT, *Letters in Applied Microbiology* **26** 333– 337.

Cheikhyoussef A, Pogori N, Chen W, Zhang H (2008) Antimicrobial proteinaceous compound obtained from bifidobacteria: From production to their application, *International Journal of Food Microbiology* **125** 215-222.

Choi N, Park H, Kim Y, Kim I, Kang H, Yoon C, Yoon H, Park S, Lee W and Chung S (2008) Utilization of monolinolein as a substrate for conjugated linoleic acid production by *Bifidobacterium breve* LMC 520 of human neonatal origin, *Journal of Agriculture Food Chemistry* **56** 10908–10912.

Chung S, Kim I, Park H, Kang H, Yoon C, Jeong H, Choi N, Kwon E and Kim Y (2008) Synthesis of conjugated linoleic acid by human derived *Bifidobacterium breve* LMC 017: Utilization as a functional starter culture for milk fermentation, *Journal of*

Agriculture Food Chemistry **56** 3311–3316.

Coakley M, Ross R, Nordgren M, Fitzgerald G, Devery R and Stanton C (2003) Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species, *Journal of Applied Microbiology* **94** 138–145.

Codex Stan 243-2003 (2003) Codex standard for fermented milks. Codex Alimantarius 13: 1-5

Collado M and Sanz Y (2007) Induction of acid resistance in Bifidobacterium: a mechanism for improving desirable traits of potentially probiotic strains, *Journal of Applied Microbiology* **103** 1147–1157.

Collado MC, Moreno Y, Cobo JM and Hernandez M (2006) Microbiological evaluation and molecular characterization of bifidobacteria strains in commercial fermented milks, *European Food Research and Technology* **222** 112-117.

Collado MC, Gonzalez A, Gonzalez R, Hernandez M, Ferrus MA and Sanz Y (2005) Antimicrobial peptides are among the antagonistic metabolites produced by *Bifidobacterium* against *Helicobacter pylori, International Journal of Antimicrobial Agents* **25** 385-391.

Crittenden RG, Martinez NR and Playne MJ (2003) Synthesis and utilization of folate by yogurt starter cultures and probiotic bacteria, *International Journal of Food Microbiology* **80** 217–222.

Crociani F, Alessandrini A, Mucci M and Biavati B (1994) Degradation of complex carbohydrates by *Bifidobacterium* spp., *International Journal of Food Microbiology* **24** 199-210.

Cruz A, Antunes A, Sousa A, Faria J and Saad S (2009) Ice cream as a probiotic food carrier, *Food Research International* **42** 1233–1239.

Cummings H, Pomare W, Branch J, Naylor P and Macfarlane T (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood, *Gut* **28** 1221–1227.

D'Souza AL, Rajkumar C, Cooke J and Bulpitt J (2002) Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis, *British Medical Journal* **324** 1361-1366.

Dave R and Shah P (1998) Ingredient supplementation effects on viability of probiotic bacteria in yougurt, *Journal of Dairy Science* **81** 2804–2816.

Dave R and Shah P (1997) Viability of yogurt and probiotic bacteria in yogurts made from commercial starter cultures, *International Dairy Journal* **7** 31–41.

De Smet I, De Boevera P and Verstraetea W (1998) Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity, *British Journal of Nutrition* **79** 185-194.

Dewanti R and Wong A (1995) Influence of culture conditions on biofilm formation by *Escherichia coli* 0157:H7, *International Journal of Food Microbiology* **26** 147–64.

DiBaise J, Zhang H, Crowell M, Krajmalnik-Brown R, Decker G and Rittmann B (2008) Gut microbiota and its possible relationship with obesity, *Mayo Clinic Proceedings* **83** 460-469.

Ding W and Shah N (2007) Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria, *Journal of Food Science* **72** 446-450.

Djordjevic D, Wiedman M and McLandsborough L (2002) Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation, *Applied and Environmental Microbiology* **68** 2950–2958.

Donlan R (2002) Biofilms: microbial life on surfaces, *Emerging Infectious Diseases* **8** 881–890.

EFSA (2008) Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance, available at (http://www.efsa.europa.eu/en/efsajournal/doc/732.pdf), accessed on 18.04.2011

EUCAST (2009) clinical MIC breakpoints, available at (http://www.srga.org/eucastwt/mictab/micaminoglycosides.html), accessed on 18.04.2011.

European Commission (2005) Opinion of the FEEDAP Panel on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance, *European Food Safety Authority Journal* **223** 1–12.

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food: Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London: FAO/WHO.

Farnworth E, Mainville I, Desjardins M, Gardner N, Fliss I and Champagne C (2007) Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation, *International Journal of Food Microbiology* **116** 174–181.

Felis G and Dellaglio F (2007) Taxonomy of lactobacilli and bifidobacteria, *Current Issues in Intestinal Microbiology* **8** 44–61.

Felley P, Theulaz I, Rivero L, Sipponen P, Kaufmann M, Bauerfeind P, Wiesel H, Brassart D, Pfeifer A, Blum L and Michetti P (2001) Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man, *European Journal of Gastroenterology and Hepatology* **13** 25–29.

Fooks J and Gibson R (2002) Probiotics as modulators of the gut flora. *British Journal of Nutrition* **88** Suppl. 1, S39–S49.

Gbassi G, Vandamme T, Yolou F and Marchioni E (2011) *In vitro* effects of pH, bile salts and enzymes on the release and viability of encapsulated *Lactobacillus plantarum* strains in a gastrointestinal tract model, *International Dairy Journal* **21** 97-102

Gevers D, Huys G and Swings J (2003) *In vitro* conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria, *Federation of European Microbiological Societies* (FEMS) *Microbiology Letters* **225** 125-130.

Gibson G and Wang X (1994) Regulatory effects of bifidobacteria on the growth of other colonic bacteria, *Journal of Applied Bacteriology* **77** 412–420.

Gilliland S (1987) Importance of bile tolerance in lactobacilli used as dietary adjunct. In: *Biotechnology in the Feed Industry,* Lyons, T. P. (Ed), 149–155. Alltech Feed Co., Lexington, KY. Cited in Succi *et al.* (2005).

Gilliland S (1978) Beneficial inter-relationships between certain micro-organisms and humans: Candidate micro-organisms for use as dietary adjuncts, *Journal of Food Protection* **42** 164–167.

Gilliland S, Reilly S, Kim G and Kim H (2002) Viability during storage of selected probiotic lactobacilli and bifidobacteria in a yogurt-like product, *Journal of Food Science* **67** 3091– 3095.

Gilliland S, Stanley T and Bush L (1984) Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct, *Journal of Dairy Science* **67** 3045–3051.

Ghoddusi H and Hassan K (2011) Selective enumeration of bifidobacteria: A comparative study, *Milchwissenschaft* **66** 149-151.

Goderska K and Czarnecki Z (2007) Characterization of selected strains from *Lactobacillus acidophilus* and *Bifidobacterium bifidum, African Journal of Microbiology Research* **1** 65-78.

Goldin B and Gorbach S (1989) *Lactobacillus* strain and methods of selection, *US Patent* 4839281.

Gomes A and Malcat F (1999) *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics, *Trends in Food Science and Technology* **10** 139-157.

Gomes A, Malcata F and Klaver F (1998) Growth enhancement of *Bifidobacterium lactis* Bo and *Lactobacillus acidophilus* Ki by milk hydrolyzates, *Journal of Dairy Science* **81** 2817-2825.

Gopal P, Prasad J, Smart J and Gill H (2001) *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli, International Journal of Food Microbiology* **67** 207-216.

Grady B and Gibson GR (2005) Microbiota of the human gut. In: *Probiotic Dairy Products,* Tamime A (Ed.), pp.120-137, **Oxford,** Blackwell Science Ltd.

Guarner F and schaafsma G (1998) Probiotic, *International Journal of Food Microbiology* **39** 237-238.

Gueimonde M, Delgado S, Mayo B, Ruas-Madiedo P, Margolles A and Gavilan C (2004) Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in commercial fermented milks, *Food Research International* **37** 839–850.

Gueimonde M, Florez A, Hoek A, Lauridsen B, Stroman P, Reyes-Gavilan C and Margolles A (2010) Genetic basis of tetracycline resistance in *Bifidobacterium animalis* subsp. *lactis, Applied and Environmental Microbiology* **76** 3364–3369.

Gurakan G, Cebeci A and Ozer B (2010) Probiotic dairy beverages: microbiology and technology. In: *Development and Manufacture of Yogurt and Other Functional Dairy Products*, Yildiz F, (Ed.),pp. 165-196, **London,** CRC Press.

Hadadji M, Benama R, Saidi N, Henni E and Kihal M (2005) Identification of cultivable *Bifidobacterium* species isolated from breast fed infant faeces, *African Journal of Biotechnology* **4** 422–430

Harmsen M, Gibson R, Elfferich P, Raangs C, Wildeboer-Veloo M, Argaiz A, Roberfroid B and Welling W (2000) Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria, *Federation of European Microbiological Societies (FEMS) Microbiology Letters* **183** 125–129.

Harris LJ, Daeschal MA, Stiles ME and Klaenhammer TR (1989) Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*, *Journal of Food Protection* **52** 384–387.

Heenan C, Adams M, Hosken R and Fleet G (2004) Survival and Sensory acceptability of probiotic microorganisms in a non fermented frozen vegetarian dessert, *Lebensmittel-Wissenschaft and Technologie* **37** 461–466.

Hickey M (2005) Current legislation of probiotic products. In: *Probiotic Dairy Products,* Tamime A (Ed.), pp.120-137, **Oxford,** Blackwell Science Ltd.

Hirayama K and Rafter J (2000) The role of probiotic bacteria in cancer prevention, *Microbes* and *Infection* **2** 681–686.

Holzapfel W, Haberer P, Snel H, Schillinger U and Veld J (1998) Overview of the gut microflora and probiotics, *Journal of Food Microbiology* **41** 85–101.

Hood S and Zottola E (1997) Adherence to stainless steel by foodborne microorganisms during growth in model food systems, *International Journal of Food Microbiology* **37**145–153.

Hopkins J, Sharp R and Macfarlane T (2001) Age and disease related changes in intestinal bacterial popular-ions assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles, *Gut* **48** 198–205.

Hopkins J, Sharp R and Macfarlane T (2002) Variation in human intestinal microbiota with age, *Digestive and Liver Disease* **34** S12–S18.

Hummel A, Hertel C, Holzapfel W and Franz C (2007) Antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria, *Applied and Environmental Microbiology* **73** 730–739.

Ibrahim S and Bezkorovainy A (1993) Inhibition of Escherichia coli by bifidobacteria, *Journal of Food Protection* **56** 713–715.

Ishibashi N (1997) Bifidobacteria: their significance in human intestinal health, *Malaysian Journal of Nutrition* **3** 149–159.

Ishibashi N and Shimamura S (1993) Bifidobacteria: research and development in Japan, *Food Technology* **47** 126–134.

ISO 29981:2010 (IDF 220: 2010) Milk products -- Enumeration of presumptive bifidobacteria -- Colony count technique at 37°C.

Iwana H, Masuda H, Fujisawa, T, Suzuki H and Mitsuoka T (1993) Isolation and identification of *Bifidobacterium* spp. in commercial yogurts sold in Europe, *Bifidobacteria Microflora* **12** 39–45.

Jayamanne V and Adams M (2009) Modelling the effects of pH, storage temperature and redox potential (Eh) on the survival of bifidobacteria in fermented milk,

International Journal of Food Science and Technology **44** 1131–1138.

Jayamanne V and Adams M (2006) Determination of survival, identity and stress resistance of probiotic bifidobacteria in bio-yogurt, *Letters in Applied Microbiology* **42** 189-194.

Jenkins J and Courtney P (2003) Lactobacillus growth and membrane composition in the presence of linoleic acid or conjugated linoleic acid, *Canadian Journal of Microbiology* **49** 51–57.

Jiang J, Bjrock L and Fonden R (1998) Production of conjugated linoleic acid by dairy starter cultures, *Journal of Applied Microbiology* **85** 95-102.

Jiang J, Wolk A and Vessby B (1999) Relation between the intake of milk fat and the occurrence of conjugated linoleic acid in human adipose tissue, *American Journal of Clinical Nutrition* **70** 21–7.

Jung Y (2005) Production of conjugated linoleic acid and conjugated linolenic acid by *Bifidobacterium breve* jkl03 and its application, Available at: http://www.google.co.uk/search?q=production+of+conjugated+linoleic+acid+and+conjugated+linolenic+acid++by+bifidobacterium+breve+jkl03&sourceid=ie7&rls=com.microsoft:en-gb:IE-SearchBox&ie=&oe=&redir_esc=&ei=NBakT_OAIonV4QS-iPCoCQ Accessed on 10 April 2012.

Kailasapathy K and Rybka S (1997) *Lactobacillus acidophilus* and *Bifidobacterium* spp. their therapeutic potential and survival in yogurt, *Australian Journal of Dairy Technology* **52** 28–35.

Kailasapathy K, Harmstorf I and Phillips M (2008) Survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in stirred fruit yogurts, *LWT-Food Science and Technology,* **41** 1317–1322.

Kapoor R, Reaney M and Westcott N (2005) Conjugated Linoleic Acid Oils, Bailey's Industrial Oil and Fat Products, Sixth Edition, Six Volume Set. Edited by Fereidoon Shahidi. **New York**, John Wiley & Sons, Inc.

Karlowsky J and Jones E (2003) Importance of using current NCCLS breakpoints to interpret cefotaxime and ceftriaxone MICs for *Streptococcus pneumoniae, Journal of antimicrobial chemotherapy* **51** 467–468.

Kasımoglu A, Goncuoglu M and Akgun S (2004) Probiotic white cheese with *Lactobacillus acidophilus, International Dairy Journal* **14** 1067–1073.

Kaufmann P, Pefferkoran A, Teuber M and Meile L (1997) Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16s rRNA-targeted probes by colony hybridization and PCR, *Applied and Environmental Microbiology* **63** 1268-1273.

Kelly D, Conway S and Aminov R (2005) Commensal gut bacteria: mechanisms of immune modulation, *Trends in Immunology* **26** 326-333.

Kheadr E, Bernoussia N, Lacroixc C and Fliss I (2004) Comparison of the sensitivity of commercial strains and infant isolates of bifidobacteria to antibiotics and bacteriocins, *International Dairy Journal* **14** 1041–1053.

Kim Y, Liu R, Bound D and Russell J (2000) Effect of linoleic acid concentration on conjugated linoleic acid production by *Butyrivibrio fibrisolvens* A38, *Applied and Environmenyal Microbiology* **66** 5226-5230.

King A and Phillips I (2001) The *in vitro* activity of daptomycin against 514 Gram positive aerobic clinical isolates, *Journal of antimicrobial chemotherapy* **48** 219-223.

Klaenhammer T and Kullen M (1999) Selection and design of probiotics, *International Journal of Food Microbiology* **50** 45-57.

Klaver F, Kingma F and Weerkamp A (1993) Growth and survival of bifidobacteria in milk, *Netherlands Milk and Dairy Journal* **47** 151–164.

Klein G, Pack A, Bonaparte C and Reuter G (1998) Taxonomy and physiology of probiotic lactic acid bacteria, *International Journal of Food Microbiology* **41** 103–125.

Kolter R, Siegele DA and Tormo A (1993) The stationary phase of the bacterial life cycle, *Annual Review Microbiology* **47** 855–74.

Kubota H, Senda S, Nomura N,Tokuda H and Uchiyama H (2008) Biofilm formation by lactic acid bacteria and resistance to environmental stress, *Journal of Bioscience and Bioengineering* **106** 381–386.

Land H, Stevens K, Woods R, Cannon L, Cnota J and Shetty K (2005) *Lactobacillus* sepsis associated with probiotic therapy, *Pediatrics* **115** 178–81.

Lankaputhra V and Shah N (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts, *Cultured Dairy Products Journal* **30** 2–7.

Lay C, Rigottier-Gois L, Holmstrom K, Rajilic M, Vaughan E, de Vos M, Collins D, Thiel R, Namsolleck P, Blaut M and Dore J (2005) Colonic microbiota signatures across five Northern European countries, *Applied and Environmental Microbiology* **71** 4153–4155.

Lebeer S, Verhoeven T, Perea Velez M, Vanderleyden J and De Keersmaecker S (2007) Impact of environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG, *Applied and Environmental Microbiology* **73** 6768–6775.

Lee Y, Yu W and Heo T (2003) Identification and screening for antimicrobial activity against *Clostridium difficile* of *Bifidobacterium* and *Lactobacillus* species isolated from healthy infant faeces, *International Journal of Antimicrobial Agents* **21** 340-346.

Leriche V and Carpentier B (2000) Limitation of adhesion and growth of on stainless steel surfaces by *Staphylococcus sciuri* biofilms. *Journal of Applied Microbiology* 88 594-605.

Licht T and Wilcks A (2005) Conjugative gene transfer in the gastrointestinal environment, *Advance Applied Microbiology* **58** 77-95.

Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR and Servin AL (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity, *Gut* **47** 646–652.

Lim K, Huh C and Baek Y (1993) Antimicrobial susceptibility of bifidobacteria, *Journal of Dairy Science* **76** 2168–2174.

Lima K, Kruger M, Behrens J, Destro M, Landgraf M and Franco B (2009) Evaluation of culture media for enumeration of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium animalis* in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus, LWT - Food Science and Technology* **42** 491–495.

Lin T (2000) Conjugated linoleic acid concentration as affected by lactic cultures and additives, *Food Chemistry* **69** 27-31.

Lin T (2006) Conjugated linoleic acid production by cells and enzyme extract of Lactobacillus delbrueckii ssp. bulgaricus with additions of different fatty acids, *Food*

Chemistry **94** 437–441.

Lin T, Lin C and Lee C (1999) Conjugated linoleic acid concentration as affected by lactic cultures and added linoleic acid, *Food Chemistry* **67** 1-5.

Lin T, Lin C and Wang Y (2003) Production of conjugated linoleic acid by enzyme extract of Lactobacillus acidophilus CCRC 14079, *Food Chemistry* **83** 27–31.

Looijesteijn J, Trapet L, de Vries E, Abee T and Hugenholtz J (2001) Physiological function of exopolysaccharides produced by *Lactococcus lactis*, *International Journal of Food Microbiology* **64** 71–80.

Macfarlane S and Macfarlane G (2006) Composition and metabolic activities of bacterial biofilms colonizing food residues in the human gut, *Applied and Environmental Microbiology* **72** 6204–6211.

Macouzet M, Lee B and Robert N (2009) Production of conjugated linoleic acid by probiotic *Lactobacillus acidophilus* La-5, *Journal of Applied Microbiology* **106** 1886–1891.

Maragkoudakis P, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B and Tsakalidou E (2006) Probiotic potential of *Lactobacillus* strains isolated from dairy products, *International Dairy Journal* **16** 189–199.

Marques M, Wall R, Ross P, Fitzgerald F, Ryan A and Stanton C (2010) Programming infant gut microbiota: influence of dietary and environmental factors, *Current Opinion in Biotechnology* **21** 149–156.

Marseglia G, Tosca M, Cirillo I, Licari A, Leone M, Marseglia A, Castellazzi A and Ciprandi G (2007) Efficacy of *Bacillus clausii* spores in the prevention of recurrent respiratory infections in children: a pilot study, *Therapeutics and Clinical Risk Management* 3 13–17.

Marteau P, Gerhardt F, Myara A, Bouvier E, Trivin F and Rambaud C (1995) Metabolism of bile salts by alimentary bacteria during transit in the human small intestine, *Microbial Ecology in Health and Disease* **8** 151–157.

Masco L, Huys G, Gevers D, Verbrugghen L and Swing J (2003) Identification of *Bifidobacterium* species using rep-PCR fingerprinting system, *Applied Microbiology* **26** 557–563.

Masco L, Ventura M, Zink R, Huys G and Swings J(2004) Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp nov and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp nov, *International Journal of Systematic and Evolutioary Microbiology* **54** 1137-1143.

Masco L, Huys E, De Brandt R, Temmerman and Swings J (2005) Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria, *International Journal of Food Microbiology* **102** 221–230.

Matsumotoa M, Ohishia H and Benno Y (2004) H^+-ATPase activity in *Bifidobacterium* with special reference to acid tolerance, *International Journal of Food Microbiology* **93** 109– 113.

Mattarelli P, Bonaparte C, Pot B and Biavati B (2008) Proposal to reclassify the three biotypes of *Bifidobacterium longum* as three subspecies: *Bifidobacterium longum* subsp. *longum* subsp. nov., *Bifidobacterium longum* subsp. *infantis* comb. nov., and *Bifidobacterium longum* subsp. *suis* comb. nov., *International Journal of Systematic and Evolutionary Microbiology* **58** 767–772.

Mattila-Sandholm T, Myllarinen P, Crittenden R, Mogensen G, Fonden R and Saarela M (2002)Technological challenges for future probiotic foods, *International Dairy Journal* **12** 173–182.

Matto J, Alakomi H, Vaari A, Virkaja I and Saarela M (2006) Influence of processing conditions on *Bifidobacterium animalis* subsp. *lactis* functionality with a special focus on acid tolerance and factors affecting it, *International Dairy Journal* **16** 1029–1037

Medina L and Jordan R (1994) Survival of constitutive micro flora in commercially fermented milk containing bifidobacteria during refrigerated storage, *Journal of Food Protection* **56** 731–733.

Meile L, Ludwig W, Rueger U, Gut C, Kaufmann P, Dasen G, Wenger S and Teuber M (1997) *Bifidobacterium lactis* sp. Nov., a moderately oxygen tolerant species isolated from fermented milk, *System Applied Microbiology* **20** 57-64.

Meghrous J, Euloge P, Junelles M, Ballongue J and Petitidemange H (1990) Screening of *Bifidobacterium* strains for bacteriocins production, *Biotechnology Letters* **12** 575–580.

Merk (2010) Do you have the right balance? TOS-MUP Medium for the enumeration of Bifidobacteria in milk products. Available at: https://de.vwr-cmd.com/bin/public/idoccdownload/10020091/Merck. Accessed on 10 April 2012

Micanel N, Haynes I and Playne M (1997) Viability of probiotic cultures in commercial Australian yogurts, *Australian Journal of Dairy Technology* **52** 24–27.

Mitsuoka T (1990) Bifidobacteria and their Role in Human Health, *Journal of Industrial Microbiology* **6** 263-268.

Modler H (1994) Bifidogenic factors - sources, metabolism and applications *International Dairy Journal* **4** 383-407.

Moubareck C, Gavini F, Vaugien L, Butel M and Doucet-Populaire F (2005) Antimicrobial susceptibility of bifidobacteria, *Journal of Antimicrobial Chemotherapy* **55** 38–44.

Munoa F and Pares R (1988) Selective medium for isolation and enumeration of *Bifidobacterium* spp., *Applied and Environmental Microbiology* **54** 1715–1718.

Nielsen D, Moller P, Rosenfeldt V, Paerregaard A, Michaelsen K and Jakobsen M (2003) Case study of the distribution of mucosa-associated *Bifidobacterium* species, *Lactobacillus* species, and other lactic acid bacteria in the human colon, *Applied and Environmental Microbiology* **69** 7545–7548.

Nieuwenhove C, Oliszewski R, González S and Chaia A (2007) Influence of bacteria used as adjunct culture and sunflower oil addition on conjugated linoleic acid content in buffalo cheese, *Food Research International* **40** 559–564.

Nighswonger BD, Brashears MM and Gilliland SE (1996) Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage, *Journal of Dairy Science* **79** 212–219.

NIH (2002) Research on microbial biofilms, available at (http://grants.nih.gov/grants/guide/pa-files/PA-03-047.html), Accessed on 18/04/12

Niku-Paavola ML, Laitila A, Mattila-Sandholm T and Haikara A (1999) New types of antimicrobial compounds produced by *Lactobacillus plantarum*, *Journal Applied Microbiology* **86** 29–35.

Nord C, Sillerstrom E and Wahlund E (2006) Effect of tigecycline on normal oropharyngeal and intestinal microflora, *Antimicrobial Agents and Chemotherapy* ***50*** 3375-3380.

Noriega L, Cuevas I, Margolles A and Reyes-Gavila C (2006) Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile, *International Dairy Journal* **16** 850–855.

Normark B and Normark S (2002) Evolution and spread of antibiotic resistance, *Journal of Internal Medicine* **252** 91-106.

O'Toole G, Kaplan H and Kolter R (2000) Biofilm formation as microbial development, *Annual Review Microbiology* **54** 49–79.

Oelschlaeger T (2010) Mechanisms of probiotic actions – A review, *International Journal of Medical Microbiology* **300** 57–62.

Ogawa J, Matsumura K, Kishino S, Omura Y and Shimizu S (2001) Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus, Applied and Environmental Microbiology* **67** 1246–1252.

O'Shea E, Cotter P, Stanton C, Ross R and Hill C (2012) Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: Bacteriocins and conjugated linoleic acid, *International Journal of Food Microbiology*

152 189-205..

Osullivan D and Kullen M (1998) Tracking of probiotic bifidobacteria in the Intestines, *International Dairy Journal* ***8*** 513-525.

Otero M, Silva C and Macias N (2007) Susceptibility of probiotic bovine vaginal Lactobacillus to antimicrobial agents, *Journal of Animal and Veterinary Advances* **6** 132-138.

Ouoba I, Lei V and Jensen L (2008) Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria, *International Journal of Food Microbiology* **121** 217-224.

Ouoba I, Parkouda C, Diawara B, Scotti C and Varnam A (2008) Identification of *Bacillus* Spp. from Bikalga, fermented seeds of *Hibiscus sabdariffa*: phenotypic and genotypic characterization, *Journal of Applied Microbiology* **104** 122–131.

Pan x, Chen F, Wua T, Tang H and Zhao Z (2009), The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT, *Food Control* **20** 598–602.

Pariza M, Park Y and Cook M (2001) The biologically active isomers of conjugated linoleic acid, *Progress in Lipid Research* **40** 283–298.

Park H, Heo W, KIM S, Kim H, Bae G, Chung S, Seo H and Kim Y (2011) Production

of conjugated linoleic acid (CLA) by *Bifidobacterium breve* LMC520 and its compatibility with CLA-producing rumen bacteria, *Journal of Agriculture Food Chemistry* **59** 984–988.

Perdigon G, Galdeano C, Valdez C and Medici M (2002) Interaction of lactic acid bacteria with the gut immune system, *European Journal of Clinical Nutrition* **56** S21-S26.

Prasad J, Gill H, Smart J and Gopal P (1998) Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics, *International Dairy Journal* **8** 993–1002.

Puupponen-Piamia R, Aura A, Oksman-Caldentey K, Myllarinen P, Saarela M, Mattila-Sandholm T and Poutanen K (2003) Development of functional ingredients for health gut, *Trends in Food Science and Technology* **13** 3-11.

Rademaker J, Aarts H and Vinuesa P (2005) Molecular typing of environmental isolates, *Molecular Microbial Ecology* **4** 97–131.

Requena T, Burton J, Matsuki T, Munro K, Simon A, Tanaka R, Watanabe K and Tannock W (2002) Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene, *Applied and Environmental Microbiology* **68** 2420–2427.

Reuter G, Klein G and Goldberg M (2002) Identification of probiotic cultures in food samples, *Food Research International* **35** 117–124.

Ritter P, Kohler C and von Ah U (2009) Evaluation of the passage of *Lactobacillus gasseri* K7 and bifidobacteria from the stomach to intestines using a single reactor model, *BMC Microbiology* **9**:87

Roberfroid M (2005) Introducing inulin-type fructans, *British Journal of Nutrition* **93** Suppl. 1 S13–S25.

Roberfroid M, Gibson G and Hoyles L (2010) Prebiotic effects: metabolic and health benefits, *British Journal of Nutrition* **104** S1-S63.

Rodriguez-Alcala L, Braga T, Malcata F, Gomes A and Fontecha J (2011) Quantitative and qualitative determination of CLA produced by *Bifidobacterium* and lactic acid bacteria by combining spectrophotometric and Ag+HPLC techniques, *Food Chemistry* **125** 1373–1378.

Romling U, Sierralta W, Eriksson K and Normark S (1998) Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter, *Molecular Microbiology* **28** 249–264.

Ronka E, Malinen E, Saarela M, Rinta-Koski M, Aarnikunnas J and Palva A (2003) Probiotic and milk technological properties of *Lactobacillus brevis*, *International Journal of Food Microbiology* **83** 63– 74.

Rosander A, Connolly E, Roos S (2008) Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938, *Applied Environmental Microbiology* **74** 6032-6040.

Rosberg-Cody E, Ross P, Hussey S, Ryan C, Murphy B, Fitzgerald G, Devery R and Stanton C (2004) Mining the microbiota of the neonatal gastrointestinal tract for conjugated linoleic acid producing bifidobacteria, *Applied and Environmental Microbiology* **70** 4635–4641.

Rosenthal I and Bernstein S (1998) The survival of a commercial culture of bifidobacteria in milk products, *Milchwissenschaft* **53** 441–443.

Roy D (2001) Media for the isolation and enumeration of bifidobacteria in dairy products, *International Journal of Food Microbiology* **69** 167–182.

Roy D, Ward P and Champagne G (1996) Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction, *International Journal of Food Microbiology* **29** 11–29.

Russell D,Ross R, Fitzgerald G and Stanton C (2011) Metabolic activities and probiotic potential of bifidobacteria, *International Journal of Food Microbiology* **149** 88–105.

Ruzauskas M, Suziedeliene E, Siugzdiniene R, Seputiene V and Povilonis J (2010) Antimicrobial resistance of *Enterococcus* spp. spread in poultry products in Lithuania, *Journal of food safety* **30** 902-915.

Saarela M, Mogensen G, Fonden R, Matto J and Mattila-Sandholm T (2000) Probiotic bacteria: safety, functional and technological properties, *Journal of Biotechnology* **84** 197–215.

Sakata S, Kitahara M, Sakamoto M, Hayashi H, Fukuyama M and Benno Y (2002) Unification of *Bifidobacterium infantis* and *Bifidobacterium suis* as *Bifidobacterium longum*, *International Journal of Systematic and Evolutionary Microbiology* **52** 1945–1951.

Salonen A, De Vos W and Palva A (2010) Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives, *Microbiology* **156** 3205-3215.

Samona A and Robinson R (1993) Effect of sweetening agents on the growth and survival of *Bifidobacterium* spp. *Journal of Dairy Science* **76** 120.

Samona A and Robinson RK (1994) Effect of yogurt cultures on the survival of bifidobacteria in fermented milks, *Journal of the Society of Dairy Technology* **47** 58–60.

Sanchez B, Verge M, Lauridsen B, Madiedo P, Anglade P, Baraige F, Gavila C, Johansen E, Zagorec M and Margolles A (2007) Adaptation and response of

Bifidobacterium animalis subsp. *lactis* to bile: a proteomic and physiological approach, *Applied and environmental microbiology* **73** 6757–6767.

Santo A, Perego P, Converti A and Oliveira M (2011) Influence of food matrices on probiotic viability - A review focusing on the fruity bases, *Trends in Food Science and Technology* **22** 377-385

Sanz Y (2007) Ecological and functional implications of the acid-adaptation ability of Bifidobacterium: A way of selecting improved probiotic strains, *International Dairy Journal* **17** 1284-1289.

Sasaki Y, Taketomo N and Sasaki T (1988) Factors affecting transfer ferequency of pAMβ1 from *Streptococus faecalis* to *Lactobacillus plantarum*, *Journal of Bacteriology* **170** 5939-5942.

Sato T and Lino T (2010) Genetic analyses of the antibiotic resistance of Bifidobacterium bifidum strain Yakult YIT 4007, *International Journal of Food Microbiology* **137** 254–258.

Schillinger U (1999) Isolation and identification of lactobacilli from novel-type probiotic and mild yogurts and their stability during refrigerated storage, *International Journal of Food Microbiology* **47** 79–87.

Schoug A, Olsson J, Carlfors J, Schnürer J and Hakansson S (2006) Freeze-drying of *Lactobacillus coryniformis* Si3—effects of sucrose concentration, cell density, and

freezing rate on cell survival and thermophysical properties, *Cryobiology* **53** 119–127.

Scotti C, Sutherland J and Varnam A (2002) Quality of fermented probiotic milks in relation to claims concerning numbers and types of starter bacteria. *British Journal of Nutrition* **88** Suppl. 1: Probiotics and health, S117.

Sela .A, Chapman J, Adeuya A, Kim H, Chen F, Whitehead R, Lapidus A, Rokhsar S, Lebrilla B, German B, Price P, Richardson M and Mills A (2008) The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome, *Proceedings of the National Academy of Sciences of the United States of America* **105** 18964–18969.

Shah N (2000) Probiotic bacteria: Enumeration and survival in dairy foods, *Journal of Dairy Science* **83** 894– 907.

Shah N (2001) Functional foods from probiotics and prebiotics, *Food Technology* **55** 46–53.

Shah N and Lankaputhra V (1997) Improving viability of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in yogurt, *International Dairy Journal* **7** 349–356.

Shah N (2006) Probiotics and fermented milks. In: *Manufacturing Yogurt and Fermented Milks*, Chandan RC, (Ed.) pp. 341-355, **Oxford**, Blackwell Publishing Ltd.

Shin H, Lee J, Pestka J and Ustunol Z (2000) Viability of bifidobacteria in commercial dairy products during refrigerated storage, *Journal of Food Protection* **63** 327–331.

Sieber R, Collomb M, Aeschlimann A, Jelen A and Eyer H (2004) Impact of microbial cultures on conjugated linoleic acid in dairy products-a review, *International Dairy Journal* **14** 1–15.

Silvi S, Rumney CL and Towland IR (1996) An assessment of three selective media for bifidobacteria in faeces, *Journal of Applied Bacteriology* **81** 561–564.

Simpson P, Ross R, Fitzgerald G and Stanton C (2004) *Bifidobacterium psychraerophilum* sp. nov., and *Aeriscardovia aeriphila gen.* nov., sp. nov., isolated froma porcine caecum, *International Journal of Systematic and Evolutionary Microbiology* **54** 401–406.

Sinde E and Carballo J (2000) Attachment of *Salmonella* spp. and *Listeriamonocytogenes* to stainless steel, rubber and polytetrafuorethylene: the infuence of free energy and the effect of commercial sanitizers, *Food Microbiology* **17** 439-447.

Stark L and Lee A (1982) The microbial ecology of the large bowel of breast fed and formula fed infants during the first year of life, *Journal of Medical Microbiology* **15** 189–203.

Stepanovic S, Vukovic D ,Hola V, Bonaventura G, Djukic S , Cirkovic I and Ruzicka F (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci, *Acta Pathologica, Microbiologica et Immunologica Scandinavica (APMIS)* **115** 891–899.

Stepanovic S, Cirkovic I, Ranin L and Svabic´-Vlahovic M (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface, *Letters in Applied Microbiology* 38 428–432.

Succi M, Tremonte P, Reale A, Sorrentino E, Grazia L, Pacifico S and Coppola R (2005) Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese, *Federation of European Microbiological Societies (FEMS) Microbiology Letters* ***244*** *129–137.*

Tabasco R, Paarup T, Janer C, Pelaez C and Requena T (2007) Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus, L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk, *International Dairy Journal* **17** 1107–1114.

Tamime A, Marshall V and Robinson R (1995) Microbiological and technological aspects of milks fermented by bifidobacteria, *Journal of Dairy Research* **62** 151-187.

Tamime AY and Robinson RK (2007) Biochemistry of fermentation, In: *Tamime and Robinson's Yogurt Science and Technology* Third edition, pp. 535-587, Cambridge,Woodhead Publishing Limited.

Tamime AY and Robinson RK (2007) Production systems of starter cultures, In *Tamime and Robinson's Yogurt Science and Technology* Third edition, pp. 626-639, Cambridge, Woodhead Publishing Limited

Tanaka H, Hashiba H, kok J and Mierau I (2000) Bile salt hydrolase of *Bifidobacterium longum*, biochemical and genetic characterization, *Applied and Environmental Microbiology* **66** 2502-2512.

Taranto P, Medici M, Perdigon G, Ruiz A and Valdez G (2000) Effect of *Lactobacillus reuteri* on the prevention of hypercholesterolemia in mice, *Journal of Dairy Science* **83** 401-403.

Temmerman R, Pot B, Huys G and Swings J (2003) Identification and antibiotic susceptibility of bacterial isolates from probiotic products, *International Journal of Food Microbiology* **81** 1–10.

Thapar N and Sanderson R (2004) Diarrhoea in children: an interface between developing and developed countries. *The Lancet* **363** 641–653.

Thitaram S, Siragusa G and Hinton J (2005) *Bifidobacterium* selective isolation and enumeration from chicken caeca by a modified oligosaccharide antibiotic-selective agar medium, *Letters in Applied Microbiology* **41** 355–360.

Tissier H (1900) Recherchers sur la flora intestinale normale et pathologique du nourisson, Thesis, University of Paris, Paris, France. Cited in Cheikhyoussef *et al.* (2008)

Toure R, Kheadr E, Lacroix C, Moroni O and Fliss I (2003) Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes, Journal of Applied Microbiology* **95** 1058-1069.

Tuomola E, Ouwehand A and Salminen S (1999) The efect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *Federation of European Microbiological Societies (FEMS) Immunology and Medical Microbiology* **26** 137-142.

Tuomola E, Salminen S (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures, *International Journal of Food Microbiology* **41** 45–51.

Turnbaugh P, Ley E, Mahowald A, Magrini V, Mardis R and Gordon I (2006) An obesity-associated gut microbiome with increased capacity for energy harvest, *Nature* **444** 1027–1131.

Turroni F, Sinderen D and Ventura M (2011) Genomics and ecological overview of the genus Bifidobacterium, *International Journal of Food Microbiology* **149** 37–44.

UKPAR (no date) available at (http://www.mhra.gov.uk/home/groups/l-unit1/documents/websiteresources/con014533.pdf) accessed on 18.04.2011.

Van Nieuwenhove C, Oliszewski R, Gonzalez S and Perez Chaia A (2007) Conjugated linoleic acid conversion by dairy bacteria cultured in MRS broth and buffalo milk, *Letters in Applied Microbiology* **44** 467–474.

Vandamme T, Lenourry A, Charrueau C, Chaumeil J (2002) The use of polysaccharides to target drugs to the colon, *Carbohydrate Polymers* **48** 219-231.

Vanderhoof J, Whitney B, Antonson L, Hanner L, Lupo V and Young J (1999) *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children, *Journal of Pediatrics* **135** 564–568.

Vasiljevic T and Shah N (2008) Cultured milk and yogurt. In: *Dairy processing and Quality Assurance,* Chandan RC, (Ed.), pp. 219-251, **Oxford**, Blackwell Publishing Ltd

Vaughan E, Heilig H, Ben-Amor K, de Vos M (2005) Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches, *Federation of European Microbiological Societies (FEMS) Microbiology Reviews* **29** 477–490.

Ventura M and Zink R (2003) Comparative sequence analysis of the *tuf* and *recA* genes and restriction fragment length polymorphism of the internal transcribed spacer region sequences supply additional tools for discriminating *Bifidobacterium lactis* from *Bifidobacterium animalis*, *Applied and Environmental Microbiology* **69** 7517–7522.

Vinderola C, Costa G, Regenhardt S and Reinheimer J (2002) Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria, *International Dairy Journal* **12** 579-589.

Vlkova E, Rada V and Trojanova I (2004) Enumeration, isolation and identification of bifidobacteria from dairy products, *Acta Agriculturae Slovenica* **841** 31-36.

Wildt S, Nordgaard I, Hansen U, Brockmann E and Rumessen J (2011) A randomised double-blind placebo-controlled trial with *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 for maintenance of remission in ulcerative colitis, *Journal of Crohn's and Colitis* **5** 115–121.

Xu K, McFeters G and Stewart P (2000) Biofilm resistance to antimicrobial agents, *Microbiology* **146** 547– 549.

Yadav H, Jain S and Sinha P (2007) Production of free fatty acids and conjugated linoleic acid in probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* during fermentation and storage, *International Dairy Journal* **17** 1006–1010.

Yaeshima T, Takahashi S, Ishibashi N and Shimamura S (1996) Identification of bifidobacteria from dairy products and evaluation of a microplate hybridization method, *International Journal of Food Microbiology* **30** 303–313.

Yildirim Z and Johnson M (1998) Characterization and antimicrobial spectrum of bifidocin B, a bacteriocin produced by *Bifidobacterium bifidum* NCFB 1454, *Journal of Food Protection* **6** 47–51.

Yildirim Z, Winters D and Johnson M (1999) Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454, *Journal of Applied Microbiology* **86** 45-54.

Yusof R, Haque F and Hassan Z (2000) Isolation of *Bifidobacteria infantis* and its antagonistic activity against *ETEC* 0157 and *Salmonella typhimurium* S-285 in weaning foods, *Asia Pacific Journal of Clinical Nutrition* **9(2)** 130-135.

Zavaglia A, Urraza P and De Antoni G (2000) Characterization of *Bifdobacterium* Strains Using Box Primers *Anaerobe* **6** 169-177.

Zhou J, Pillidge C, Gopal P and Gill H (2005) Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains, *International Journal of Food Microbiology* **98** 211 – 217.

Zhu W, Liu W and Wu D (2000) Isolation and characterization of a new bacteriocin from *Lactobacillus gasseri* KT7, *Journal of Applied Microbiology* **88** 877–886.

Zouhir A, Kheadr E, Fliss I and Ben Hamida J (2011) Partial purification and characterization of two bacteriocin-like inhibitory substances produced by bifidobacteria, *African Journal of Microbiology Research* **4** 411-418.

Zubillaga M, Weill R, Postaire E, Goldman C, Caro R, Boccio J (2001) Effect of probiotics and functional foods and their use in different diseases, *Nutrition Research* **21** 569-576.

APPENDICES

APPENDIX 1

Growth media and diluents

DeMan, Rogosa and Sharp broth and agar (MRS, CM0359 and CM0361, Oxoid Ltd)	g/l
'Lab-Lemco' Powder	8
Yeast extract	4
Pepton	10
Glucose	2
Tween 80	1
Di-potassium phosphate	2
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.2
Manganese sulphate	0.05
Agar	10

Tryptone Soya Agar (TSA, CM131, Oxoid Ltd)	g/l
Pancreatic digest of casein	15
Papaic digest of soybean	5
Sodium chloride	5
Agar	15

Tryptone Soya Broth (TSB, CM129, Oxoid Ltd)	g/l
Pancreatic digest of casein	17
Papaic digest of soybean	3
Sodium chloride	5
Di-basic potassium phosphate	2.5
Glucose	2.5

Brain Heart Infusion (BHI, CM1135, Oxoid, Ltd)	g/l
Beef heart infusion solid	5
Proteose peptone	10
Glucose	2
Sodium chloride	5
Di-sodium phosphate	2.5

Reinforced Clostridia Medium and Agar (RCM, CM0149 and CM0151, Oxoid Ltd)	g/l
Yeast extract	3
'Lab-Lemco' Powder	10
Peptone	10
Glucose	5
Soluble starch	1
Sodium chloride	5
Sodium acetate	3
Cysteine hydrochloride	0.5
Agar	15

Maximum Recovery Diluent (MRD, CM0733, Oxoid Ltd)	g/l
Pepton	1
Sodium chloride	8.5

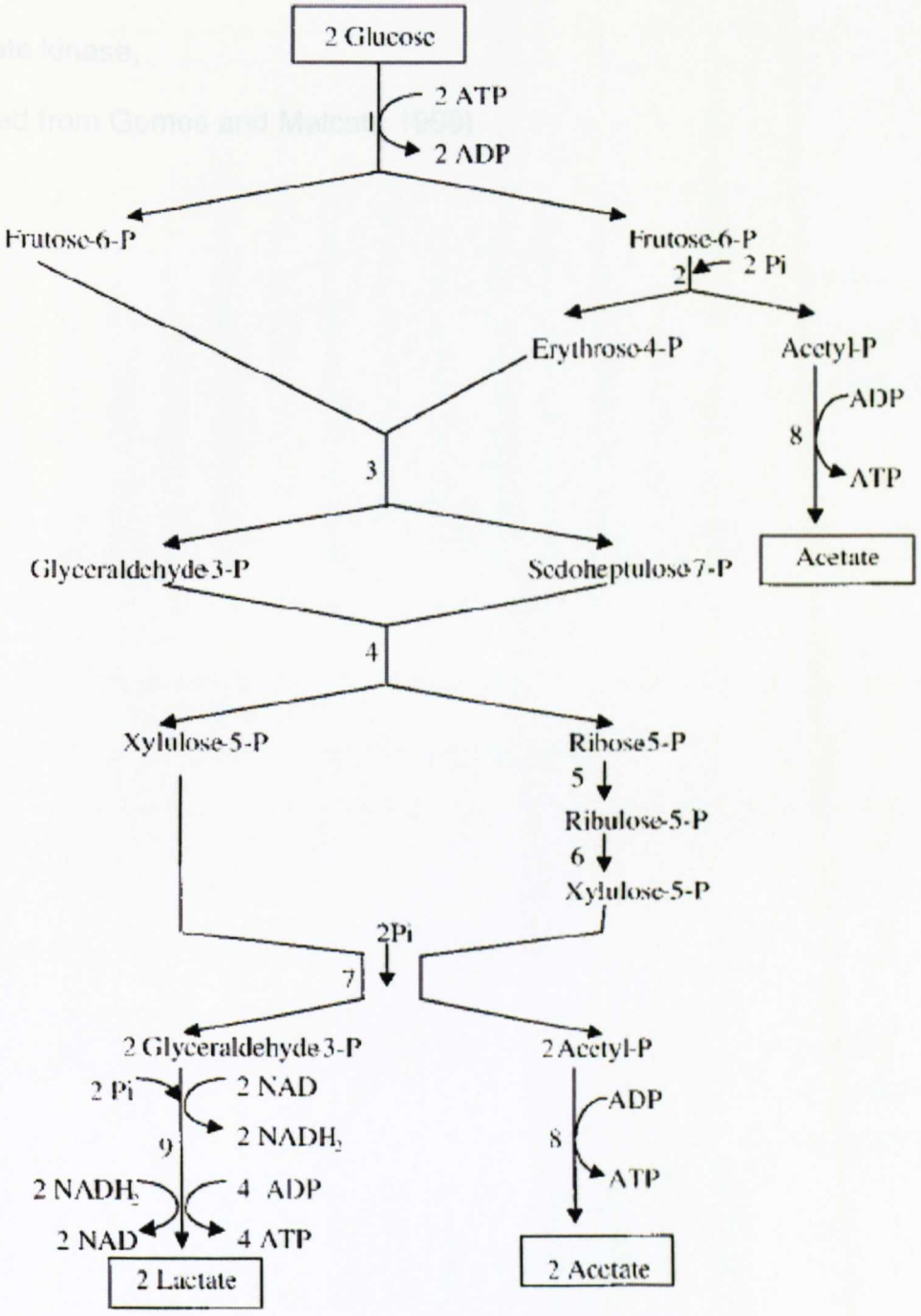
Phosphate Buffered Saline	g/l
Sodium chloride	8
Potassium chloride	0.2
Di-sodium hydrogen phosphate	1.15
Potasium dihydrogen phosphate	0.2

Nutrient agar	g/l
'Lab-Lemco' Powder	1
Yeast extract	2
Pepton	5
Sodium chloride	5
Agar	15

In general, media and solutions were sterilised at 121 °C for 25 min according to manufacturer's instructions except otherwise stated. Where required, filter sterilisation was performed either using a vacuum pump system (model 2552C-02, Welch Thomas, Skokie, IL, USA) with sterile membrane filter (0.2 µm, 7187, Whatman Filters, Maidstone, UK) or with sterile syringe-driven membrane filters (0.2, 190-2545, Nalgene, Rochester, USA).

APPENDIX 2

Formation of acetate and lactate from glucose by the bifidobacterium pathway



1-hexokinase and fructose-6-phosphate isomerase,

2-fructose-6-phosphate phosphoketolase,

3-transaldolase,

4-transketolase,

5-ribose-5-phosphate isomerase,

6-ribulose-5-phosphate-3-epimerase,

7-xylulose-5-phosphoketolase,

8-acetate kinase,

(Adapted from Gomes and Malcata 1999).

APPENDIX 3

Evaluation of cell growth of bifidobacteria cultivated in four different media (MRSC broth, MRS broth, RCM and TSB) and dilution (1/2 and 1/20), before determination of biofilm

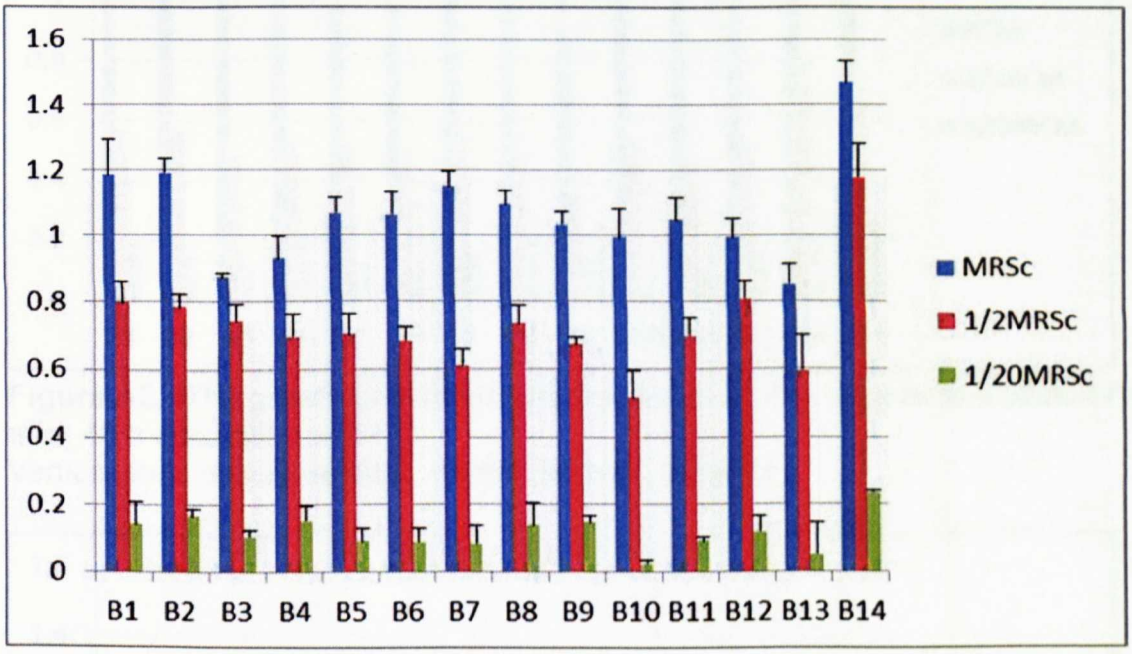


Figure A3.1 The growth of isolates (expressed as OD) in original and diluted MRSc broth after 48 h incubation at 37°C
Vertical axis: optical density, Horisontal axis: isolates

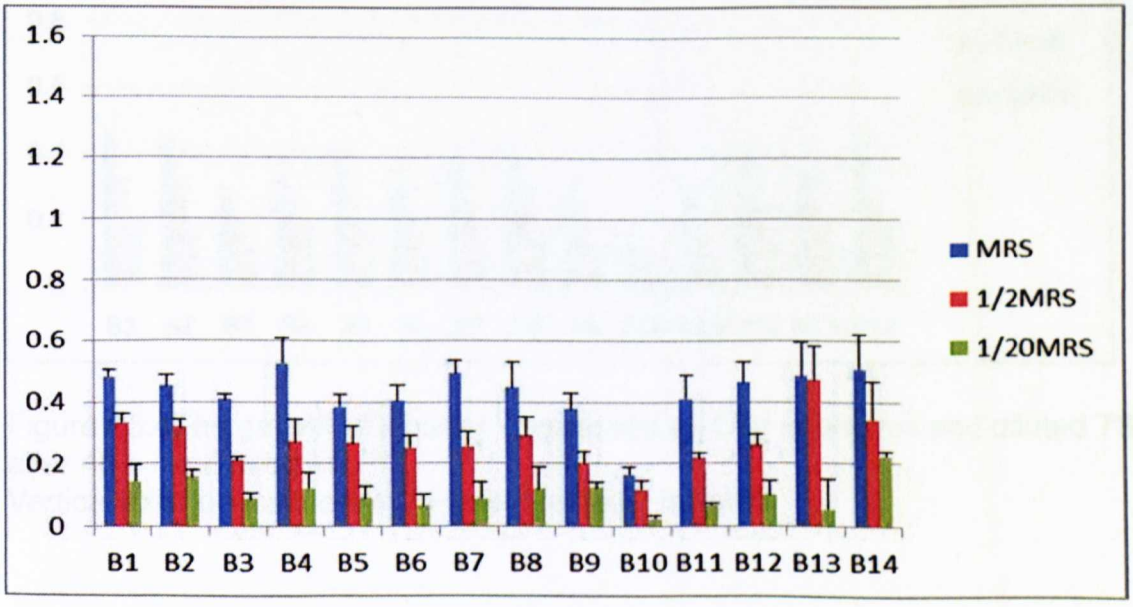


Figure A3.2 The growth of isolates (expressed as OD) in original and diluted MRS broth after 48 h incubation at 37°C
Vertical axis: optical density, Horisontal axis: isolates

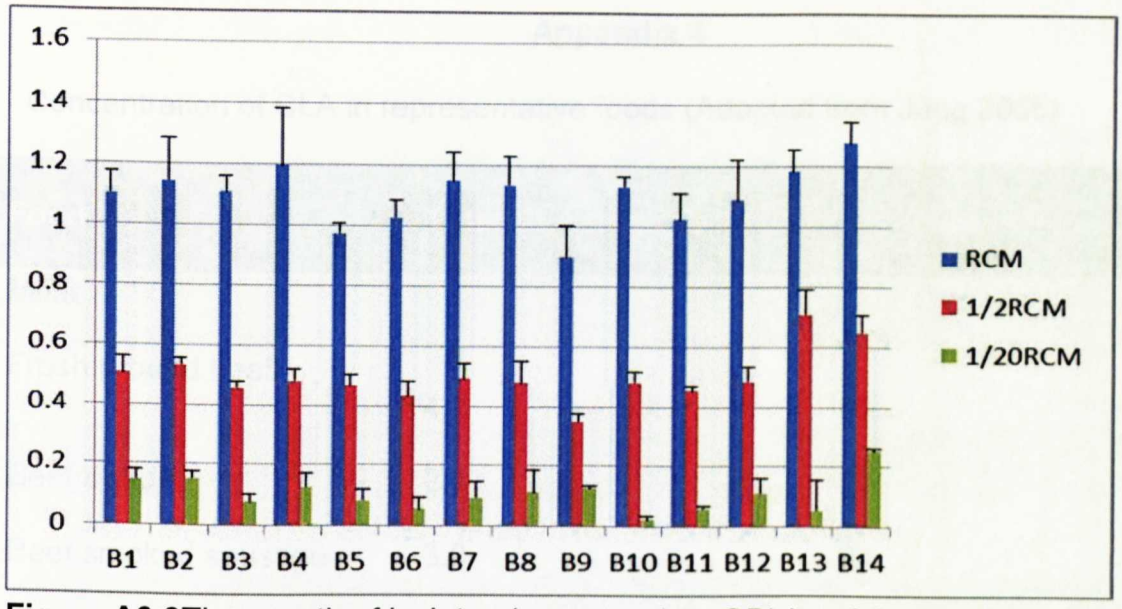


Figure A3.3 The growth of isolates (expressed as OD) in original and diluted RCM after 48 h incubation at 37°C
Vertical axis: optical density, Horisontal axis: isolates

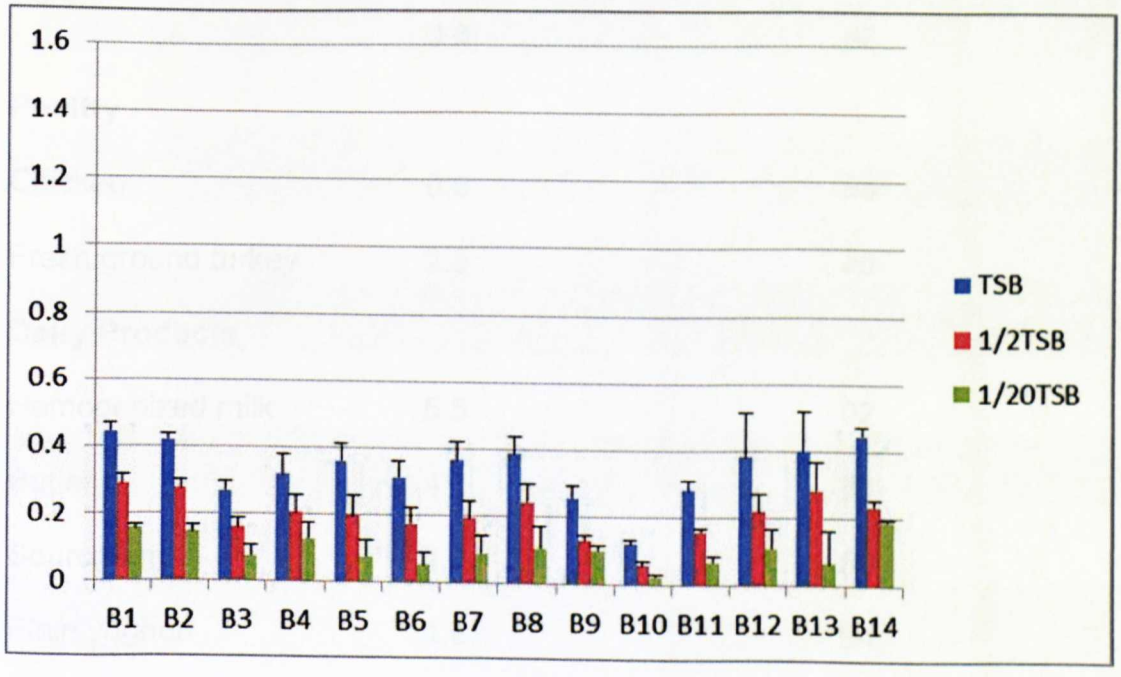


Figure A3.4The growth of isolates (expressed as OD) in original and diluted TSB after 48 h incubation at 37°C
Vertical axis: optical density, Horisontal axis: isolates

Appendix 4

Concentration of CLA in representative foods (Adapted from Jung 2005)

Food	Total CLA (mg/g fat)	*Cis-9, trans-11* isomer (%)
Meat		
Fresh ground beef	4.3	85
Beefround	2.9	79
Beef smoked sausage	3.8	84
Veal	2.7	84
Lamb	5.6	92
Pork	0.6	82
Poultry		
Chicken	0.9	84
Fresh ground turkey	2.5	76
Dairy Products		
Homogenized milk	5.5	92
Butter	4.7	88
Sourcream	4.6	90
Plain yoghurt	4.8	84
Ice cream	3.6	86
Processed cheese	6.7	93
Sharp cheddar cheese	3.6	93
Colby cheese	4.9	95
Reduced fat Swiss	4.5	83
Cottage cheese	6.1	92

Appendix 4 *continued:* Concentration of CLA in representative foods (Adapted from Jung 2005)

Food	Total CLA (mg/g fat)	*Cis-9, trans-11* isomer (%)
Seafood		
Salmon	0.3	ND
Lake trout	0.5	ND
Shrimp	0.6	ND
Vegetable Oils		
Safflower	0.7	44
Sunflower	0.4	38
Canola	0.5	44

ABBREVIATIONS

API= Analytical Profile Index

B. lactis= *Bifidobacterium lactis*

B. animalis subsp. *lactis*= *Bifidobacterium animalis* subsp. *lactis*

BIM-25= Bifidobacterium Iodoacetate Medium

BLAST = Basic Local Alignment Search Tool

BLIS = Bacteriocin like inhibitory substance

bp= base pair

BSH = Bile salt hydrolase

CFU = Colony forming unit

Cis-9, trans-11= c9,t11

CLA= Conjugated linoleic acid

DNA= Deoxyribonucleic acid

dNTP= Deoxyribonucleotide

DVI = Direct-to-vat inoculation

EFSA= European food safety authority

EUCAST= European Committee on Antimicrobial Susceptibility Testing

FAO= Food and Agriculture Organisation

F6PPK = Fructose-6-phosphate phosphoketolase

GIT= Gastero intestinal tract

GRAS = generally recognized as safe

H= Hour

IBD = Inflammatory bowl diseases

IDF= International Dairy Federation

LA= Linoleic acid

LAB =Lactic acid bacteria

MIC = Minimal Inhibitory Concentration

Min= Minute

MRD = Maximum Recovery Diluent

MRS = De Man Rogosa Sharpe

MRSC= De Man Rogosa Sharpe plus cystein

MRU= Microbiology Research Unit

NA= Nutrient Agar

NCIMB= National Collections of Industrial, Marine and Food Bacteria

NCTC = National Collection of Type Cultures

NDRI = National Dairy Research Institute of India

OD= Optical Density

OD_C= Optical Density cut off

PBS = Phosphate Buffered Saline

PCR= Polymerase Chain Reaction

S= Second

TBE = Tris Borate Ethylene diamine tetra acetic acid

TPY =Trypticase Phytone Yeast extract

TSB= Tryptone Soya Broth

RCA = Reinforced Clostridial Agar

RCM= Reinforced Clostridial Medium

Rep-PCR= repetitive extragenic palindromic polymerase chain reaction

RNA= Ribonucleic acid

SCFA= Short chain fatty acid

Trans-10, cis-12 = t10,c12

TTC = 2, 3, 5-triphenyltetrazolium chloride

WHO= World Health Organization