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Characterization of probiotic *Lactobacillus* spp. isolates from commercial fermented milks

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Declaration

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

(Signature)

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ABSTRACT

The aim of this project was to study the identity of probiotic lactobacilli in fermented milk products from the United Kingdom/European markets during their survival during shelf-life. This *in vitro* study was also aimed at undertaking studies on some of the physiological probiotic criteria, such as resistance to stomach/intestine conditions and also possible functional properties of the isolates, such as antimicrobial activities, antibiotic resistance/susceptibility and antibiotic resistance genes, biofilm formation and production of conjugated linoleic acid (CLA).

Primarily, a comparative study was carried out on selectivity of MRS-Clindamycin, MRS-Sorbitol and MRS-IM Maltose, to select the right medium for enumeration of probiotic *Lactobacillus*. Based on selectivity of medium for recovery of the targeted lactobacilli and also simplicity of preparation, MRS-Clindamycin was chosen as the best medium for enumeration of probiotic *Lactobacillus* in fermented milks. The results of enumeration of lactobacilli showed that 22 out of a total 36 tested products contained more than 10^6 colony forming units/g at the end of their shelf-life, which comply with the recommended minimum therapeutic level for probiotics. Rep-PCR using primer GTG-5 was applied for initial discrimination of isolated strains, and isolates, which presented different band profile, were placed in different groups. The isolated *Lactobacillus* spp. were identified mainly as *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus paracasei* by analysis of partial sequences of the 16S ribosomal RNA and *rpoA* genes.

In order to characterize the isolates for probiotic properties, this study was focused on six *Lactobacillus* isolates along with two commercial *Lactobacillus* cultures from

Chr. Hansen (*Lactobacillus acidophilus* La5 and *Lactobacillus casei* C431) and three *Lactobacillus* type strains (*Lactobacillus casei* subsp*. casei, Lactobacillus paracasei* subsp. *paracasei and Lactobacillus acidophilus*) which were purchased from NCIMB.

The stomach and intestine conditions were mimiced using a batch culture fermentation system, and the combined effects of pH, enzymes and bile salts on survival of tested isolates was tested. The tested isolates were able to survive at low pH environment and also high concentrations of bile salts of the upper digestive tract.

The potential of tested isolates for biofilm formation was determined in different conditions of nutritional and physiological stresses. The capability of tested isolates to produce biofilm in nutrient rich medium was recorded. However, the growth limitation, such as nutrient shortage in diluted media and also using inulin rather than glucose in synthetic medium, did not induce biofilm formation.

Antimicrobial activities of tested bacteria against indicator bacteria namely *Escherichia coli* NCTC 12900*, Salmonella enterica* serovar Typhimurium DT124 and *Salmonella enterica* serovar Enteritidis PT4 and *Lactobacillus delbruckii* subsp. *bulgaricus* were studied. The production of organic acids and bacteriocin was considered as key mechanisms for antimicrobial activity of tested strains.

Screening the isolates competence for production of CLA demonstrated that this feature is species dependent and also entirely related to the level of initial linoleic acid in the medium.

Eleven tested isolates were also assessed for their antibiotic resistance profile by determination of minimum inhibitory concentration (MIC). The acquired resistance to cefoxitin, ceftriaxone, chloramphenicol, erythromycin, gentamycin, kanamycin, lincomycin, streptomycin, tylosin tartarate, tetracycline and vancomycin was observed in all tested isolates. Also their genetic background of antibiotic resistance genes was studied by PCR reactions and none of the tested isolates showed positive bands for investigated resistance genes.

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Abbreviations

°C= degrees Centigrade

µg/ml= Microgram per mililiters

µg= Micrograms

µl= Microliters

µm= Micrometres

µM= Micromol

AHL= Acyl-homoserine lactone

AI= Autoinducer

ATCC= American Type Culture Collection

ATP = Adenosine triphosphate

BHT= Butylated hydroxytoluene

BLAST = Basic Local Alignment Search Tool

BLIS = Bacteriocin like inhibitory substance

bp= base pair

BSH = Bile salt hydrolase

CFU = Colony forming unit

c9,t11 = *Cis-9, trans-11*

CLA= Conjugated linoleic acid

CLSI= Clinical and Laboratory Standards Institute

DNA= Deoxyribonucleic acidl

dNTP= Deoxyribonucleotide

e.g.= Example

ECPS= Extracellular polymeric substances

EFSA= European food safety authority

EPS= Exopolysaccharide

et al = and others

EUCAST= European Committee on Antimicrobial Susceptibility Testing

FAME= Fatty acid methyl ester

FAO= Food and Agriculture Organisation

GC= Gas chromatography

GCA= Glycochenodeoxy cholic acid

GIT= Gastero intestinal tract

g= Gram

GRAS = generally recognized as safe

H= Hour

H_2O_2= Hydrogen peroxide

HCl= Hydrochloric acid

HDL= High density lipoprotein

HIP= Hexhane/isopropanol

i.e.= that is

IDF= International Dairy Federation

ISO= International Organization for Standardization

kDa= Kilodalton

L= Litre

LA= Linoleic acid

LAB = Lactic acid bacteria

LAMVAB= Anaerobic MRS agar supplemented with vancomycin and bromocresol green

LDL= Low density lipoprotein

M= Molar

MIC= Minimal Inhibitory Concentration

Min= Minute

ml= Millilitres

mm= Millimetres

mM= Millimolar

MRD = Maximum Recovery Diluent

MRS = De Man Rogosa Sharpe

MRS-LC= MRS-*Lactobacillus casei*

MRS-LP= MRS agar with lithium chloride and sodium propionate

MRS-NPNL= Nalidixic paromomycin neomycin lithium

MRU= Microbiology Research Unit

n-3= Omega 3

n-6= Omega 6

n-9= Omega 9

NA= Nutrient Agar

NaCl= Sodium chloride

NCIMB= National Collections of Industrial, Marine and Food Bacteria

NCTC= National Collection of Type Cultures

OD= Optical Density

OD_C= Optical Density cut off

PBS = Phosphate Buffered Saline

PCR= Polymerase Chain Reaction

pmol/µl= Picomolar per microliter

ppm= Part per million

PUFA= Poly unsaturated fatty acids

QPS= Qualified presumption of safety

RAPD= Randomly amplified polymorphic DNA

RCA = Reinforced Clostridial Agar

RCABC= Reinforced Clostridial Agar with bromocresol green and clindamycin

Rep-PCR= repetitive extragenic palindromic polymerase chain reaction

RNA= Ribonucleic acid

RPM= Revolutions per minute

SCAN= Scientific Committee for Animal Nutrition

SCFA= Short chain fatty acid

SD= Standard deviation

Sec= Second

spp.= species

subsp.= sub-species

TBE = Tris Borate Ethylene diamine tetra acetic acid

TCA= Taurochenodeoxy cholic acid

TGV agar= Tryptone, glucose, and meat extract

Trans-10, cis-12 = t10,c12

TSB= Tryptone Soya Broth

TTC = 2, 3, 5-triphenyltetrazolium chloride

U= Unit

UKPAR= United Kingdom Public Assessment Report

v/v= Volume per volume

w/v= Weight per volume

WHO= World Health Organization

CHAPTER ONE: INTRODUCTION

1.1 Introduction

During the past years, among different functional foods, the foods containing probiotic microorganisms have been given great attention (Granato *et al*. 2010a). Probiotic bacteria are "live microorganisms, which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO 2002). Apart from this widely used definition of probiotics, other reports have clarified this concept. In general terms, a probiotic food substance must contain a taxonomically defined microorganism and be shown in human studies to provide considerable benefits for the host. The FAO/WHO (2002) definition embraces utilization of all live microbes, not only those for intestinal benefits (Anukam and Reid 2007). In addition to the incorporation of the probiotics in food and dietary supplements, probiotic microorganisms may be used in pharmaceutical applications (tablet, capsule, sachet and powder forms) and microbial feed. A variety of food products, such as fermented milk, plant derived food and meat products, have been used as a food matrix for probiotics. The therapeutic effects of these microorganisms include balancing the intestinal flora, absorption of calcium, synthesis of vitamins, enhancing lactose tolerance, decreasing cholesterol level and modulating the immunological system (Margoles and Garcia 2003).

Several endogenous and exogenous factors, such as age, stress, immunological status, diet and medical treatments, might affect the composition and activities of the intestinal microbiota (Isolauri *et al*. 2004). Most probiotics belong to the lactic acid-producing bacteria, including lactobacilli and bifidobacteria. There are also non-bacterial and non-lactic acid bacterial organisms, such as *Saccharomyces*

boulardii lyo and *Escherichia coli (E. coli*) strain Nissle, have been suggested as probiotic (Anukam and Reid 2007). Lactobacilli are among the bacteria mostly used as probiotics in animal feeds and human foods (Coeuret *et al*. 2004).

Lactobacillus spp. which is one of the main genera found in the intestine, is most frequently chosen as a probiotic. The main commercial species used for the production of functional foods belong to this genus (Gibson *et al.* 2003; Vinderola *et al*. 2011).

The most important species of *Lactobacillus* spp. used as probiotics include *Lactobacillus acidophilus (Lb. acidophilus), Lactobacillus casei (Lb. casei), Lactobacillus paracasei (Lb. paracasei), Lactobacillus rhamnosus (Lb. rhamnosus), Lactobacillus plantarum (Lb. plantarum), Lactobacillus gasseri (Lb. gasseri), Lactobacillus johnsonii (Lb. johnsonii), Lactobacillus reuteri (Lb. reuteri), Lactobacillus crispatus (Lb. crispatus)* and *Lactobacillus fermentum (Lb. fermentum)*. The concentrates of probiotic lactobacilli used for functional foods are usually supplied in freeze-dried, spray dried or microencapsulated form.

1.2 History of the probiotic

The term probiotic means "for life" in Greek language, and is used to name bacteria associated with benefits for humans and animals (FAO/WHO 2002). Elie Metchnikoff in 1907 hypothesised that substituting the putrefactive bacteria in the gut with lactic acid bacteria (LAB) results in normalising the bowel health and

prolonging the life. He observed that the regular consumption of fermented dairy products is the main reason of health and longevity in Bulgarian people.

Metchnikoff attributed the health effects of these bacteria and indicated how health-promoting bacteria in yogurt might be helpful in digestion and also in better functionality of immune system. He stated that ‘the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes’ (Metchnikoff 1907). Another investigation about the benefits of LAB was made by French paediatrician Henry Tissier who reported that some bacteria could be used for individuals with diarrhoea to restore their well gut flora (Tissier 1906). In 1935, a probiotic product, which was named Yakult, was produced by a Japanese scientist Minoru Shirota (Sako 2011).

Lilly and Stillwell (1965) used the term “probiotics” and mentioned that there are some compounds produced by some organisms, which stimulate the growth of others.

Parker (1974) stated that probiotics are the organisms that play an important role in intestinal microbial balance. In 1989, Fuller reported that probiotics are "live microbial supplements which have beneficial effects on the host by improving its microbial balance". According to another definition suggested by Havenaar and Huis in't Veld (1992), probiotics are "viable mono or mixed cultures of bacteria which, when applied to animal or human, beneficially affects the host by improving the properties of the indigenous flora".

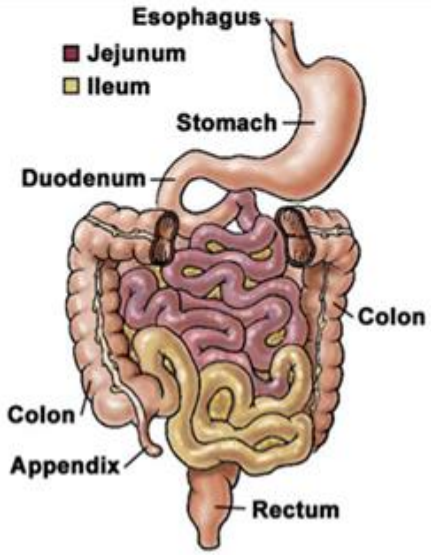
The definition by FAO/WHO (2002), which defined probiotics as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" is not acceptable to the European Food Safety Authority (EFSA), because it includes a health claim, which is not measurable (Rijkers *et al.* 2011). In recent years, scientific advances using molecular and genetic studies have been obtained in the selection and characterization of different probiotic microorganisms and health claims have been also substantiated regarding their use in life (Anukam and Reid 2007).

1.3 Ecological considerations of the gastrointestinal tract

More than 400 bacterial species have been identified in the GIT of human (Tannock 1999). The bacterial concentration in the stomach is $<10^2$ CFU /ml of gastric juice and in the colon is about 10^{12} CFU/g of colonic material (Table 1.1) (Mitsuoka 1992). Some preliminary studies on the environment of the human gut have indicated that consuming multiple probiotic strains presents more benefit than a single strain (Timmerman *et al*. 2004; Williams *et al*. 2008). Extensive 16S rRNA gene sequencing has indicated the high diversity of the intestinal microbiota (Ley *et al*. 2006).

Table 1.1 Numbers and species of the most common bacteria of the alimentary tract

Oral cavity	Stomach	Duodenum	Distal ileum	Colon
10^5-10^8 CFU/ml	<100 CFU/ml	10^4 CFU/ ml	10^8 CFU/ml	10^{10}– 10^{12} CFU/ml
saliva *Streptococcus* *Actinomyces* *Veillonella* *Fusobacterium* *Porphromonas* *Prevotella* *Treponema* *Neisseria* *Haemophilus* *Eubacteria* ***Lactobacillus*** *Capnocytophaga* *Eikenella* *Leptotrichia* *Peptostreptococcus* *Staphylococcus* *Propioni bacterium*	*Helicobacter* ***Lactobacillus*** *Veillonella* *Clostridium*	***Lactobacillus*** *Streptococcus* *Veillonella*	***Lactobacillus*** *Streptococcus* *Enterobacteriacae* *Bacteroides* *Clostridium*	*Bacteroides* *Eubacterium* *Bifidobacterium* *Fusobacterium* *Clostridium* *Peptococcus* *Peptostreptococcus* *Ruminococcus* *Actinomyces* ***Lactobacillus*** *Coprococcus* *Faecalibacterium* *Megasphaera* *Veillonella* *Collinsella* *Eggertella* *Enterobacteriacae* *Enterococcus*



Source: Lonnermark (2010)

Gut microbiota present extensive effect on the host physiology, biochemistry, immunology and resistance to intestinal infections (Berg 1996). Different compartments of the GIT have variable chemical and physical characteristics; therefore, only certain microbial communities are able to survive and grow in the stomach, small intestine, and large intestine (Tannock 1995). The human stomach and the first two-third of the small bowel contain relatively a small number of

microbes due to the pH of the stomach contents (pH 2, when empty). The toxicity of bile salts, and also the swift flow of the digesta might be the other reasons for low microbial population in the upper digestion systems (Tannock 1995).

The microbial population of the proximal small intestine, ileum and colon are 10^3, 10^8 and 10^{10}-10^{12} CFU/ml, respectively. In addition to density, microbial diversity also increases from proximal small intestin to the colon (Finegold *et al*. 1983; Lonnermark 2010). Lactobacilli grow optimally between the temperatures of 30 and 40 °C. Therefore, they have a diverse generation time, e.g. the generation time of *Lb*. *acidophilus* is in the range of 22 and 84 minutes in 40 and 30°C, respectively (Cho *et al.* 1996). Lactobacilli are commonly associated with the GIT of animals and humans, but some of them are originated from plant (Walter 2008).

1.4 Lactobacilli

Lactobacillus is a genus of Gram-positive facultative anaerobic or micro-aerophilic, non-spore-forming and rod-shaped bacteria (Makarova *et al*. 2006). Usually lactobacilli are straight rods, but under certain conditions, they can be in spiral or coccobacillary forms. Lactobacilli are found in pairs or chains of different length. Almost all required energy for lactobacilli is obtained from the conversion of glucose to lactate during homolactic fermentation and, 85-90% (w/w) of the sugar utilised, is converted to lactic acid. Lactobacilli are a main part of the LAB due to the ability to convert lactose to lactic acid, and is traditionally identified on the basis of morphology, ability to use various carbohydrates, fermentation products and

associated enzyme activities. Testing for absence of catalase activity, and also the presence of lactic acid as the major acid produced from the fermentation of glucose, might be a confirmation result that the isolates belong to the genus *Lactobacillus* (Tannock 1999).

Lactobacilli constitute a small part of the intestinal flora (Walter 2008). They are benign in GIT, but are associated with dental caries (Piwat *et al*. 2010). Generally, *Lb. fermentum* has been reported to be the most predominant species in dental caries of children (Piwat *et al.* 2010). Another study has revealed that *Lb. gasseri* and *Lactobacillus ultunensis* are prevalent species colonizing carious dentine (Byun *et al*. 2004).

The genus *Lactobacillus* was identified by Martinus Beijerinck in 1901(Barinov *et al.* 2011). According to the type of metabolism, lactobacilli species can be classified into three groups including obligatory homofermentative (such as *Lb. acidophilus, Lb. bulgaricus, Lactobacillus helveticus (Lb. helveticus)* and *Lactobacillus salivarius*), facultative heterofermentative (such as *Lb. casei, Lactobacillus curvatus (Lb. curvatus), Lb*. *planetarum, Lactobacillus sakei (Lb. sakei)*) and obligatory heterofermentative (such as *Lb. brevis, Lactobacillus bunchneri (Lb. bunchneri), Lb. fermentum, Lb. reuteri*) (Felis and Dellaglio 2005).

Homofermentative lactobacilli in the fermentation process of glucose produce mainly lactic acid. Heterofermentative lactobacilli, in addition to lactic acid, produce acetate, ethanol and carbon dioxide (Karpinski and Szkaradkiewicz 2013).

1.5 Criteria for selection of probiotic bacteria

1.5.1 Viability and effects of processing, food matrix and storage on probiotic lactobacilli

Viability and functionality of probiotic lactobacilli should be maintained during processing, handling and storage of the product. Probiotic lactobacilli must be alive during the passage through the digestive tract and also they should be able to proliferate in the gut (Holzapfel *et al*. 2001). *In vivo* techniques to assess the survival of probiotics are performed using different methods such as faecal collection, intestinal intubation and identification of strain on mucosal biopsies (Marteau 2001).

Animal and clinical studies are two forms of *in vivo* research. *In vivo* testing is often used over *in vitro* because it is more appropriate to monitor the overall impacts of an experiment on a living subject.

It is necessary to note that extrapolation of results gained in animals to humans is dangerous, may be unrealistic and the results should be treated with caution, due to the variability of reactions among species and strains.

Food matrix, physicochemical properties and functional ingredients of product affect the performance of lactobacilli (Ranadheera *et al*. 2010). Higher protective effect of bovine milk compared to soy milk on survival of *Lb. casei* in simulated gastric and intestinal juice has indicated the importance of the food matrix regarding the physiological properties of lactobacilli (Wang *et al*. 2009). Dairy products need refrigeration in the distribution channels but, in dried formulations, lactobacilli can

survive without refrigeration, if they are retained in proper vials kept in a cool and dry place with suitable desiccants (Anukam and Reid 2007).

1.5.2 Adhesion of probiotic lactobacilli in gastrointestinal tract

It has been hypothesized that *Lactobacillus* strains with a high value of cell surface hydrophobicity have a better adhesion to the epithelial cells of intestine (Kotzamanidis *et al*. 2010). Adherence of lactobacilli to epithelial cells can block adherence of pathogens. It has been reported that production of mucin by *Lb. plantarum* 299v or *Lb. rhamnosus* GG prevented the adhesion of entero-pathogenic *E. coli* strain E2348/69 in cell culture assays (Mack *et al.* 2003). *Lactobacillus rhamnosus* GG can reduce adhesion of pathogenic *Salmonella* spp., *Clostridium* spp. and *E. coli* in pig intestinal mucus (Collado *et al.* 2007).

In vitro studies have shown that *Lb. casei* DN-114001 (Ingrassia *et al*. 2005), *Lb. plantarum* 423 (Botes *et al*. 2008) and *Lactobacillus kefir (*Golowczyc *et al*. 2007) can interfere with invasion of host epithelial cells by *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis.

1.5.3 Antimicrobial properties of probiotic lactobacilli

Different antimicrobial substances, such as short chain fatty acids**,** hydrogen peroxide, lactic acid and bacteriocins are produced by lactobacilli. Lactic acid prevents the growth of microorganisms sensitive to acidic conditions by decreasing the local pH (De Keersmaecker *et al*. 2006). It also permeates the outer membrane of Gram-negative bacteria (Alakomi *et al*. 2000). Lactobacilli can produce low and

high molecular weight bacteriocins including lantibiotics, heat stable non-lantibiotics and cyclic antimicrobial peptides (Maqueda *et al*. 2008).

Interactions among different lactobacilli have been reported during fermentation (Giraffa *et al.* 1996). Beneficial or unfavorable microbial interactions among different mixtures of probiotic and lactic starter cultures generate undesirable changes in the composition of the bacterial population throughout the fermentation and storage of dairy products (Bellengier *et al*. 1997). The inhibitory, stimulatory or neutral interactive effects among probiotics and starter cultures may be related to the production rate of lactic acid, other organic acids or secondary metabolites (Rattanachaikunsopon and Phumkhachorn, 2010). There is an associative growth between the yogurt starter cultures (*Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus)* (Michaylova *et al*. 2007). However, in some co-cultures the proportion of probiotic to starter culture becomes unbalanced, so neither functions properly (Goderska and Stanton 2010). To avoid the competition among the strains, it has been suggested to use starter cultures and probiotic strains from the same supplier (Cruz *et al*. 2009b). Apart from the difference between the starter and probiotic strains, it seems that this advice can be due to this fact that two types of strains undergo the similar environmental factors, which exist during preparation process, and subsequently impact the functional behaviours of the strains. In another study, it has been also reported that it can be solved by use of cultures from the same supplier (Granato *et al*. 2010b).

It has been reported that interaction among species is an important factor affecting the viability of *Lb*. *acidophilus* (Kailasapathy and Rybka 1997). In some cases,

synergistic growth-promoting effects have been reported between *Lb. acidophilus* and *Bifidobacterium bifidum* strains (Kneifel *et al*. 1993). In other cases, growth inhibition has been found among probiotic species and it has been attributed to the production of bacteriocin (Yildirim and Johnson 1998).

Lactobacillus acidophilus is a thermophilic starter which grows slowly in dairy products, and has a high acid tolerance (Tamime and Marshall 1997). As lactobacilli are the most acid tolerant of LAB, they can lower the pH of milk from initial values of (6.6-6.8) to below 4.0. Therefore, they are usually used in combination with *S. thermophilus* (Hassan and Frank 2001). H_2O_2, which is generated throughout the storage of yogurt, is considered as a main compound for reduction of the viability of *Lb. acidophilus*, and does not have catalase to hydrolyze H_2O_2. The presence of *S. thermophilus* helps decompose H_2O_2 into water and oxygen (Ng *et al.* 2011).

It was demonstrated that the inhibitory effect of *Lb. acidophilus* CNRZ 1881 on *Lb. delbrueckii* subsp. *bulgaricus* Ab1 was attributed to the presence of a bacteriocin-like compound, because antibacterial activity of *Lb. acidophilus* did not decrease after neutralization or heating at 121 °C for 15 min of the concentrated cell-free supernatant, while it entirely disappeared after treatment with proteinase K and pepsin (Vinderola *et al*. 2002b).

In general, the interactions between species of probiotic bacteria and lactic acid starter include stimulation, delay or completely inhibition of growth, although in some cases no interactive effects between them.

Considering the interactions among the mentioned cultures, it is important to select the best combination in order to optimize their survival in the products and their performance during the fermentation process (Vinderola *et al*. 2002b). Hence, interaction among lactic acid starter and probiotic bacteria is needed to be well investigated, to provide an optimal combination of cultures for manufacturing probiotic dairy products (Timmerman *et al*. 2004; Kosin and Rakshit 2006).

1.5.4 Safety

Although some epidemiological surveillance have been conducted to assess the safety of lactobacilli (Saxelin *et al*. 1996), potential probiotic lactobacilli should be periodically assessed for different characteristics, including antibiotic resistance gene transfer, enzymatic properties and metabolic activities, such as D-lactate production and bile salt deconjugation, toxicity and post-market incidents. Other side-effects of probiotics include systemic infections and excessive immune stimulation in susceptible people. Generally, safety evaluation of probiotics can be based on the intrinsic characteristics, their pharmacokinetics and interactions among different strains and the host (Marteau, 2001).

1.5.4.1 Antimicrobial (antibiotic) resistance genes and their transferability potential

Another main concern of the use of probiotic microorganisms in foods is the fact that they may contain specific antimicrobial resistance genes (FAO/WHO 2006) and, therefore, it is recommended that bacteria with transferable antibiotic resistance genes should not be used in foods. Unless, certain antibiotics may not be useful for treatment of infectious diseases.

The transferability of genetic elements to other intestinal and foodborne bacteria is one of the important criteria in investigation of probiotic safety (Saarela *et al*. 2000).

Plasmids in isolated lactobacilli from the intestine may have genes which encode antibiotic resistance. In selection of probiotic strains, it is strongly advised that probiotic microorganisms should not contain transferable antibiotic resistance genes which encode resistance to clinical antibiotic, because resistance genes might be transferred by probiotics to the intestinal flora. Factors affecting the transferability of antibiotic resistance gene include nature of the genetic elements, their concentrations and exposure and the nature of the donor and recipient strains (Marteau 2001). Intrinsic resistance of lactobacilli is encoded by chromosome and is not transferable (Bernardeau *et al*. 2008). Thus, manufacturer has to prove that the strain used cannot acquire or transfer antibiotic resistance or be virulent and induce infection (FAO/WHO 2006).

1.5.4.2 Infections and deleterious metabolites

Although no pathogenic or virulence features of lactobacilli have been reported, some *Lactobacillus* spp. have been related to adverse effects, such as bacteremia under particular conditions (Saxelin *et al*. 1996). However, no increased incidence/frequency of bacteremia was reported with enhanced usage of lactobacilli (Salminen *et al*. 2001). Rare reports of local or systemic infections such as septicemia and endocarditis due to lactobacilli have been published (Husni *et al*. 1997).

1.5.4.3 Mortality

In a randomised, double-blind, placebo-controlled trial in University Medical Center Utrecht, Netherlands, patients with diagnosed severe acute pancreatitis received a multispecies preparation of probiotics or placebo given twice daily via a jejunal catheter for 28 days.

Multispecies preparation of probiotics (Ecologic® 641) consisted of 6 strains of viable and freeze-dried bacteria, including *Lb. acidophilus, Lb. casei, Lb. salivarius*, *Lactococcus lactis* subsp. *lactis, B. bifidum* and *Bifidobacterium animalis* subsp. *lactis* in a total daily dose of 10^{10} bacteria. Infections occurred in 30% and 28% of patients in the probiotics and placebo group, respectively. Also, the mortality rate was 16% and 6% in the probiotics and placebo group, respectively. In patients with predicted severe acute pancreatitis, the probiotic prophylaxis was associated with a more than two-fold increase in mortality (Besselink *et al*. 2008). There are concerns over recommending probiotic for sever conditions. Further *in vivo* studies are needed in order to characterize the safety and probiotic properties of different species.

1.5.5 Functionality

The viability of probiotic lactobacilli need to be controlled to monitor their functionality. Various concentrations of viable bacteria to guarantee the functional quality of the food have been viewed from different perspective (Saxelin, 2008; Degnan, 2008; Amagase, 2008). Vinderola *et al*. (2011) have stated that the control of cell viability is not sufficient to guarantee the probiotic functionality, because different conditions of production and storage have different effects on

susceptibility, adherence to the epithelial cells of the intestinal, without changes in viability of probiotic. In other words, there are some variations in the induced beneficial effect of a particular *Lactobacillus* species without changes in the viable cell counts.

1.5.6 Health effects of lactobacilli

Potential effects of probiotics include decreasing the risk of diseases of the gastrointestinal, urogential, respiratory, kidney and cardiovascular tract and allergic symptoms. Although many products with claim of probiotic on the label are available in the market, their efficacy has not been clinically proved. Thus, in some cases, the healthcare providers and consumers are not aware which to take and what to expect from the use of probiotic products (Anukam and Reid 2007). It is necessary to mention that molecular techniques are needed for identification of probiotics and selection of the most suitable strains for the prevention and treatment of an illness, and explanation of host-microbe interactions.

1.5.6.1 Effect of lactobacilli on diarrhoea

Some studies have reported the effect of probiotics on diarrhoeal diseases (Johnston *et al*. 2007; Szajewska and Mrukowicz 2005). Generally, the mechanisms of action of probiotic lactobacilli against intestinal pathogens include production of antimicrobial compounds, competition with them for nutrients and also exclusion of pathogen binding (Sullivan and Nord 2005; FAO/WHO 2006). The effectiveness of certain lactobacilli in diarrhoea is based on their ability to protect the host against toxins.

Oral administration of a fermented product containing *Lb. gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711 resulted in the production of short chain fatty acids. Also, it increased the volume, frequency and moisture of stool (Olivares *et al*. 2006).

The influence of *Lb. rhamnosus* GG has been reported for prevention (Szajewska *et al*. 2001) and treatment (Guandalini *et al*. 2000) of acute diarrhoea. It has been reported that *Lb. rhamnosus* GG is capable of decreasing the duration of diarrhoea and reducing length of hospital stay in developed countries (Szajewska and Mrukowicz, 2005). It has also been shown that *Lb. rhamnosus* GG decreases the daily stool frequency and the duration of the diarrhoea in infants and toddlers (Canani *et al*. 2007; Henker *et al*. 2007).

Lactobacillus GG is able to ameliorate ulcerative colitis (Zocco *et al*. 2006), but had no effect in extension of remission in Crohn's disease patients (Schultz *et al*. 2004; Bousvaros *et al*. 2005).

Effectiveness of *Lb. reuteri* 55730 in the treatment of rotavirus-associated diarrhoea has been proven (Weizman *et al*. 2005). In a double-blind and randomized controlled study, preventive effect of probiotics on antibiotic associated diarrhoea (Camras 2008; Wenus *et al*. 2008) and traveler's diarrhoea (MacFarland 2007) has been reported.

1.5.6.2 Effect of lactobacilli on inflammatory bowl disease

There are also some studies regarding the efficacy of probiotics on inflammatory bowel disease, especially ulcerative colitis and pouchitis (Hart *et al*. 2003; Geier *et*

al. 2007), gastric ulcers, reduction of colorectal cancer risk (Geier *et al*. 2006) and irritable bowel syndrome (Verdu and Collins 2005; Aragon *et al*. 2010).

Anti-inflammatory activity of *Lb. plantarum* has been attributed to the teichoic acid, which is a constituent of the Gram-positive cell wall (Grangette *et al*. 2005). The anti-inflammatory properties of *Lb. fermentum* has been attributed to a produced phospholipid, because it has been shown that inhibitory effect of *Lb. fermentum* is abolished during treatment of microorganism supernatants with phospholipase C (Frick *et al*. 2007).

1.5.6.3 Effect of lactobacilli on lactose intolerance

There are some investigations regarding correlation between oral supplementation with lactobacilli and lactose intolerance (Levri *et al*. 2005; Montalto *et al.* 2006). Lactobacilli with potential of high β-galactosidase activity might be a suitable treatment for the lactose intolerance. The effect of the probiotics administration on lactose intolerance is assessed via measurement of hydrogen levels (mg/kg) at the end of treatment. Lactose intolerance test is usually carried out using 50 g oral lactose, then breath hydrogen for three hours is analyzed. The 20 ppm hydrogen is considered as diagnosing level for lactose intolerance (Matthews *et al*. 2005).

During lactose breath test, *Lb. reuteri* influences excretion of hydrogen in the breath and gastrointestinal symptoms in lactose intolerant patients (Ojetti *et al.* 2010). Another study has indicated that consumption of *Lb. casei* Shirota improve symptoms and diminish hydrogen breath level and these positive effects continued for three months after probiotic consumption (Almeida *et al*. 2012). In terms of

lactose intolerance, some studies have reported that although probiotic bacteria can alleviate lactose intolerance, they are not able to supply enough quantity of lactase for the consumers (Ouwehand *et al.* 2003). There is a high variability in the amount of lactase activity in different lactobacilli (Arola and Tamm 1994).

1.5.6.4 Effects of probiotic lactobacilli on blood cholesterol level and cardiovascular disease

A reduction of blood cholesterol level has been reported as a consequence of consumption of dairy foods containing different probiotic lactobacilli such as *Lb. fermentum* (Simons *et al*. 2006). Lactobacilli may reduce total plasma cholesterol and low-density-lipoprotein (LDL) cholesterol (Sanders 2000). Bile salt deconjugation ability by some lactobacilli is used to decrease serum cholesterol level in hypercholesterolemic patients and prevent hypercholesterolemia. Lactobacilli can excrete the bile salt hydrolase, which accelerate the hydrolysis of taurine and glycine conjugated bile salts, and generate amino acid residues and free bile salts (Liong and Shah 2005).

1.6 Labelling of foods and supplements and declared counts of lactobacilli

The microbial species and identity of the strain need to be declared on the food label. Also, it is necessary to accurately enumerate the probiotic cell count in the products, and state the viable count on their label. Labeling should be clear and comprise minimum dosage of probiotic count and health claims. Various differential and selective culture media has been proposed in the last decades for the microbiological analysis of probiotic lactobacilli (Vinderola *et al*. 2009). Selective

enumeration of lactobacilli at the level of species and strains is challenging, because they are phylogenetically related together and also show similar metabolic responses (Vinderola *et al*. 2011).

As lactobacilli have a strain-specific response to some culture media, it is not possible to select one culture medium for all probiotic lactobacilli and for all the food matrices. Also, it is not easy to differentiate *lactobacillus* strains of the same species by the plate count method. Regardless of plate counts techniques which are used in routine quality control of laboratories at industrial level, more advanced methods may also be used, such as flow cytometry (Ben Amor *et al*. 2007) and real-time quantitative polymerase chain reaction (PCR) (Friedrich and Lenke 2006) to provide a better estimation of the number of bacteria in the products.

1.7 Consumption dosage of probiotic lactobacilli and duration of use

Probiotics should be adminstrated in adequate amounts to be able to exert the health effects on the host. Different studies have indicated that the concentration of probiotics should be at least 10^7 to 10^8 CFU/g with daily serving size around 100 to 200 g (Rijkers *et al.* 2010). According to the other studies, the minimum daily quantity of probiotic for any beneficial effect on the host is considered to be 10^8–10^9 CFU/day, which can be provided by intake of 100 g probiotic product containing 10^6–10^7 CFU/g (Lourens-Hattingh and Viljoen 2001; Talwalkar and Kailasapathy 2004).

Each probiotic product should state the minimum daily quantity required for it to confer specific health benefits. The minimum level of probiotic cells in commercialized probiotic products should be counted at the expiration date (Araujo *et al*. 2012).

1.8 Non-*Lactobacillus* probiotic microorganisms

Other probiotic microorganisms include different species of *Bifidobacterium* and *Streptococcus*. Other microorganisms, which are considered as probiotics, include *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, *Clostridium butyricum, Enterococcus faecalis* and *Enterococcus faecium* (Heyman and Menard 2002).

A few years ago, some medicinal supplements containing *Bacillus* species (Enterogermina®) were used as probiotic products in Italy (Cutting 2011). It has been reported that spore forming bacilli have higher capability of surviving the low pH of the gastric barrier and higher viability at room temperature compared to non-spore formers such as lactobacilli (Barbosa *et al*. 2005).

Some *Bacillus* species have shown probiotic effects as lactobacilli, including immune stimulation, antimicrobial activities and competitive exclusion (Cutting 2011). Examples of such species include *Bacillus clausii* (Senesi *et al.* 2001), *Bacillus coagulans* (Mandel *et al*. 2010) and *Bacillus subtilis* (Hosoi and Kiuchi, 2004). *Bacillus clausii* has been reported to have positive effects on respiratory infection (Ciprandi *et al*. 2004; Marseglia *et al*. 2007) and urinary tract infections (Fiorini *et al*. 1985). *Bacillus coagulans* produce a bacteriocin against enteric

microbes; it is named coagulin (Hyronimus *et al*. 1998). *Bacillus coagulans* also present some positive effects on rheumatoid arthritis (Mandel *et al*. 2010).

1.9 The Market

The market for foods, especially dairy products including fermented milks, yogurts, cheeses, and beverages containing probiotic cultures is on the rise (Viana *et al*. 2008). The global market for probiotic ingredients, supplements and foods have been reported to be worth $14.9-16 billion in 2007-2008. The global probiotic products market was estimated at $26.12 billion in 2012 (Anon 2014).

Products containing the genus *Lactobacillus* has approximately accounted for the 62% of total sales of probiotic products in 2007. Although various products, such as probiotic cheese, ice cream, nutrition bars, infant formula and breakfast cereal and soy-based drinks have emerged, yogurt has accounted for the largest share of sales, representing 36.6% in 2008 (Anon 2009).

Non-dairy probiotic products are also important in the global market, because there are some drawbacks related to the intake of dairy products including lactose intolerance, cholesterol content, and allergenic milk proteins (Granato *et al*. 2010b). Also, non-dairy probiotic products, such as fortified fruit and vegetables matrices with modified pH can be great substrates for probiotic cells, because they contain nutritional substances, such as antioxidants, minerals, dietary fibre and vitamins, without any dairy allergens (Betoret *et al*. 2003; Sheehan *et al*. 2007).

Consumer surveys are extremely important in the food industry. Increasing the general knowledge and awareness of probiotic products might be the key element contributing to the probiotic market growth. Probiotic products normally are purchased by people who pay attention to their health (FAO/WHO 2006). In some studies the level of knowledge of probiotic foods was related to educational level (Babajimopoulos *et al.* 2004), but in other investigations no relationship between consumption of probiotics and education level was established (Viana *et al.* 2008).

Data on the perception and consumption of probiotic products and the type of action of these products need to be provided to enhance awareness of consumers. As previously mentioned, probiotic products are considered as one of the largest functional food markets in the world. The future success of probiotic foods containing lactobacilli in the market is related to various factors. One of the important elements in this regard is the fact that consumers should be convinced by health claims of probiotic foods through clear, truthful, and unambiguous messages (Granato *et al*. 2010b). Regardless of the use of probiotics in food additives, it is worth the efforts to introduce probiotics more frequently into medication (Oelschlaeger 2010).

1.10 Rationale for this study

Probiotic microorganisms should meet certain criteria based on physiological, safety and functional characteristics and to be present in high numbers in the product to be able to provide health benefits. One needs to bear in mind that,

probiotic functionalities and health benefits are strain dependent. Therefore, the strain needs to be noted on the product label (Saad *et al*. 2013; Rajasekaran and Kalaivani 2013). It has been reported that a large number of commercial products do not contain the specified strains nor they state the number of probiotic microorganism(s) present in the product (Hamilton-Miller and Shah 2002), or contain species other than what has been claimed on the label (Weese 2002). Hence, it was deemed necessary to review the status of the available probiotic products on UK and some other European markets and screen their quality in terms of the true strain and number of viable probiotic cells. In the absence of any international standard, the minimum therapeutic level of 10^6 to 10^7 CFU/ml probiotic cells is widely acceptable.

Viability of the cells in the product aside, functionality of the probiotics also needs to be monitored (Vinderola *et al*. 2011). However, before that the organisms should prove fit for purpose from a physiological point of view and be able to survive the harsh condition of the upper digestive tract. Therefore, potential probiotic microorganisms are usually screened *in vitro* for acid and bile tolerance. Acid stress may occur in the stomach, but also during the fermentation, due to the production and accumulation of organic acids, such as lactic acid. Bile stress could also occur in the small intestine, due to the secretion of bile salts. Therefore, the effects of sequential treatment with acid, bile salts and gastric enzymes on the viability of probiotic bacteria are justified.

Some probiotic bacteria are able to form biofilms in the gut and produce antagonistic (antimicrobial) compounds to out-compete undesirable bacteria. This perhaps is one less studied area of probiotics.

The probiotic organisms are expected to produce functional ingredients, such as conjugated linoleic acid (CLA). Again this is an area which is not well studied and deserves further investigation.

Safety is also one of the most important selection criteria for probiotics (Gueimonde *et al.* 2013). Probiotic bacteria should be able to tolerate antibiotics in order to aid re-colonisation of the gut, during antibiotic therapy; however, they should not contain transferable antibiotic resistance genes to other bacteria. Thus, studying the antibiogram of selected lactobacilli was included in this project. The study is extended to include assessment of the antibiotic resistance gene transferability amongst the isolates.

1.11 Purpose of this research

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

- Monitoring survival of the lactobacilli in the carrier foods at the time of purchase and at the end of their shelf-life
- Isolation and identification of presumptive lactobacilli with 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 the acidic condition of the stomach and the presence of bile salts in the small intestine
- Antimicrobial activities and potential for bacteriocin production and 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.

CHAPTER TWO: ENUMERATION AND IDENTIFICATION OF *LACTOBACILLUS* SPP. IN FERMENTED DAIRY PRODUCTS

2.1 INTRODUCTION

Certain dairy products are vehicles by which consumers receive adequate counts of probiotic lactobacilli (Vinderola and Reinheimer 2000). Probiotic effects are dependent on the number of viable microbial cells that reach the human gut (Leahy *et al*. 2005). Therefore, their viability in the product is considered as an important prerequisite for achieving health effects.

There are various reports regarding the adequate number of probiotic microorganisms in different products in order to ensure the probiotic effects. Some of the suggested minimum levels of viable cells in dairy products are 10^5 CFU/g (Shah *et al*. 1995), 10^6 CFU/g (Talwalkar and Kailasapathy 2004) and 10^7 CFU/g (Korbekandi *et al*. 2011). It should be noted that it is not simple to keep a high number of viable probiotic bacteria in fermented milk throughout the shelf life, because the viability of probiotic bacteria in the product matrix is influenced by numerous factors. These parameters include temperature of storage condition, hydrogen peroxide (H_2O_2) which might be produced by other existing bacteria, dissolved oxygen content due to process conditions, pH of final product and finally, strain variation that is considered the most important factor for survivability of probiotic cultures in final product (Shah and Lankaputhra 1997; Vinderola *et al*. 2002).

Probiotic lactobacilli may be incorporated alone or in combination with commercial cultures into specific dairy products. Interactions between microorganisms in co-cultured products cause difficulties in enumeration. *Lactobacillus acidophilus, Lb.*

casei and *B. animalis* subsp. *lactis* are the most frequently used strains in commercial probiotic products (Tabasco *et al.* 2007).

Reliable determination of viable probiotic bacteria in food is important and selective/differential media are necessary to count the probiotic population. Different parameters such as medium, time, temperature and also incubation conditions influence the effective isolation of probiotic bacteria (Lima *et al*. 2009).

Generally, de Man, Rogosa and Sharpe (MRS) agar is used for enumeration of LAB; however, MRS is a general medium and is not suitable for selective enumeration of probiotic lactobacilli in the presence of other starter or probiotic cultures.

Numerous selective media have been developed for accurate enumeration of *Lactobacillus* spp. in fermented milks. However, due to presence of closely related species of *Lactobacillus* in probiotic products, the differential enumeration seems challenging and relies directly on differences in colonial morphology (Van de Casteelea *et al.* 2006).

Variable results have been reported for enumeration of *probiotic* lactobacilli in the presence of bifidobacteria, S*. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus,* e.g. *Lb. acidophilus* (Van de Casteele *et al.* 2006; Tabasco *et al*. 2007; Lima *et al*. 2009), *Lb. casei* (Tharmaraj and Shah 2003; Talwalkar and Kailasapathy 2004; Lima *et al*. 2009), *Lb. paracasei* (Van de Casteele *et al*. 2006; Tabasco *et al*. 2007) *Lb. plantarum* and *Lb. rhamnosus* (Tharmaraj and Shah 2003; Van de Casteele *et al*. 2006).

Selectivity and/or differentiation ability of the culture media depends on the type and concentration of inhibitory compounds added.

Elective media promote the growth of certain microorganisms, while selective media inhibit the growth of certain microorganisms. The additives including bile (Bergamini *et al.* 2005), gentamycin (Lima *et al*. 2009), clindamycin (Van de Casteele *et al.* 2000) penicillin (Bielecka *et al*. 2000) and salicin (Bielecka *et al*. 2000) are added to the media because they have suppressive effects against some species, but other compounds, such as lithium chloride and sodium propionate (Tharmaraj and Shah 2003; Lima *et al*. 2009), sorbitol (Tharmaraj and Shah 2003), maltose (de Souza *et al*. 2008; Lima *et al*. 2009) trehalose (Lima *et al*. 2009), fructose (Tabasco *et al*. 2007), ribose (Ravula and Shah 1998) are added to the media because of inability of some species to metabolise them.

There are various instructions regarding the probiotic enumeration, but few are official protocols for enumeration of lactobacilli (ISO 2006). Enumeration in co-cultured products is more complicated than in products made with single culture. In mixed cultures, inhibitory agents are needed to suppress the interfering species in order to recover the target lactobacilli.

Anaerobically-incubated MRS with vancomycin and bromocresol green (LAMVAB) is another selective medium suggested for enumeration of lactobacilli. Two selective agents in LAMVAB agar are vancomycin and low pH. Bromocresol green was considered as a differentiative agent, Lactobacilli colonies appear in blue or

green colour, but other genera produce yellow or white colonies (Hartemink *et al*. 1997).

One real concern is that some culture media that contain antibiotics might also restrict the growth of target lactobacilli and the counts may not be representative of the real number of viable cells present in the product (Ashraf and Shah 2011). In addition, some antibiotics cannot inhibit the growth of all non-target bacteria (Novik *et al*. 2007). The variation among the minimum inhibitory concentration (MIC) ranges of antibiotics for lactobacilli can be exploited in selective enumerative media to allow discrimination between different probiotic lactobacilli species used in the same product (D'Aimmo *et al*. 2007).

2.1.1 Enumeration of lactobacilli using plate count techniques

As *Lb*. *acidophilus* and *Lb. casei* were found to be the most commonly used probiotic species in probiotic products, it is essential that they are properly enumerated and isolated from those products, hence an appropriate selective medium is always needed.

2.1.1.1 Enumeration of *Lactobacillus acidophilus*

MRS-bile agar has been originally proposed for detection and enumeration of *Lb. acidophilus* (IDF 1945). For enumeration of *Lb. acidophilus* in the presence of the dairy cultures or bifidobacteria, MRS-bile agar, incubated aerobically at 37 °C or at 42 °C can be used (Lima *et al*. 2009). Gentamycin in MRS agar has been shown to inhibit *Lb. casei* Lc1 incubated at 42 °C without affecting *Lb. acidophilus* La5. It is necessary to mention that gentamycin presented no inhibitory effect at an

incubation temperature of 37 °C, in neither aerobic nor anaerobic conditions (Lima *et al*. 2009).

In the study of Gomes and Malcata (1999) TGV agar (tryptone, glucose, and meat extract) containing 2% (w/v) NaCl was used to enumerate *Lb*. *acidophilus* Ki. Van de Casteele *et al*. (2006) used Nutrient agar and MRS supplemented by salicin and clindamycin, respectively, for enumerating *Lb. acidophilus*. Acidified MRS agar with pH 5.4 and anaerobic incubation at 37 °C for 3 days can be useful for counting *Lb. acidophilus* (Kasımoglu *et al*. 2004). Darukaradhya *et al*. (2006) have stated that Reinforced Clostridium Agar (RCA) with bromocresol green and clindamycin (RCABC) with pH 6.2 is the best culture medium for selective counting of *Lb. acidophilus* in Cheddar cheese.

Replacement of glucose in MRS agar by maltose or trehalose negatively affects the growth of the yogurt bacteria, indicating that glucose is essential for their growth (Lima *et al*. 2009). Glucose in MRS agar might be substituted by maltose in order to modify the MRS for *Lb. acidophilus* recovery (Buriti *et al.* 2005). The same procedure has been used by Cardarelli *et al*. (2008) for counting *Lb. acidophilus* after 72 h anaerobic incubation at 37 °C in petit-suisse cheese.

MRS-D-sorbitol agar is one of the suitable media which need to undergo membrane sterilization because D-sorbitol is structurally deformed by heat treatment (Ozer *et al.* 2008).

2.1.1.2 Enumeration of *Lactobacillus casei*

Vinderola and Reinheimer (2000) have used MRS agar with lithium chloride and sodium propionate (MRS-LP) and MRS-bile for selective and differential enumeration of *Lb. casei* from *Lb. acidophilus*, bifidobacteria and LAB starters in fermented milk and yogurt. This medium has been reported as inhibitory for *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. They used catalase testing and cell morphology recording to confirm the identity of the colonies. In their study, the colonies of *Lb. casei* were round, creamy- white and measured 1.7-2.4 mm and 0.9-1.3 mm on MRS-LP and MRS-bile, respectively. Also, the colonies of *Lb. acidophilus* were irregular, light brown on MRS-bile with a diameter of 0.9-1.5 mm. According to their results, both MRS-LP and MRS-bile can be utilized for the enumeration of *Lb. casei*, but in presence of other probiotics, MRS-LP and MRS-bile allow the growth of bifidobacteria and *Lb. acidophilus*, respectively.

Bile has been shown to be inhibitory to *Lb. casei* Lc1 when anaerobically incubated at 42 °C, but not under aerobic condition (Lima *et al*. 2009). However, it has been recently indicated that bile, like lithium chloride and sodium propionate, is not a selective agent for differentiation of *Lb. casei* and *Lb. acidophilus* colonies (Colombo *et al*. 2014).

Another proposed medium for the selective counting of *Lb. casei* in yogurts and fermented milk drinks is MRS-*Lactobacillus casei* (MRS-LC) medium (Ravula and Shah 1998; Shah and Ravula 2000). MRS-LC agar inhibits the growth of yogurt bacteria and bifidobacteria, due to the adjusted pH of the medium (pH 5.1) and

inability to ferment ribose, but *Lb. acidophilus* also can grow on this medium (Talwalkar and Kailasapathy 2004).

It has been observed that nalidixic acid in nalidixic paromomycin neomycin lithium MRS agar (MRS-NPNL) allows the growth of *Lb. casei* at the incubation temperatures of 30 and 37 °C. Furthermore, nalidixic acid in that medium inhibits *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *Lb. acidophilus*, *Bifidobacterium* and *Propionibacterium freudenreichii* subsp. *Shermanii* strains (Tharmaraj and Shah 2003).

Colombo *et al*. (2014) reported that MRS with nalidixic acid is not able to inhibit LAB, except *S. thermophilus* TH-4® and ST 066. They concluded that nalidixic acid is not a selective agent for *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*.

2.1.2 Molecular methods for identification of lactobacilli

In addition to the plate count enumeration, lactobacilli need to be identified and characterized (Dalezios and Siebert 2001). Different characteristics including phenotype, physiological and biochemical features and sequence comparisons of 16S rRNA gene have been suggested to make the identification and characterization of *Lactobacillus* spp. more reliable (Kwon *et al*. 2004). It should be noted that there is taxonomic dispute and ambiguity among some lactobacilli due to the differences at nucleotide level in the 16S rRNA gene (Singh *et al*. 2009). It is hard to differentiate between some species and strains of lactobacilli (Song *et al*. 1999), and some closely related groups of lactobacilli species are indistinguishable on the basis of phenotype.

Several reports have revealed the misidentification of a number of strains belonging to some groups of lactobacilli (Yeung *et al.* 2002). As probiotic ability is often strain dependent, accurate detection, characterization and identification of probiotic lactobacilli is required.

Advanced methods like DNA-based molecular techniques are needed for proper identification and characterization of lactobacilli added in functional products.

Also, to assure quality management, it is necessary to unambiguously identify probiotic lactobacilli at genus, species and strain levels using genotyping methods (Herbel *et al.* 2013). Molecular identification methods are consistent, rapid, reliable and reproducible, compared to phenotypic methods.

There are some studies in which species-specific oligonucleotide probes have been employed to identify various *Lactobacillus* spp. (Ampe 2000; Chagnaud *et al.* 2001; Park and Itoh 2005). Most genetic probes have been designed based on 16S rRNA or 23S rRNA (Briengel *et al.* 1996; Sghir *et al*. 1998).

Some of the most utilized molecular techniques for identification of *Lactobacillus* spp. are as follows:

a) Non-PCR-based methods

DNA dot blot techniques (Rachman *et al*. 2003), ribotyping (Kitahara *et al*. 2005) and pulsed field gel electrophoresis (Bouton *et al*. 2002) are common non-PCR-based methods.

b) PCR-based methods

PCR-based techniques including simple PCR, randomly amplified polymorphic DNA (RAPD) and multiplex PCR have been employed to identify lactobacilli.

In general, there is an ambiguity in differentiation of specific lactobacilli. According to the study of the Singh *et al*. (2009) there are simillarity at nucleotide level in the 16S rRNA gene in some lactobacilli, such as *Lb. acidophilus*, *Lb. casei*, *Lb. plantarum* and *Lb. bulgaricus*, making them hard to distinguish. It has been reported that sometimes *Lb. gasseri* and *Lb. johnsonii* are difficult to differentiate from each other even by molecular methods (Walter *et al.* 2000). *Lactobacillus plantarum* and *Lactobacillus pentosus* have greater than 99% similarity with only 0.3% difference in their 16S rRNA sequences (Quere *et al*. 1997). However, some alternative molecular markers have been used for discrimination among these species (Torriani *et al.* 2001).

The aim of the work described in this chapter was to isolate, enumerate and identify *Lactobacillus* spp. in commercial probiotic dairy products in UK and European markets.

The specific objectives were:

a) Evaluation of 36 commercial probiotic dairy products for the number of probiotic *Lactobacillus* spp. by enumerating at the time of purchase and at the end of shelf-life.

b) Isolation and preservation of probiotic *Lactobacillus* spp. from these commercial probiotic products.

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

In addition, accuracy of the label descriptions for fermented milk products was assessed.

2.2 MATERIALS AND METHODS

2.2.1 Media

2.2.1.1 General media

MRS agar (CM0361, Oxoid, Basingstoke, UK) was prepared according to the manufacturer's instructions and autoclaved at 121 °C for 25 min, cooled in water bath to 55 °C and poured into petri dishes.

Also, Maximum Recovery Diluent (MRD) (CM0733, Oxoid, Basingstoke, UK) was used as diluent for serial dilutions.

2.2.1.2 Selective/elective media

Three selective/elective media were used for comparative enumeration of lactobacilli:

a) MRS-clindamycin:

MRS supplemented with clindamycin hydrochloride for selective, isolation and enumeration of *Lb. acidophilus* and *Lb. casei* was developed by ISO (2006). MRS medium supplemented with clindamycin (C5269-10mg, Sigma, UK) was evaluated

herein as a medium for enumeration of *Lb. acidophilus, Lb. rhamnosus, Lb. casei* and *Lb. paracasei.*

The medium was prepared as follows:

Two mg of clindamycin hydrochloride was dissolved in 10 ml distilled water and sterilized through a 0.2 µm syringe filter (Nalgene, Fisher Scientific, Loghborough, UK), and kept as a stock solution at -20 °C. MRS agar was prepared according to the manufacturer's instructions, autoclaved at 121 °C for 25 min and cooled to 44 °C and 47 °C. Aliquots of 250 µl of the clindamycin stock solution (0.02% w/v) were added to 500 ml of MRS agar and mixed carefully to avoid bubbles and poured into petri dishes.

b) MRS-Sorbitol

In this medium sorbitol was added to the MRS agar instead of dextrose. A 100 mg/ml concentration of sorbitol solution was prepared and added at the rate of 10 ml to 90 ml molten MRS (Ozer *et al*. 2008; Ong and Shah 2009).

Only *Lb*. *acidophilus* grows on MRS-D-sorbitol agar. It forms small, rough, brownish, dull colonies of 0.1 to 0.5 mm in MRS-Sorbitol agar.

c) MRS-IM agar with Maltose

Based on sugar fermentation patterns, maltose was used instead of glucose (dextrose) was used. MRS-IM agar with added Maltose (Ref: 610067,Liofilchem, Italy) was prepared according to the manufacturer's instructions, autoclaved at 121 °C for 25 min, cooled to 55 °C and poured into petri dishes.

In this medium, *Lb*. *acidophilus* and *Lb*. *casei* display growth. *Lactobacillus acidophilus* gives star formed, irregular small colonies while *Lb. casei* gives larger regular colonies in this medium.

2.2.2 Reference microorganisms

Reference microorganisms used in the study included three commercial cultures (*Lb. acidophilus* La5, *Lb. delbrueckii* subsp. *bulgaricus* Lb12 and *Lb. casei* C431) which were kindly provided by Chr. Hansen and four type strains of *Lactobacillus* from National Collections of Industrial, Marine and Food Bacteria (NCIMB). These were *Lb. delbrueckii* subsp. *bulgaricus* 11778, *Lb. acidophilus* 701748, *Lb. casei* subsp. *casei* 11970 and *Lb. paracasei* subsp. *paracasei* 700151.

2.2.3 Probiotic products

Thirty six commercial fermented milks claiming to contain *Lactobacillus* strains/spp. were purchased from different supermarkets and retailers in the UK and Europe, transported to laboratory and stored at 4 °C. Samples from countries outside of the UK were purchased and sent to the UK in a cool box; they were received in very good condition. Table 2.1 shows details of the tested products.

2.2.4 Measurement of pH value

The pH was measured with a pH meter (Whatman PHA 2000), which was calibrated using pH buffer at pH 4.0 and 7.0 according to the operating manual.

Table 2.1 Details of tested probiotic products

Sample Code	Commercial Name	Product Description	Manufacture/Supplier	Days To Expiry	Claimed Culture	Initial pH*	Final pH**
P1	Waitrose	Stirred yogurt	Waitrose	10	*Bifidobacterium, Lactobacillus acdophilus, Streptococcus thermophilus*	4.16	3.99
P2	Yeo vally	Organic natural yogurt	OMSCO	12	*Lactobacillus acidophilus, Bifidobacterium*	4.10	4.00
P3	Onken	Natural fresh and mild yogurt	Dr. Oetker	17	*Lactobacillus acidophilus, Bifidobacterium longum, Streptococcus thermophilus*	4.12	4.01
P4	Rachel's	Fruit yogurt	Rachel's	13	*Lactobacillus acidophilus, Bifidobacteria*	4.05	3.92
P5	Waitrose	Thick and creamy yogurt	Waitrose	11	*Bifidobacterium, Lactobacillus acidophilus, Streptococcus thermophilus*	4.08	4.01
P6	Morrison	Fruit yogurt	Morrison	13	*Bifidobacterium animalis* subsp.*lactis Lactobacillus acidophilus*	4.22	4.10
P7	Delamere	Natural goat yogurt	Delamere	25	*Bifidobacterium longum, Lactobacillus acidophilus*	3.95	3.66
P8	Delamere	Goat fruit yogurt	Delamere	26	*Streptococcus thermophilus, Lactobacillus casei* ***	3.80	3.80
P9	Sheep milk yogurt	Natural greek style	Woodland Dairy	15	*Lactobacillus acidophilus, Lactobacillus bulgaricus,**** *Streptococcus thermophilus*	4.28	4.20
P10	Low fat fruit yogurt	Fruit yogurt	Stapleton	11	*Lactobacillus bulgaricus,**** *Lactobacillus acidophilus, Streptococcus thermophilus, Bifidobacterium*	4.70	4.61

Table 2.1continued

Sample Code	Commercial Name	Product Description	Manufacture/Supplier	Days To Expiry	Claimed Culture	Initial pH*	Final pH**
P11	Actimel	Fat free yogurt drink	Danone	19	*Lactobacillus casei****	4.06	4.01
P12	Count Lodge	Bio pouring yogurt	OMSCO	11	Probiotic	3.94	3.96
P13	Multi fruit yogurt drink	Fruit yogurt drink	Morrison	4	*Lactobacillus casei****	4.24	4.02
P14	Yakult	Fermented milk drink	yakult	23	*Lactobacillus casei* Shirota	3.62	3.76
P15	Diet Fat free probiotic yogurt	Fruit yogurt	Irish yogurt	19	probiotic	3.95	3.95
P16	Natural probiotic goats milk yogurt	Gout milk yogurt	St. Helen's farm	17	*Lactobacillus acidophilus, Lactobacillus bulgaricus,**** *Streptococcus thermophilus, Bifidobacterium*	4.16	3.99
P17	Waitrose Deliciously fruity low fat yogurt	Fruit yogurt	Waitrose	14	*Lactobacillus acidophilus, Bifidobacterium, Streptococcus thermophilus*	3.95	3.86
P18	Lassi	Fruit Yogurt smoothie	Tesco	20	*Yogurt culture, Lactobacillus acidophilus, Bifidobacterium*	3.85	3.61
P19	Tesco Fat free natural defences	Yagurt drink	Tesco	11	*Lactobacillus casei****	4.06	3.97
P20	Apple + spice gourmet probiotic yogurt	Fruit yogurt	The collective great dairy	11	*Streptococcus thermophilus, Lactobacillus acidophilus, Bifidobacterium, Lactobacillus casei****	3.97	3.55

Table 2.1continued

Sample Code	Commercial Name	Product Description	Manufacture/Supplier	Days To Expiry	Claimed Culture	Initial pH*	Final pH**
P21	Little swallow Bi grade plus with casei cultures	Live natural yogurt	House of westphalia (Germany)	23	*Lactobacillus acidophilus, Lactobacillus casei,*** *Bifidobacterium*	3.94	4.21
P22	Organic natural fromage frais	Fromage frais blanc	Vrai	27	*Bifidobacterium, Lactobacillus acidophilus*	4.21	4.24
P23	Sainsbury's Crunch tip &mix chocolate probioticyogurt	yogurt	Sainsbury's	12	Sainsbury's probiotic bacteria	3.79	3.8
P24	Tesco (value) Probiotic yogurt drink	Yogurt drink	Tesco	26	*Lactobacillus acidophilus* La5	3.98	4.05
P25	Sainsbury's active health	Yogurt drink	Sainsbury's	19	Sainsbury's probiotic bacteria, *Lactobacillus casei****	3.76	3.46
P26	Taste the diffrence (Sainsbury's) West country yogurt	Probiotic yogurt selection	Sainsbury's	11	probiotic	3.92	3.81
P27	Sojade Bio organic Fresh soya specialty	Fermented soya drink	Sojade. (France)	11	*Bifidus, Lactobacillus acidophilus*	4.07	3.78
P28	Biona fresh Demeter	Organic kefir	Windmill organics Ltd.	17	Probiotic	3.99	3.85
P29	Nourish kefir	Natural probitic drink	Nourish kefir	19	Rich in probitic	4.29	4.45
P30	Optifit active Full of natural ingredients	Fruit layer Yogurt	Aldi	20	*Lactobacillus acidophilus* La5 *Bifidobacterium animalis* subsp.*lactis* BB12	3.9	3.82

Table 2.1continued

Sample Code	Commercial Name	Product Description	Manufacture/Supplier	Days To Expiry	Claimed Culture	Initial pH*	Final pH**
P31	Minus L (Lactosefrei) Joghurt mild himbeere	Probiotic yogurt	OMIRA MILCH (Germany)	15	probiotic	3.99	3.77
P32	Probiotic joghurt	Stirred yogurt	Hergestellt Fur (Germany)	10	*Bifidobacterium animalis* subsp. *lactis* BB12 *Lactobacillus acidophilus*	3.97	3.95
P33	LC1	Stirred yogurt	Nestle. (Germany)	8	*Lactobacillus* lc1 *****	4.11	4.16
P34	BIAC probiotic kulturen	fruit yogurt	BIAC (Germany)	4	*Lactobacillus casei****	3.92	3.85
P35	Be light (Leichte Linie)	Probitic yogurt drink	Be light (Leichte Linie) (Germany)	3	*Bifidobacterium animalis* subsp. *lactis* BB12 *Lactobacillus acidophilus* La5 *Lactobacillus casei****	4.04	3.94
P36	Proviact	Fruit yogurt	Lidl (Germany)	7	*Lactobacillus casei****	4.05	3.88

*Initial pH was measured upon their arriving date to the lab
**Final pH was measured at their expiry date
*** *Lactobacillus casei* subsp. *casei*
**** *Lactobacillus delbrueckii* subsp. *bulgaricus*
***** *Lactobacillus johnsonii* La1

2.2.5 Determination of viable cell count of *Lactobacillus* spp. in the fermented milks

All products were analysed on the day of purchase and again on their expiry date using unopened product each time. Four pots of each product were purchased. Two of them were tested for presumptive *Lactobacillus* spp. sampled on the day of receipt in laboratory and two on the expiry date. One gram of homogenised sample was mixed with 9 ml of MRD and vortexed (homogenised using Whirlimixer). Several dilutions were made using MRD up to 10^{-8}. Agar plates were divided into 4 sections using a marker and 25 µl of each dilution was spread on to each quarter of MRS, MRS-Maltose, MRS-Sorbitol and MRS-Clindamycin in duplicate. The plates were then incubated at 37 °C in anaerobic cabinet (Don Whitley, Skipton, UK) containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide for three days.

2.2.6 Isolation and storage of the isolates

Two to four typical colonies grown on MRS-Clindamycin were harvested per each product and cultured on MRS agar. Following overnight anaerobic incubation at 37 °C, the colonies were streaked on MRS agar and incubated in the same conditions. A pure isolate was picked up and inserted aseptically into a cryovial (Micro Bank, Pro-Lab Diagnostics, Neston, UK) and stored at - 20 °C.

2.2.7 Identification of the isolates by genotyping methods

Initially, six selected *Lactobacillus* spp. isolates, which were provided by microbiology research unit (MRU) culture collection were identified using phenotypic methods (API 50 CHL), and results were compared with genotypic identification. The results did not match together. Tested isolates were not part of this study, and they were used only for validation of phenotypic test and accuracy of identification. Therefore, the rest of experiments were based on the genotypic techniques.

2.2.7.1 DNA Extraction

The presumptive *Lactobacillus* spp. isolates, which have been kept on the beads at -20 °C, were grown on MRS agar anaerobically at 37 °C for 24 h, restreaked and incubated under the same conditions. A fresh colony was picked and resuspended in 1 ml of sterile water in a sterile Eppendorf tube and centrifuged for 1 min at 6700 × *g*, then the supernatant was discarded and, after addition of 100 µl of Instagene matrix (BioRad, Hemel Hempstead, UK), the mixture was incubated at 56 °C on a heating block (TECHNE, Stone, UK) for 30 min. After mixing the tube content for 10 seconds, incubation was continued at 100 °C for 10 min. The tubes were then vortexed for 10 seconds, centrifuged at 9700 × *g* for 3 min and the supernatant was collected in a sterile Eppendorf tube, which was stored at -20 °C and used as DNA template for future use.

2.2.7.2 Differentiation of isolates using rep-PCR

This method was used for initial screening and grouping of the isolates by comparing their DNA profiles with commercial and type strains. Rep-PCR was

applied for differentiation of all isolates by the method of Ouoba *et al*. (2008a). Rep-PCR was undertaken in 25 µl of reaction mixture containing 2 µl of DNA template, 2.5 µl of PCR buffer (10 X; N808-0161, Applied Biosystems), 4 µl of dNTP (1.25 mmol/l; U1511, Promega, Southampton, UK), 2 µl of MgCl2 (25 mmol/l; AM9530G, Applied Biosystems), 4 µl of primer GTG5 (5'-GTG GTG GTG GTG GTG-3') (5 pmol/µl), 0.25 µl of Taq polymerase (5 U; N808-0161, Applied Biosystems) and 10.25 µl of autoclaved high purity water (Sigma, Gillingham, UK). Amplification consisted of 30 PCR cycles in a thermocycler (GeneAmp PCR 2700 system, Applied Biosystems, Singapore). 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 (AM8556, Applied Biosystems) to 1.5% agarose gel (Bio-rad, Hemel Hempstead, UK).

2.2.7.3 Agarose gel electrophoresis

The products of rep-PCR were analysed using agarose gel electrophoresis, followed by staining with ethidium bromide and visualisation of the gel under UV irradiation of the gel. A volume of 10 µl of PCR products was mixed with 2 µl loading dye (Sigma, UK) and loaded in to a 1.5% (w/v) agarose gel. A DNA molecular marker (D7058, 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 2 h at 120 V. The gel was stained by placing in a solution of ethidium bromide 0.5 µl/ml

(Sigma, UK) for 20 min and excess stain was rinsed off with distilled water. PCR products were viewed under a UV light at 260 nm.

The DNA profiles were observed and all bacteria showing the same profile were clustered in the same group. Profiles were analyzed using the Bionumerics system (Bio-Numerics 4.50, Applied Maths, Sint-Martens-Latem, Belgium).

2.2.7.4 Identification of the isolates by sequence analysis of 16S ribosomal RNA gene

2.2.7.4.1 First PCR cycle sequencing

Following the rep-PCR screening and arranging the isolates into different groups, further identification was carried out using 16S rRNA gene sequencing. 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.

2.2.7.4.2 Purification of PCR products

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

2.2.7.4.3 Second PCR Cycle Sequencing

The second PCR was undertaken to generate 550 base pair (bp) of nucleotides, using the primer pD (5` GTA TTA CCG CGG CTG CTG 3`, Sigma, UK). This was done with a reaction mixture which consisted of:

PCR product	4	µl
Primer pD (20 ng/µl)	2	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.

2.2.7.4.4 Precipitation

Second PCR products were precipitated with 1 µl of 3 M sodium acetate (pH 4.6) and 50 µl of absolute ethanol and centrifuged at 9700 × *g* for 20 min. Pellets were rinsed with 250 µl of 70% v/v ethanol and centrifuged for 10 min at 9700 × *g*. Supernatants were discarded and pellets were air dried and sent for sequencing (Gene servicing, Cambridge, 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.

2.2.7.5 Identification of bacteria by *rpoA* gene sequencing

Primarily, all randomly selected isolates were identified by 16S rRNA gene sequencing, where it was not possible to distinguish between closely related species (i.e. *Lb. casei* and *Lb*. *paracasei*), amplification and sequencing of *rpoA* gene was carried out.

The amplification of *rpoA* gene was carried out using the forward primer *rpoA*- 21-F (5`ATG ATTC GAGA TTT GAA AAA CC 3`) and reverse primer *rpoA*-23-R (5`ACACT GTGA TTGA ATD CCGAT GCGA CG 3`) (Anyogu *et al*. 2014).

The reaction mixture consisted of:

Water (autoclaved high purity)	36.8	µl
10 X PCR Buffer (with MgCl2)	5	µl
dNTP (1.25 mM)	5	µl
Primer rpoA-21-F (21 µM/L)	0.5	µl
Primer rpoA-23-R (21 µM/L)	0.5	µl
AmpliTaq DNA Polymerase (5U)	0.25	µl
Chromosomal DNA	2	µl

Amplification of DNA was performed under the following thermal programme,

(I) 5 min at 95 °C.

(II) 3 cycles of 1 min at 95 °C + 2 min 15 s at 46 °C + 1 min 15 s at 72 °C.

(III) 30 cycles of 35 s at 95 °C + 1 min 15 s at 46 °C + 1 min 15 s at 72 °C.

(IV) A final 7 min at 72 °C.

All PCR products were checked by gel electrophoresis and products of positive reactions purified as described in section 2.2.7.4.1, and the samples were sent for sequencing using 3.2 pmol/µl of the same primers.

2.2.8 Statistical analysis

Results are the mean±standard deviation. Data were analysed using the independent-samples t-test was performed using SPSS 21 software (Chicago, IL: SPSS Inc.) to determine the statistical significance of differences. Data were considered significantly different when $p<0.05$.

2.3 RESULTS

In the present study MRS, MRS-Maltose, MRS-Sorbitol and MRS-Clindamycin were used for enumeration of probiotic lactobacilli in 36 probiotic dairy products (Figure 2.1 a, b, c, d, e and f). MRS was used as a non-selective culture medium and a reference medium to assess the growth of probiotic lactobacilli. MRS-Maltose did not give a good recovery of the target strain even when compared with that obtained on the control medium (MRS). Comparison of the viable counts indicated that in eight products (P8, P9, P11, P13, P14, P31, P32 and P35) the viable counts on MRS-Sorbitol were higher than viable counts on MRS-Clindamycin, while in six products (P15, P17, P26, P29, P34 and P36) viable counts on MRS-Clindamycin were higher than viable counts on MRS-Sorbitol.

2.3.1 Enumeration of *Lactobacillus* spp. in commercial fermented milk

Thirty one out of 36 fermented milks contained more than 6 $\log_{10}$ CFU/g on at least one medium at the time of purchase (Figures 2.1 a, b, c and d, e and f). According to the results of the present study and those suggested by Van de Casteele *et al.* (2006), MRS-Clindamycin seems to be a valuable selective culture medium for enumeration of probiotic lactobacilli. The following figures (2.2 a, b, c, d) show numbers recovered on MRS-Clindamycin at the end of expiry dates compared to the numbers at initial sampling.

Depending on the tested product, the number of *Lactobacillus* spp. declined between 0.00 and 2.62 $\log_{10}$ on the expiry date (Figure 2.2 a, b, c, d). However, at the end of the shelf life, 22 (61.1%) of tested samples contained greater than 6 $\log_{10}$ CFU/g viable *Lactobacillus* spp. Products P3, P4, P15, P18, P21, P22 and P23 contained an initial *Lactobacillus* spp. population of more than 6 $\log_{10}$ CFU/g, which had significantly decreased to less than 6 $\log_{10}$ CFU/g by the expiry date ($p < 0.05$). However, products P8, P11, P14, P27 and P32 contained less than 6 $\log_{10}$ CFU/g viable *Lactobacillus* spp. at the time of purchase.

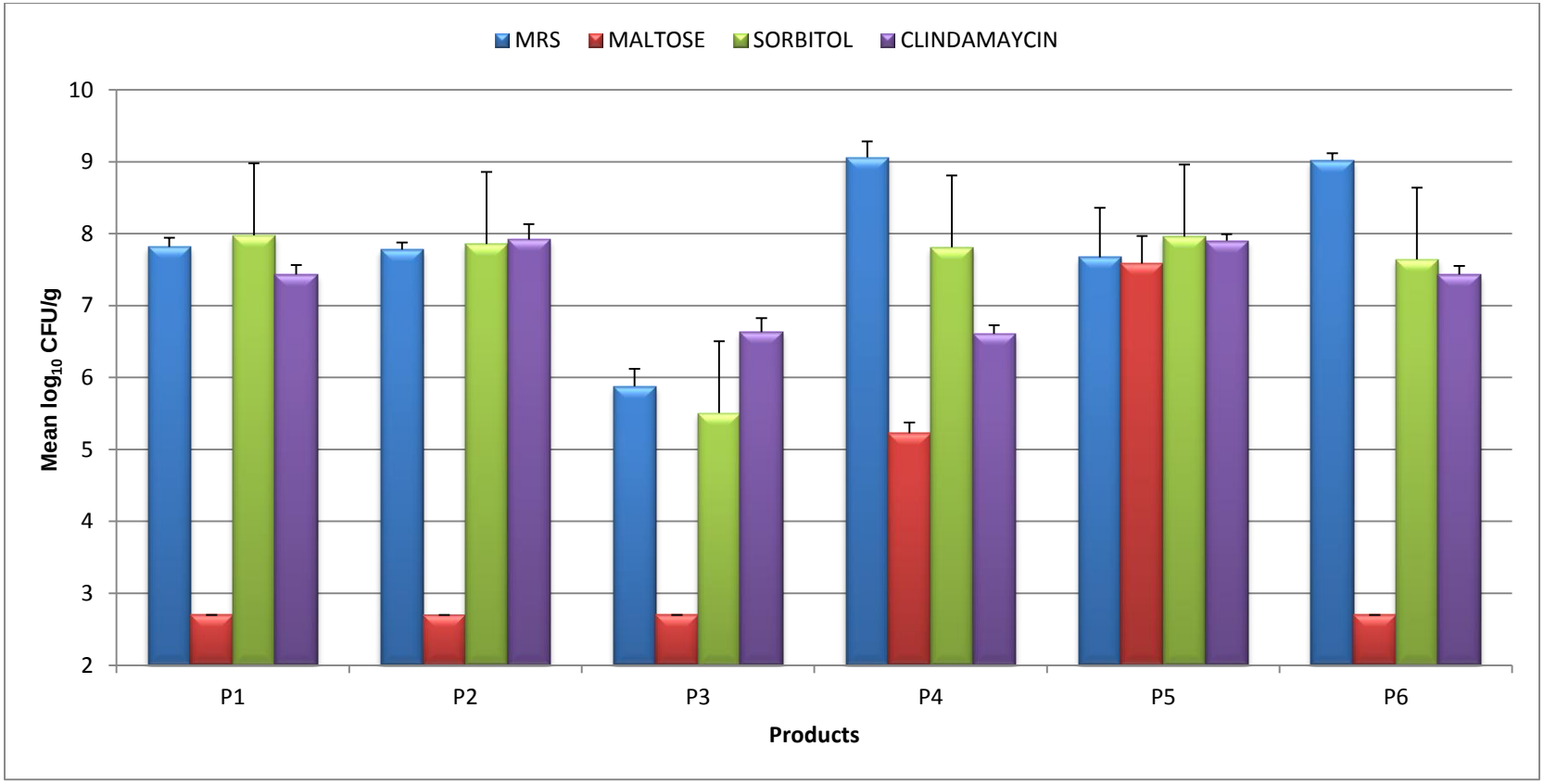


Figure 2.1 a The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on four different media (MRS, MRS Maltose, MRS-Sorbitol, MRS-Clindamaycin)

Data are means ± SD (n=4)

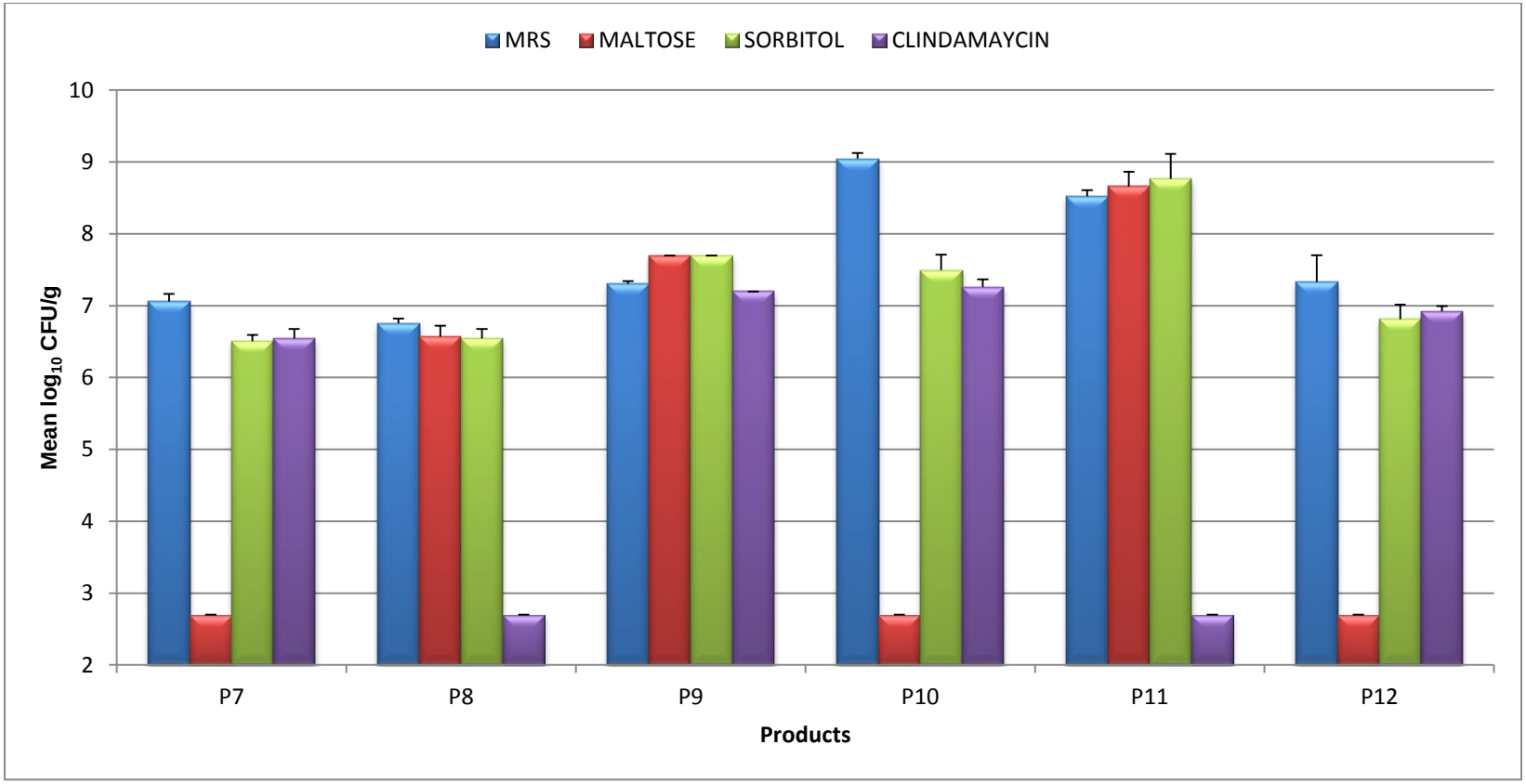


Figure 2.1 b The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on four different media (MRS, MRS-Maltose, MRS-Sorbitol, MRS-Clindamaycin)

Data are means ± SD (n=4)

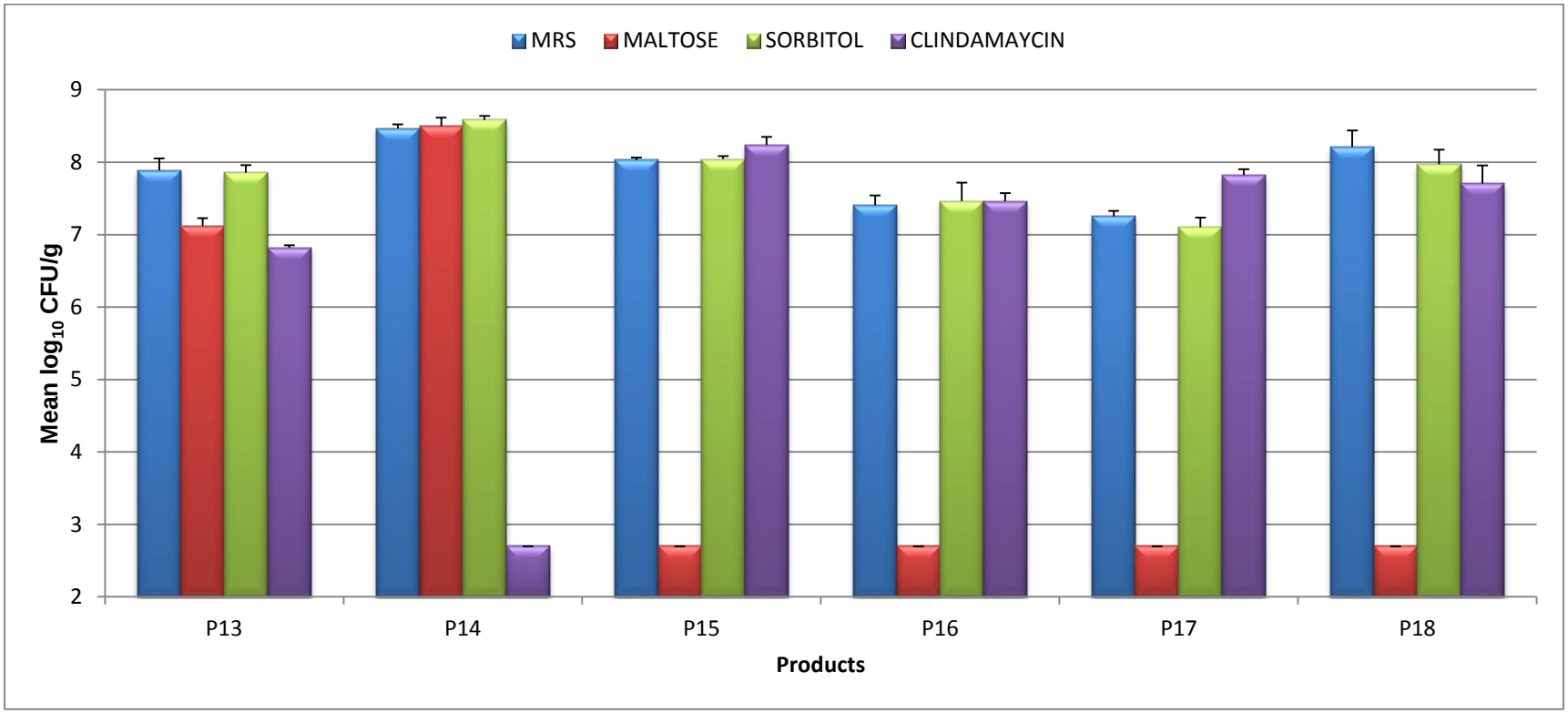


Figure 2.1 c The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on four different media (MRS, MRS-Maltose, MRS-Sorbitol, MRS-Clindamaycin)

Data are means ± SD (n=4)

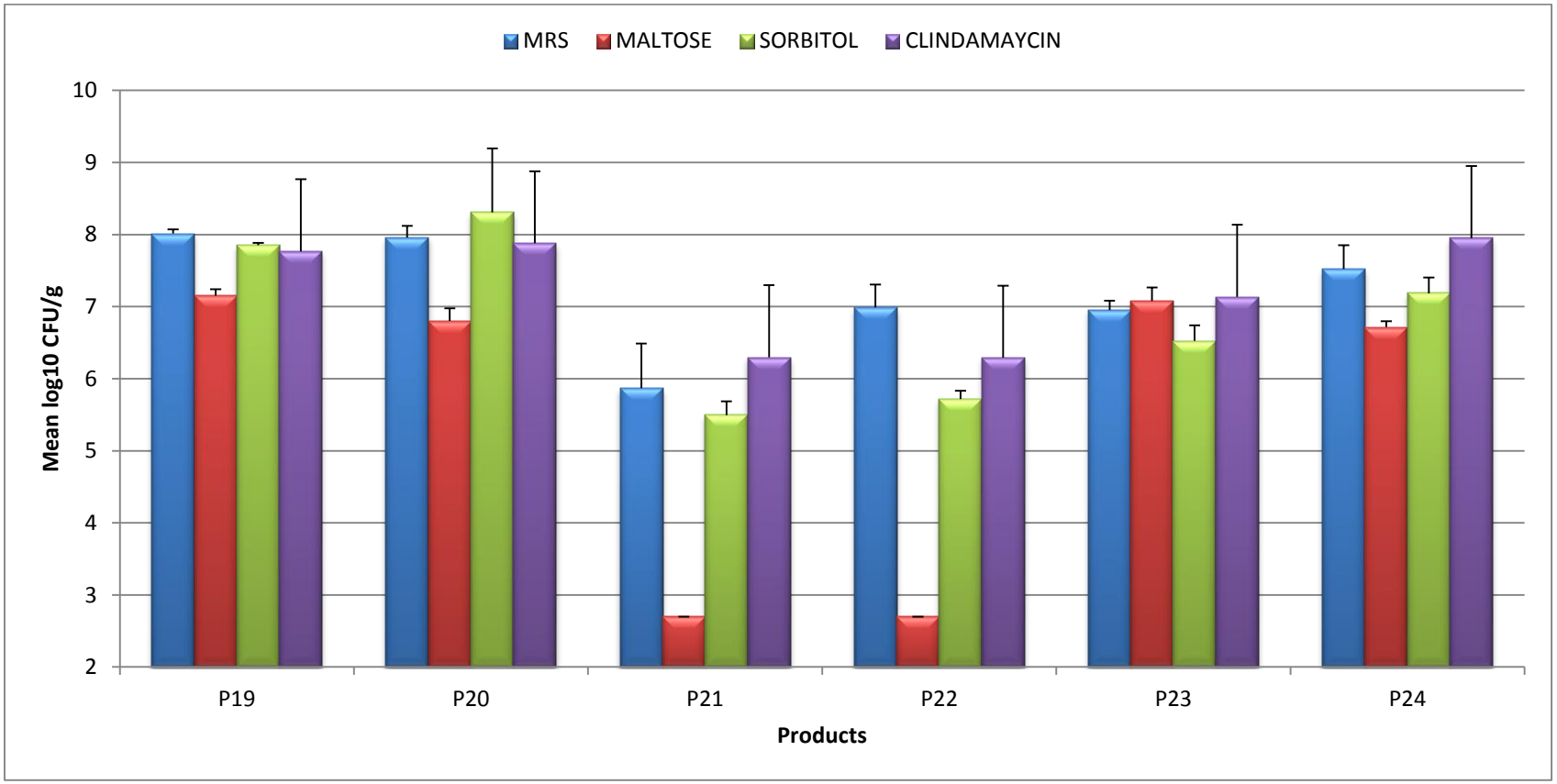


Figure 2.1 d The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on four different media (MRS, MRS-Maltose, MRS-Sorbitol, MRS-Clindamaycin)

Data are means ± SD (n=4)

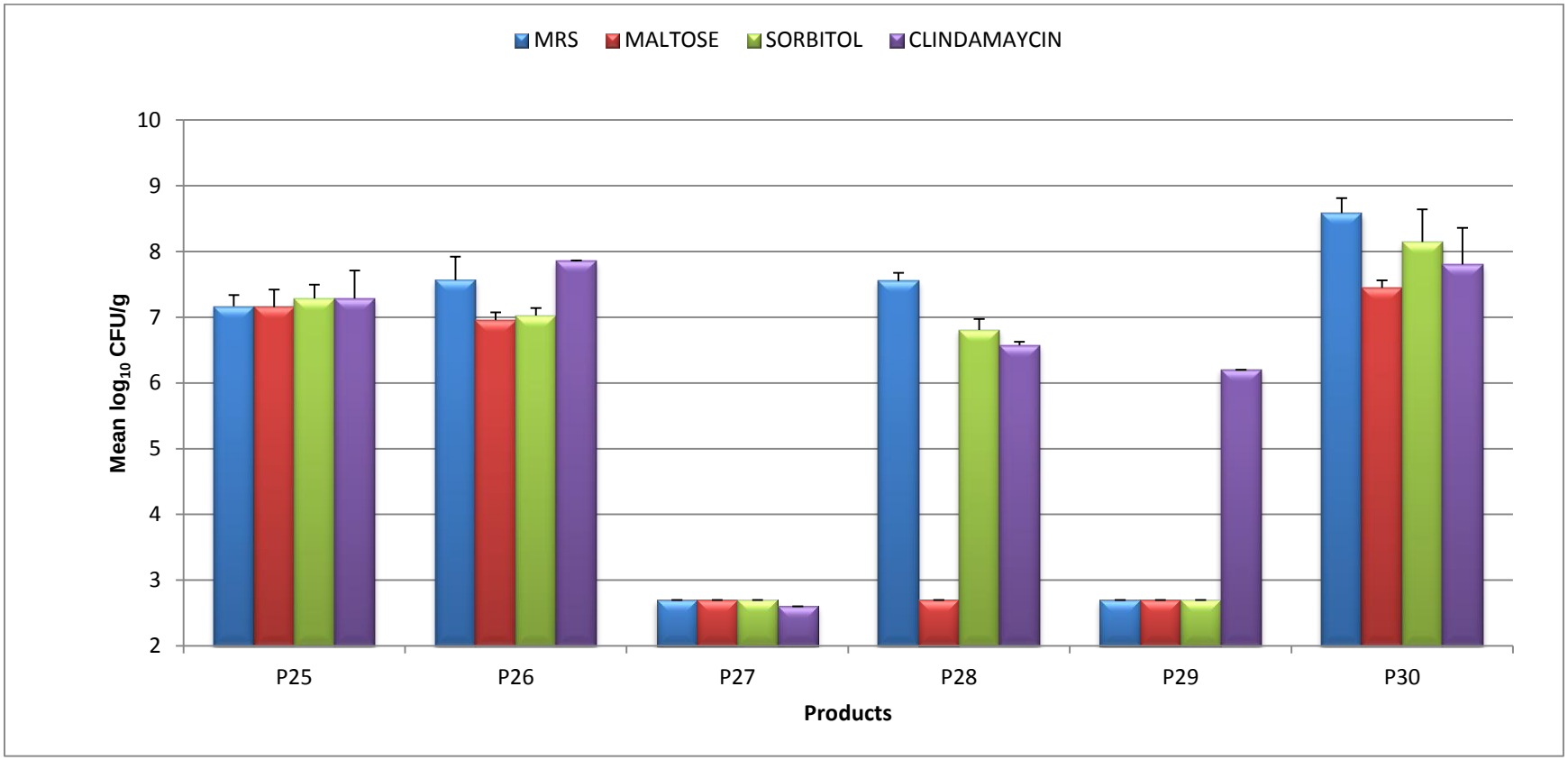


Figure 2.1 e The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on four different media (MRS, MRS-Maltose, MRS-Sorbitol, MRS-Clindamaycin)

Data are means ± SD (n=4)

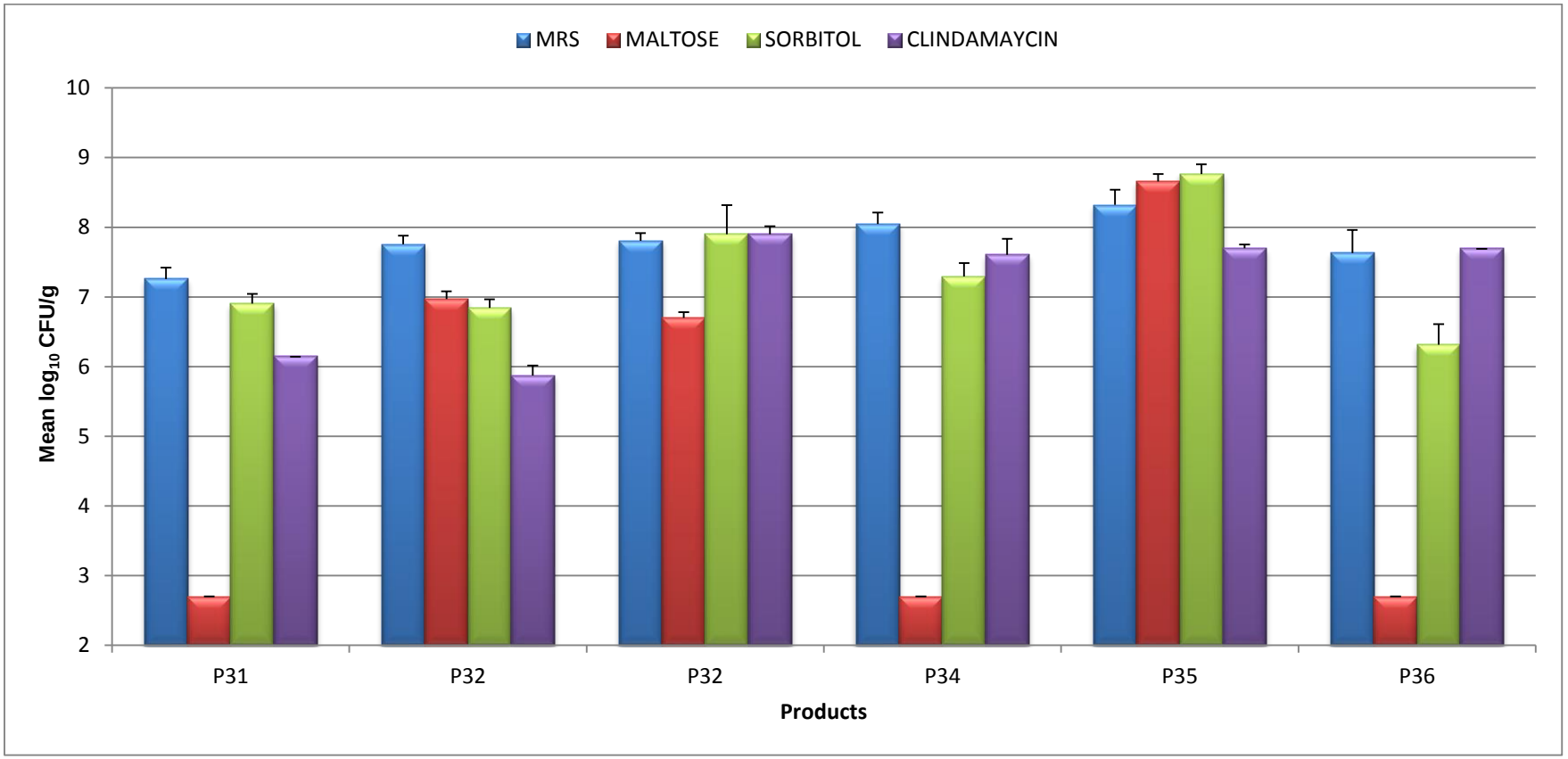


Figure 2.1 f The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on four different media (MRS, MRS Maltose, MRS-Sorbitol, MRS-Clindamaycin)

Data are means ± SD (n=4)

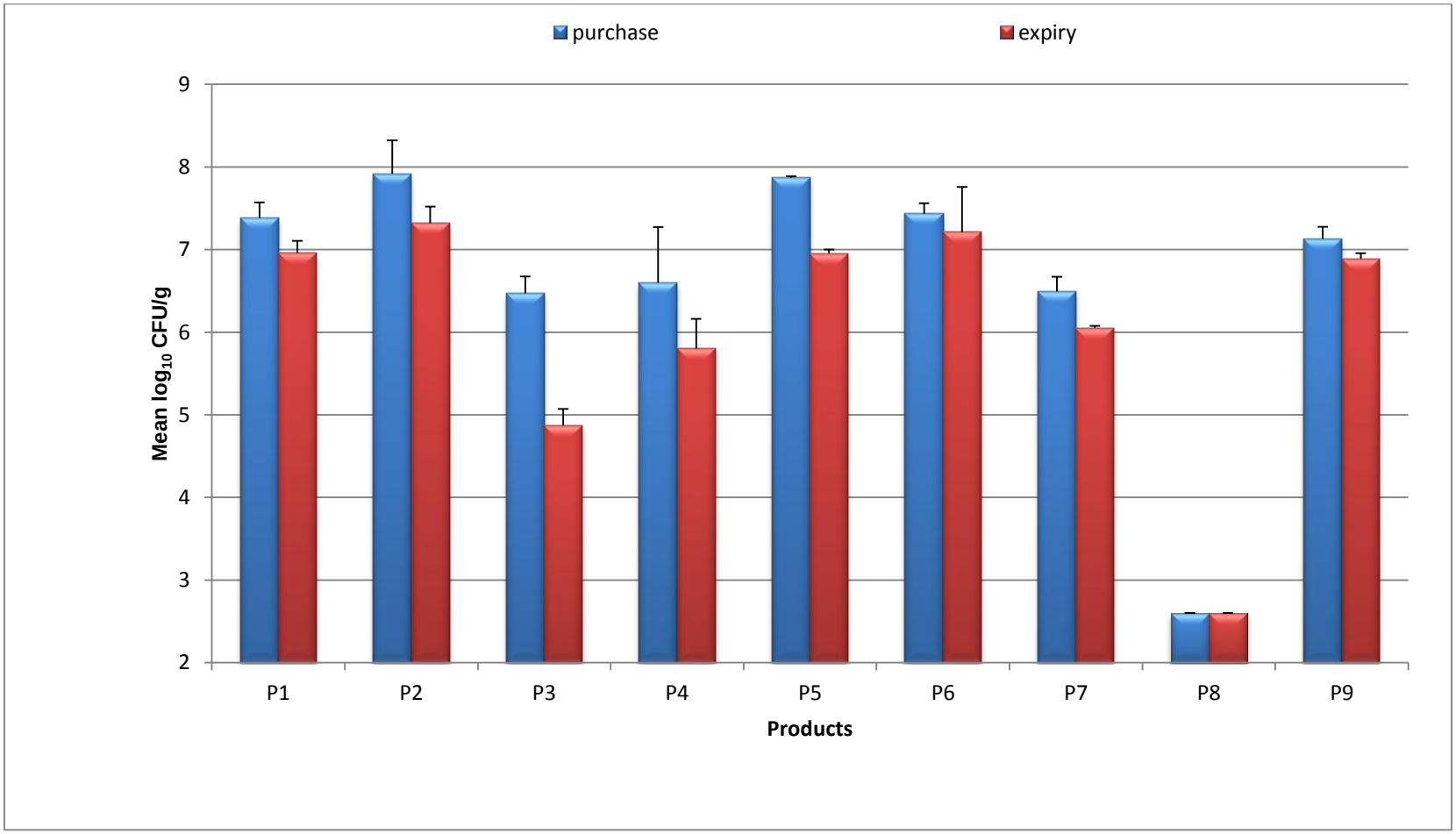


Figure 2.2 a The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on MRS-Clindamycin agar in tested products at the time of purchase and at the end of expiry date

Data are means ± SD (n=4)

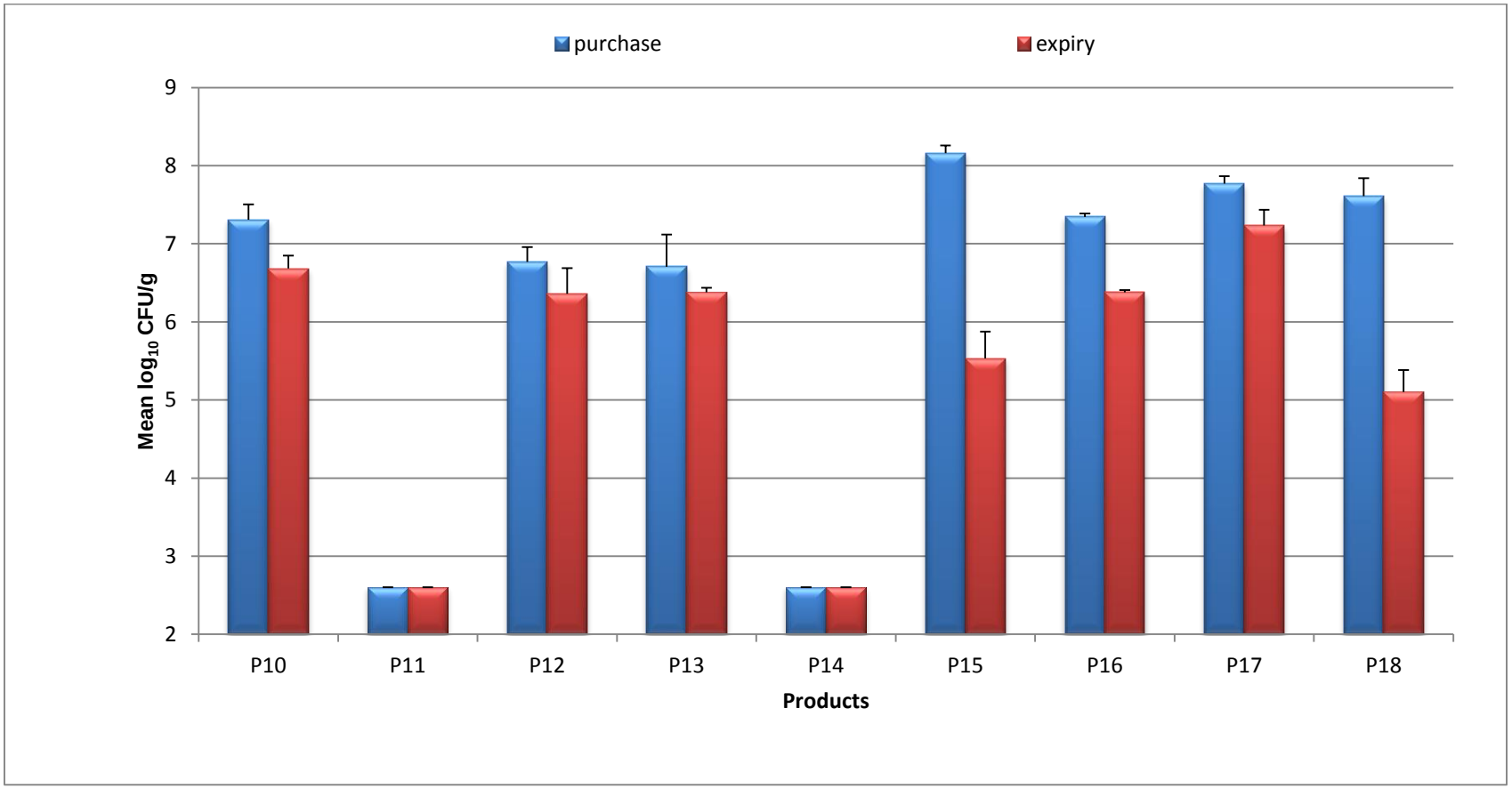


Figure 2.2 b The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on MRS-Clindamycin agar in tested products at the time of purchase and at the end of expiry date

Data are means ± SD (n=4)

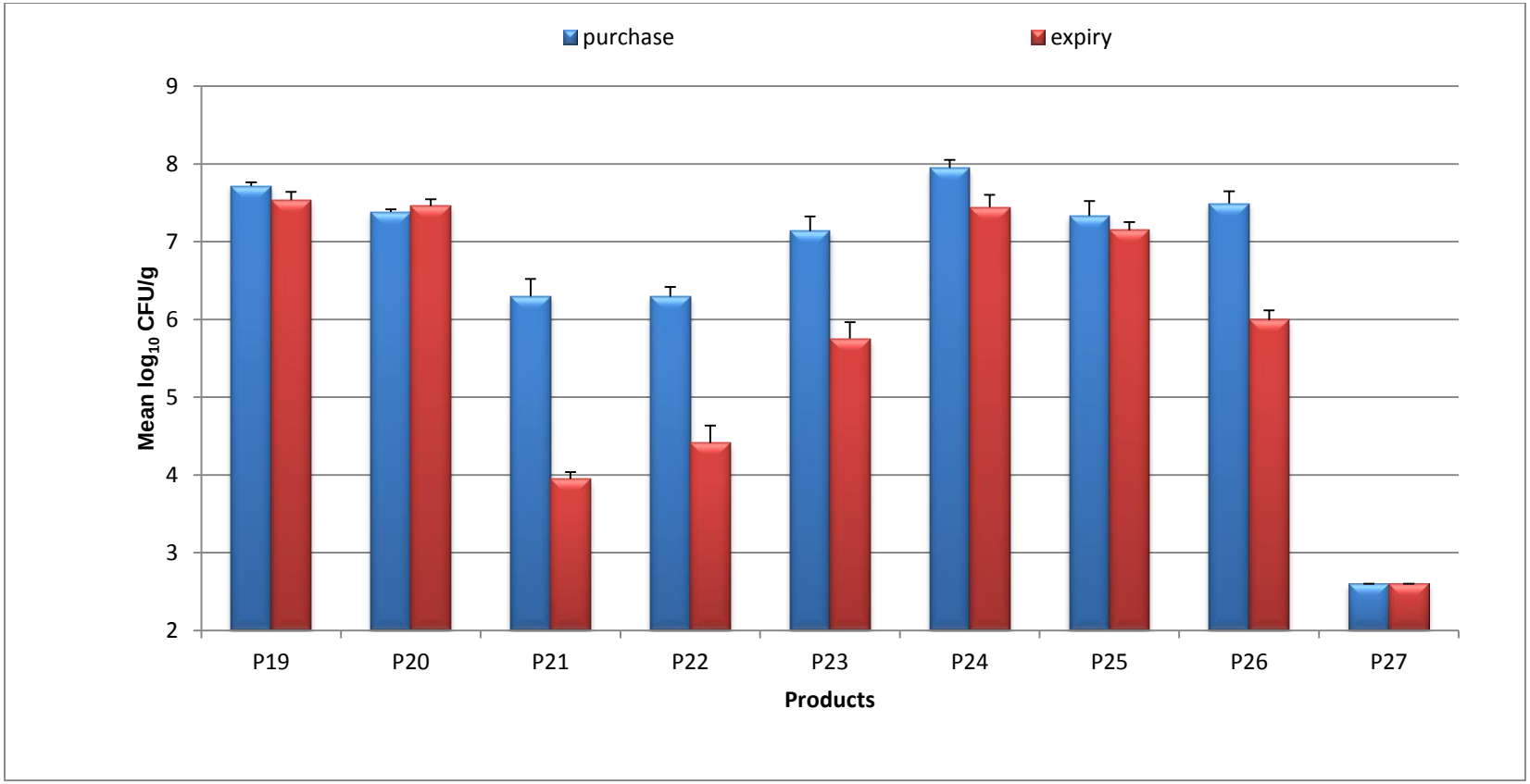


Figure 2.2 c The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on MRS-Clindamycin agar in tested products at the time of purchase and at the end of expiry date

Data are means ± SD (n=4)

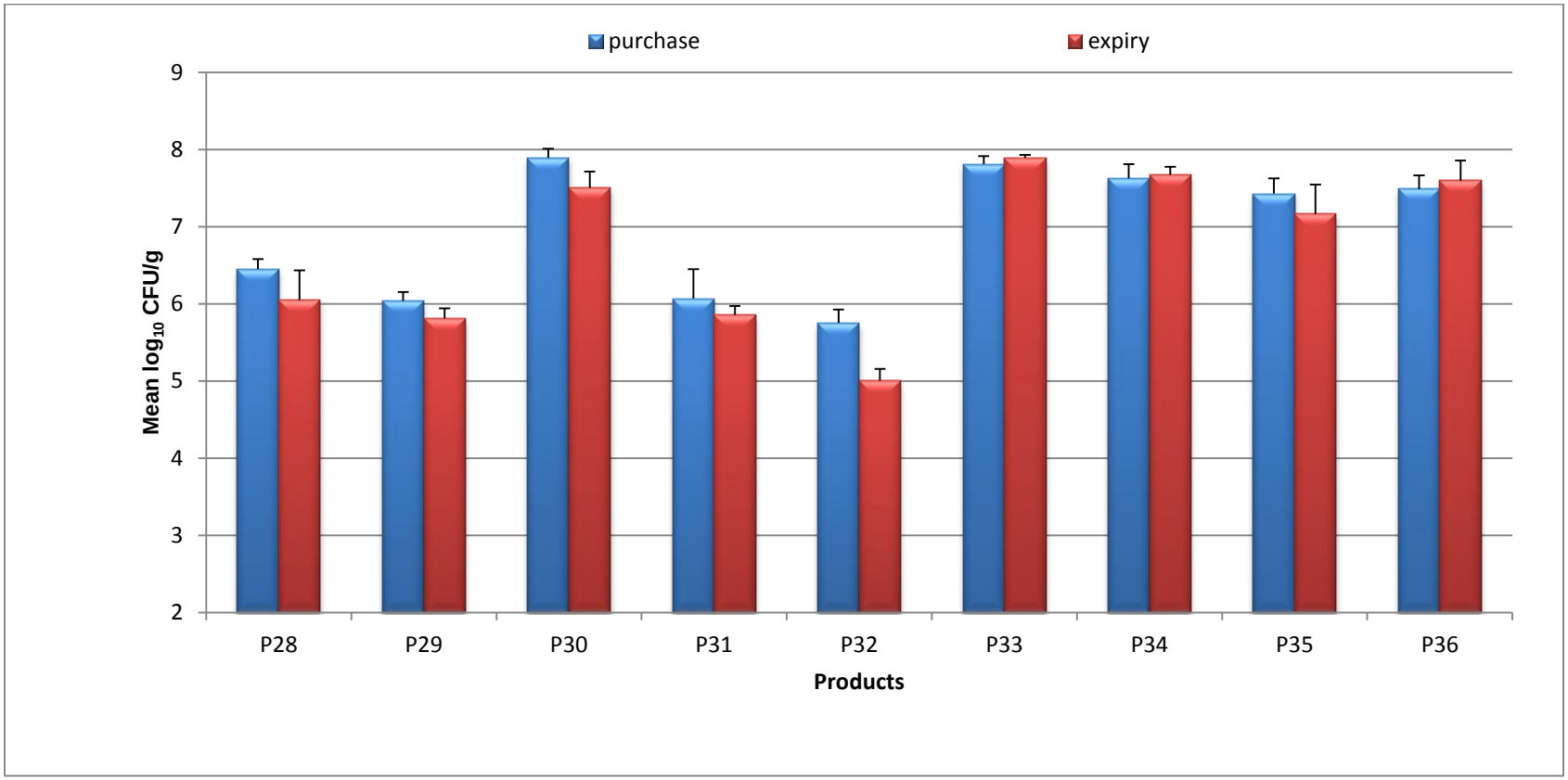


Figure 2.2 d The average viable counts of presumptive *Lactobacillus* spp. (log_{10} CFU/g) on MRS-Clindamycin agar in tested products at the time of purchase and at the end of expiry date

Data are means ± SD (n=4)

2.3.2 Changes in pH value during storage of samples

The pH of all samples declined during the shelf life (Table 2.1). The initial pH on the day of purchase ranged between 3.62 and 4.7 and at the end of shelf life ranged from 3.46 to 4.61.

2.3.3 Differentiation of isolates by rep-PCR

At the end of the enumeration procedure, with regard to shape and size of visible colonies on MRS-Clindamycin in section 2.2.1.2, a total of 85 isolates were isolated and purified. All selected isolates were categorized along with commercial and type strains of *Lactobacillus* by using rep-PCR. All tested isolates were categorized in 8 different groups A, B, C, D, E, F, G and H (Figure 2.3). Group A, as the major group, contained 51 isolates presenting the same DNA band profile. Others included 22 isolates in group B, six isolates in group C, five isolates in group D, four isolates in group E, two isolates in group H and one isolate in each group of F and G.

Sequencing of 16S rRNA and *rpoA* genes was applied for further identification of isolates. In total, 20 isolates were randomly selected from the above groups, and identified by partial sequencing of 16S rRNA and *rpoA* genes.

2.3.4 Identification of lactobacilli isolates recovered from fermented milk products by partial sequencing of 16S rRNA and *rpoA* genes

Random representatives of each group {A (6), B (3), C (4), D (2), E (2), F (1)} were analysed using the 16S rRNA gene and where needed examined further with *rpoA* gene sequencing. Table 2.2 presents the results of identification using 16S rRNA and *rpoA* genes sequencing of isolates.

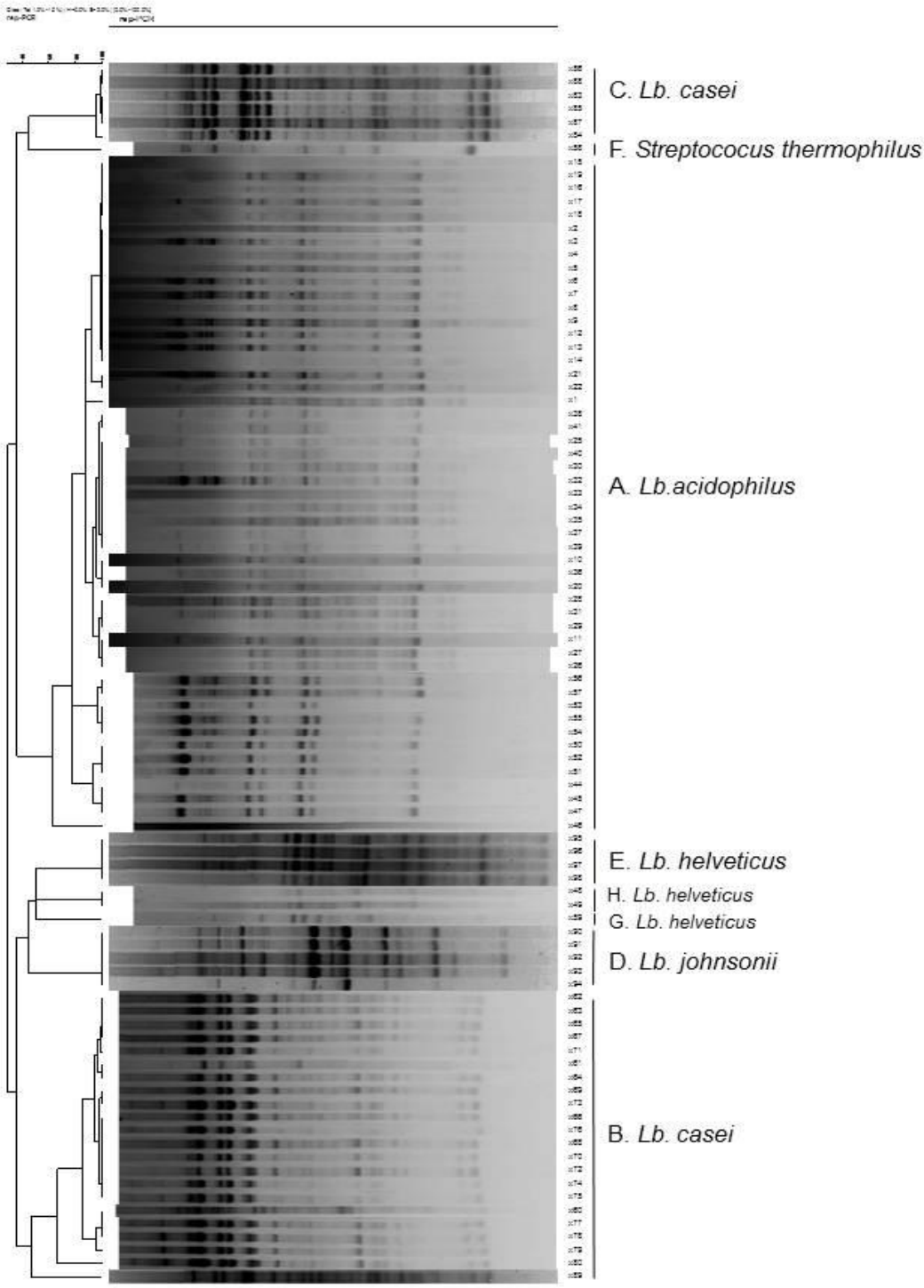


Figure 2.3 Dendrogram obtained by cluster analysis of rep-PCR fingerprints from isolated strains.

The six isolates selected as being representative of the 51 isolates in group A were identified as *Lb. acidophilus* (100% similarity to GenBank sequences) by 16S rRNA sequencing.

The identity of the isolates in group B was revealed as *Lb. casei/paracasei* (98%) by 16S rRNA gene sequences. As the 16S rRNA gene sequencing could not differentiate between *Lb. casei* and *Lb. paracasei*, sequencing of *rpoA* gene was used to discriminate the two species.

Similarly, isolates in group C were identified as *Lb. casei/paracasei* (99% similarity) by both, 16S rRNA and *rpoA* gene sequencing.

Isolates of group D, were identified as *Lb. Johnsonii* (100% similarity) by 16S rRNA gene sequencing. The isolates in group E were identified as *Lactobacillus helveticus*, *gallinarum*, *suntoryeus* (99% similarity) by both 16S rRNA and rpoA gene sequencing. Group F included one isolate and was identified as *S. thermophilus* (96% similarity) by 16S rRNA gene sequencing. Groups G and H were identified as *Lb. helveticus, gallinarum, suntoryeus* (99% similarity) by both 16S rRNA and *rpoA* gene sequencing.

Sequencing of *rpoA* genes in addition to sequencing of 16S rRNA did not discriminate between isolates in groups B, C, E, G and H (Table 2.2).

Due to failure of the 16s rRNA and *rpoA* genes sequencing to differentiate closely related species, the DNA banding profiles of unconfirmed isolates was compared with those of type strains and their identy confirmed according to their similarities to the type strains.

Table 2.2 Identification of probiotic lactobacilli isolated from commercial fermented milks by sequence analysis of 16S rRNA

Isolate code and group	Identification 16S rRNA sequencing	Similarity	Identification *rpoA* sequencing	Similarity
A-1	*Lactobacillus acidophilus*	100%	_____	_____
A-2	*Lactobacillus acidophilus*	100%	_____	_____
A-3	*Lactobacillus acidophilus*	97%	_____	_____
A-4	*Lactobacillus acidophilus*	100%	_____	_____
A-5	*Lactobacillus acidophilus*	99%	_____	_____
A-6	*Lactobacillus acidophilus*	99%	_____	_____
B-1	*Lactobacillus casei*	95%	*Lactobacillus casei*	99%
	Lactobacillus paracasei	95%	*Lactobacillus paracasei*	99%
B-2	*Lactobacillus casei*	100%	*Lactobacillus casei*	99%
	Lactobacillus paracasei	100%	*Lactobacillus paracasei*	99%
B-3	*Lactobacillus casei*	98%	*Lactobacillus casei*	100%
	Lactobacillus paracasei	98%	*Lactobacillus paracasei*	100%
C-1	*Lactobacillus casei*	99%	_____	_____
	Lactobacillus paracasei	99%		
C-2	*Lactobacillus casei*	99%	*Lactobacillus casei*	100%
	Lactobacillus paracasei	99%	*Lactobacillus paracasei*	100%
C-3	*Lactobacillus casei*	100%	*Lactobacillus casei*	100%
	Lactobacillus paracasei	100%	*Lactobacillus paracasei*	100%
C-4	*Lactobacillus casei*	99%	*Lactobacillus casei*	99%
	Lactobacillus paracasei	99%	*Lactobacillus paracasei*	99%
D-1	*Lactobacillus johnsonii*	100%	_____	_____
D-2	*Lactobacillus johnsonii*	100%	_____	_____
E-1	*Lactobacillus helveticus*	99%	*Lactobacillus helveticus*	99%
	Lactobacillus gallinarum	99%	*Lactobacillus gallinarum*	99%
	Lactobacillus suntoryeus	100%	*Lactobacillus suntoryeus*	100%
E-2	*Lactobacillus helveticus*	98%	*Lactobacillus helveticus*	98%
	Lactobacillus gallinarum	97%	*Lactobacillus gallinarum*	97%
	Lactobacillus suntoryeus	98%	*Lactobacillus suntoryeus*	98%
F-1	*Streptococcus thermophilus*	95%	_____	_____

Table 2.2 Continued

Isolate code and group	Identification 16S rRNA sequencing	Similarity	Identification *rpoA* sequencing	Similarity
G-1	*Lactobacillus helveticus*	95%	*Lactobacillus helveticus*	99%
	Lactobacillus gallinarum	95%	*Lactobacillus gallinarum*	98%
	Lactobacillus suntoryeus	95%	*Lactobacillus suntoryeus*	99%
H-1	*Lactobacillus helveticus*	95%	*Lactobacillus helveticus*	99%
	Lactobacillus gallinarum	95%	*Lactobacillus gallinarum*	98%
	Lactobacillus suntoryeus	95%	*Lactobacillus suntoryeus*	99%

2.4 DISCUSSION

The use of food as a carrier probiotic organism has attracted considerable attention among food manufacturers because of the claimed health-associated benefits. However, maintaining a large amount of viable probiotics in fermented milks is not easy, and a large quantity of probiotic culture should be added to the carrier food to compensate for the likely losses of probiotics during the shelflife (Wang *et al.* 2009). The recommended quantity of probiotic lactobacilli that needs to be consumed for a health benefit varies in different studies (Guarner *et al*. 2008). Information concerning the expected viable concentration of each probiotic present at the end of shelflife should be available to the consumer (Reid *et al*. 2001).

In order to ensure that the consumers benefit from commercial probiotic products, it is necessary to confirm the identity of the claimed organisms at species/strain level and that they are present in the product in appropriate numbers before consumption.

Although there are no universally established standards for microbial content and label claims on the labels of probiotic products, the manufacturers should at least clearly express the genus, species and strain of the probiotic microorganism and also the minimum viable count of each probiotic strain at the end of shelf-life (Guarner *et al.* 2008).

To have any health benefits and functional properties, it is suggested that at least 10^{6-7} CFU/g of live probiotic should be present in the product at the end of expiry date (Samona and Robinson 1994; Gueimonde *et al.* 2004; Wang *et al.* 2010). Current research findings show that at the time of purchase 86% of tested samples contained the minimum recommended quantity, but on the expiry date only 61% of tested products met the minimum therapeutic requirement.

Other researchers have also reported commercially probiotic dairy products with inadequate amounts of viable probiotic (Iwana *et al.* 1993; Schillinger 1999; Gilliland *et al.* 2002). The difference between actual included and counted number of probiotic lactobacilli may also be attributable to disruption of the cold chain (Nayra *et al.* 2002; Godward and Kailasapathy 2003), or inadequate proliferation of lactobacilli throughout processing due to shortage of probiotic culture enhancer (Perko *et al.* 2002). In this study during cold storage, the number of *Lactobacillus* spp. in eight samples decreased considerably more than in others. The most important contributing factors for loss of cell viability are decreasing pH during product storage, presence of dissolved oxygen and also presence of preservatives in final products (Vinderola *et al.* 2002a). In this study, the pH decline between the purchase and expiry date was in some cases

considerable. It could be due to continued fermentation process by LAB even in low temperatures known as post acidification.

The presence of dissolved oxygen might be the other important reason for drop in viability of cell count in fermented milk (Dave and Shah 1997; Shah 2000). The majority of tested products in this study were stirred yogurts, in which air could have been incorporated when the yogurt was mixed with the fruit compote. Also, some of the commercial fruit products contain preservative agents to control contamination and this might affect the viability of the probiotic cells (Kailasapathy *et al.* 2008).

Based on these results, which are in line with those of Jayamanne and Adams (2006), higher numbers of probiotic would be ingested if probiotic fermented products are consumed earlier than their expiry date. The viability of the bifidobacteria in bio-yoghurts available in the UK market was decreased during storage at 4°C. On expiry (3 weeks) only five products retained viability $>10^6$ CFU/g.

It was found that MRS-Maltose is not a good choice for selective enumeration of lactobacilli; but, MRS-Clindamycin and MRS-Sorbitol media are both reliable in their ability to recover accurately numbers of *Lb*. *acidophilus* and *Lb*. *casei* in fermented dairy products. This study reports that MRS-Sorbitol exhibits higher viable count than MRS-Clindamycin, suggesting that MRS-Sorbitol might allow the growth of additional LAB. In other words, MRS-Sorbitol agar could not be used for selective enumeration of *Lb. casei* and *Lb. acidophilus* in products containing both these bacteria (Shah 2000). MRS-Clindamycin has been proposed to enumerate lactobacilli in different studies (Van de Casteele *et al*.

2006). Furthermore, the International Organization for Standardization (ISO 2006) has recommended MRS-Clindamycin agar for the enumeration of *Lb. acidophilus* in dairy products where *Lb. acidophilus* strains are present along with other LAB and bifidobacteria. Additionally, another study reportes that MRS-Clindamycin could be used for the selective enumeration of *Lb. acidophilus* in yogurt-related milk products containing a mixed microflora of lactobacilli, streptococci and bifidobacteria (Ashraf and Shah 2011). MRS-Clindamycin has the advantage of being simple (only one composite antibiotic supplement) and easier to prepare. However, it has proved not to be a perfect selective medium as other LAB, such as *S. thermophilus* (difficult to different from lactobacilli based on colony morphology) are able to grow on the medium.

Oberg *et al*. (2011) reported that although MRS-Sorbitol is a medium designed for *Lb. acidophilus* in which sorbitol is the sole sugar constituent and, *Lb. casei* can grow on the medium only at elevated incubation temperature (42 °C). At this temperature, the MRS-Sorbitol medium gave higher bacterial counts compared to the *Lb. casei* specific medium (LC agar), indicating that it could be used to obtain the total LAB count at different temperature. However, in this study colonies of both *Lb. acidophilus* and *Lb. casei* were recovered on MRS-Sorbitol agar.

Some of the tested products in this study presented inadequate information on their labels. Microbial investigations of probiotic products have indicated that the number and identity of recovered species do not always correspond to those stated on the labels of products (Hamilton-Miller and Shah 2002; Temmerman *et al*. 2003).

Identification of probiotic species used in carrier products is considered an important issue, which should be verified in support of health benefits claimed for the products. To obtain accurate and reliable identification of the probiotic species, molecular techniques should be applied. DNA profiling by PCR based methods are the best applicable means for identification of probiotic bacteria to strain level (Gueimonde *et al*. 2004; Tabasco *et al.* 2007). Many mis-identifications of probiotic microorganisms maybe due to the use of only phenotypic methods for taxonomic characterization (Ouwehand *et al*. 2006).

The rep-PCR fingerprinting profile revealed relative genetic differences between the tested isolates. Rep-PCR genomic fingerprinting is able to differentiate bacteria at species, subspecies and even strain level. In addition, many repetitive sequence primers are available, and a different primer could generate different band patterns, which would assist identification (Rademaker *et al.* 2005).

In this study, 85 isolates from fermented milks were grouped based on their DNA patterns by rep-PCR, and 20 isolates out of 85 were selected for identification by sequence analysis of 16S rRNA. Amplification of the 16S rRNA gene often provides a rapid and reliable tool for bacterial identification without the need for phenotypic characterization. However, 16S rRNA sequencing cannot discriminate closely related species. Thus, sequencing of alternative genes such as *rpoA* with more reliability has been proposed (Koo *et al*. 2003; Santos and Ochman 2004; Naser *et al.* 2007).

However, in this research, amplification and sequencing of the *rpoA* gene could not provide enhanced discriminatory information for identifying tested isolates

compared to use of 16S rRNA gene sequences. Sequencing of other genes such as *rpoB* and *pheS* with more discriminatory potential to differentiate strains with close genetic profiles could be more interesting. Anyogu *et al*. (2014) stated that sequencing of the *pheS*, *rpoA* and *rpoB* genes along with 16S rRNA gene sequencing provide a better identification of LAB and *Bacillus* isolates.

The results of identification revealed that *Lb. acidophilus* and *Lb. casei* were the most frequently recovered species from the fermented milks.

2.5 CONCLUSION

Out of 20 identified isolates of probiotic *Lactobacillus*, six were selected for screening for functional properties. These isolates were carefully chosen to include all major brands of probiotic products. These isolates along with two commercial cultures of *Lactobacillus* from Chr. Hansen (La5 and C431) and also three different type strains from National Collection of Industrial Food and Marine Bacteria (NCIMB) (*Lb. casei* subsp. *casei*, *Lb. paracasei* subsp. *paracasei* and *Lb. acidophilus*) were used throughout this study.

The potential of MRS-Maltose, MRS-Sorbitol and MRS-Clindamycin as selective medium for isolation of *Lactobacillus* spp. from commercial fermented milks was examined. It was concluded that MRS-Maltose was not a good choice for selective enumeration of lactobaciili, MRS-Sorbitol might allow the growth of additional LAB regardless of the intended lactobacilli and at last MRS-Clindamycin not to be perfect selective medium as *S. thermophilus* was able to grow on it and it is relatively difficult to differentiate from colonies of lactobacilli.

However, MRS-Clindamycin has the advantage of being simple (only one composite antibiotic supplement) and easier to prepare.

The total viable count of *Lactobacillus* spp. in most tested products remained over 10^6 CFU/g at the end of shelflife. This could mainly be due to technical processing and strain selection improvement. Also, most *Lactobacillus* spp. were isolated from commercial fermented milks were identified as *Lb. acidophilus, Lb. casei* and *Lb. paracasei* by analysis of partial sequences of the 16S rRNA and *rpoA* genes and discrimination by rep-PCR.

CHAPTER THREE: *IN VITRO* ASSESSMENT OF *LACTOBACILLUS* SPP. FOR PHYSIOLOGICAL PROPERTIES: TOLERANCE TO ACID, BILE SALT AND ENZYMES

(Criteria for selection: Resistance to upper digestive tract conditions)

3.1 INTRODUCTION

In order to sustain certain organisms, such as probiotics, some requirements need to be fulfilled; viability and survival of probiotic bacteria are the most important parameters to provide therapeutic functions. Probiotic lactobacilli need to survive during the manufacturing process of food and then in the upper GIT.

A number of factors have been claimed to affect the viability of probiotic bacteria in dairy foods, such as yogurt and fermented milks, including low pH and refrigerated storage (Dave and Shah 1998; Shah 2000). Moreover, the resistance to human gastric transit constitutes an important selection criterion for probiotic bacteria (Goldin and Gorbach 1989). Microorganisms ingested with food begin their journey to the lower intestinal tract via the mouth and are exposed to stress factors that influence their survival.

Resistance to gastric acid and physiological concentrations of bile is one of the *invitro* tests that have been frequently suggested for the evaluation of the probiotic potential of *Lactobacillus* spp. (Vijendra and Prasad 2005; Mathara *et al*. 2008).

3.1.1 Effect of pH on viability of lactobacilli

Lactic acid production is an important index of adaptation of LAB, which secrete lactic acid as end-product of lactose fermentation. The viability of *Lactobacillus* spp. depends on a number of factors (Marteau *et al*. 1997). Acidity is believed to be the most detrimental factor affecting the viability of lactobacilli, because their growth is reduced considerably below pH 4.5 (Lankaputra and Shah 1995).

The time reported from entrance to release of food from the stomach is about 2-4 h (Berrada *et al*. 1991), but dwell time in the stomach depends on the nature of food, e.g. fatty food may lead to longer residence times. Cellular stress begins in the stomach. Depending on whether the subject is in a fasting or fed state, the pH of gastric juice is about 0.9, but the presence of food in the stomach raises the pH value to the level of pH 3 (Vandamme *et al*. 2002).

So, food matrix would buffer probiotic *Lactobacillus* spp. to facilitate survival at the pH of the stomach (Prasad *et al*. 1998). Among different foods, milk and dairy products play a critical role in the survival of probiotic lactobacilli through the GIT and in the colonization of intestine (Ritter *et al*. 2009).

It has been demonstrated that milk proteins are able to neutralize the harmful effects of different substances on bacterial growth (Boyaval *et al*. 1995; Kim and Liu 2002). Rodriguez *et al*. (2012) found that selected lactobacilli isolates in their work had good viability during the simulated GIT digestion, with reductions of 0.87– 2.89 $\log_{10}$ units. They concluded that *Lb. paracasei* INIA P272 and *Lb. rhamnosus* INIA P344 were the most resistant to simulated gastric digestion and best adhesive bacteria during the *in vitro* test. Corsetti *et al.* (2008) evaluated the resistance of the *Lb. casei* strains isolated from Pecorino cheese to gastric acidity (pH 2.0) and bile salts (0.5% w/v bovine bile) in simulated gastric and intestinal fluid, respectively. *Lactobacillus casei* strains were highly susceptible to low pH and their viability decreased after the exposure to simulated gastric fluid with an average reduction of 4.42 $\log_{10}$ CFU/ml. In other reported study, *Lb. acidophilus* La5 reduced only 1 og_{10} cycle after 2 h of exposure to pH 2, but were entirely destroyed after 1 h at pH 1 (Favaro and Grosso 2002). Pan *et al.* (2009) observed that with an increased incubation

time, the count of viable lactobacilli was decreased. They evaluated the resistance of *Lb. acidophilus* NIT to pH 2–4 and 1–3% bile. In their study, at the lowest pH of 2, the counts of lactobacilli were reduced to an undetectable level after 2 h. The lactobacilli survival at pH 3 was higher than pH 2, and after 3 h interaction, 10% viable count of *Lb. acidophilus* NIT was achieved. A higher survival of these bacteria (70%) was seen after 3h exposure at pH 4.

3.1.2 Effect of bile on viability of lactobacilli

In accordance with guidelines for the evaluation of probiotics described by WHO experts (FAO/WHO 2006), bile salt hydrolase activity is a property to be considered in the screening of potential probiotic organisms. The expression of bile salt hydrolase has been proposed as a protective system to allow the potential probiotic bacteria to survive and colonize the intestine after oral consumption (Parvez *et al*. 2006).

Cholesterol is the main component of primary bile salts, which are formed in the liver and are accumulated as conjugated bile salts in the gall bladder (Corzo and Gilliland 1999). Conjugated bile salts are released into the duodenum after ingestion of fatty meals and improve the emulsification of lipids as well as the absorption of lipid nutrients. They are in the form of N-acyl compounds conjugated with glycine or taurine (Hofmann and Mysels 1992).

Bile secretion is one of the important pathways of eliminating serum cholesterol (Turley and Dietschy 1988). About 97% of conjugated bile salts are reabsorbed to the liver from the duodenum (MacDonald *et al*. 1983).

Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes. Bile is able to affect the phospholipids and proteins of cell

membranes and disrupt cellular homeostasis, as well as disturbing macromolecule stability (Begley *et al*. 2005).

It has been shown that some lactobacilli could lower total plasma cholesterol and low-density lipoprotein (LDL) cholesterol (De Smet *et al*. 1994; Ahn *et al*. 2000).

The study by Ahn *et al.* (2000) revealed that serum cholesterol levels of the volunteers decreased significantly after the intake of acidophilus milk, containing *Lb. acidophilus* SNUL01 for 4 weeks. This hypocholesterolaemic effect of acidophilus milk has been attributed to the deconjugation of bile salts by *Lb. acidophilus* SNUL01.

Some probiotics secrete bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), the enzyme that catalyzes the hydrolysis of glycine and taurine conjugated bile salts into amino acid residues and free bile salts (bile acids) (Liong and Shah 2005). Free bile salts are less soluble than conjugated bile salts, resulting in lower absorption in the GIT, and therefore are lost in faeces (Center 1993). Bile salt hydrolysis was reported in *Lactobacillus* spp., *Bifidobacterium longum*, *Clostridium perfringens* and *Bacteroides fragilis* subsp. *fragilis* (Corzo and Gilliland 1999).

The solubility and emulsifying capacity of conjugated bile salts decrease when they are deconjugated in the intestine (Hofmann and Mysels 1992; Tannock 1995).

Gilliland *et al*. (1984) considered 0.3% bile salts as a critical concentration to screen for resistant strains. Goldin and Gorbach (1992) stated that the same

level should be critical when selecting human probiotics.

Moreover, different bile salt hydrolase activities have been reported for different strains of the same bacterial species under similar pH values (Lunden and Savage 1990).

3.1.3 Bile salt deconjugation properties of lactobacilli

It is necessary to examine the bile salt deconjugation properties and bile salt hydrolase activities of *Lactobacillus* strains, before further usage in *in vivo* studies. Unconjugated bile salts are considered more toxic than conjugated forms (Vinderola and Reinheimer 2003), and glycoconjugates are more toxic towards probiotic bacteria than their tauroconjugates counterparts (Noriega *et al*. 2006) and the toxicity may be attributed to the different dissociation constants (De Smet *et al*. 1995). Differences in sensitivity among strains could be related to the presence of bile salt hydrolases with preference to glycoconjugated bile acids, which are a common trait in intestinal isolates (Begley *et al*. 2006).

However, acid resistance and bile tolerance of *Lactobacillus* spp. are considered as the main criteria for selection as probiotics and these characteristics should be examined by *in vitro* and *in vivo* methods.

One useful tool in the selection of a probiotic strain would be *in vitro* tests to determine the survivability of bacteria in the upper gastrointestinal conditions.

The aim of this experiment was to determinate the survival of *Lactobacillus* isolates in simulated gastric juice and intestinal conditions. The specific objectives included:

a) *In vitro* assessment of the resistance of the isolates to various pH level (i.e. 2.0, 3.0 and 4.0).
b) *In vitro* assessment of the resistance of the isolates to the different concentration of bile salts.
c) *In vitro* assessment of protective role of gastric enzymes for the isolates.
d) Comparing the isolates to type strains and commercial cultures for the above.

3.2 MATERIALS AND METHODS

3.2.1 Acid resistance

An acid resistance study of 11 *Lactobacillus* strains was carried out in normal Phosphate Buffered Saline (PBS) (pH 7.4) (as control) and PBS with the pH adjusted to 4, 3 and 2 using 2M hydrochloric acid.

Strains tested for this experiment were as follows (Table 3.1):

a) Two commercial cultures of *Lactobacillus* spp. kindly provided by starter culture supplier, Chr. Hansen (Lb1 and Lb2).

b) Three different lactobacili type strains purchased from NCIMB (National Collection of Industrial Food and Marine Bacteria, Aberdeen, UK) (Lb3, Lb4 and Lb5).

c) Six *Lactobacillus* spp. which were isolated from yogurt and other fermented milk products (Lb6-Lb11).

Acid tolerance was determined with and without addition of 3 g/l of pepsin (P7000, Sigma, UK) to the PBS to evaluate any protective or damaging effect of pepsin at low pH.

Acid resistance test was performed according to the method of Prasad *et al*. (1998). After two consecutive streaking of cultures, individual colonies from MRS (De Man, Rogosa and Sharp agar) (CM0361, Oxoid Ltd., UK) were inoculated into 1 ml of MRD (Maximum Recovery Diluent) (CM0733, Oxoid Ltd., UK) until a heavy microbial suspension was obtained and this suspension was diluted in 5 ml of sterile PBS. The final concentration of cells was adjusted to 0.5 MacFarland solutions using a calibrated sensititrenephelometer (Trek, diagnostic systems Ltd., East Grinstead, UK). This microbial suspension was used as an inoculum for the further experiments. An aliquot of each culture (1 ml) was inserted into control and experimental tubes (9 ml) and were incubated at 37 ^{o}C for 0, 0.5, 1, 1.5, 2 and 3 h under anaerobic conditions. The resistance to acid was measured by viable cells counts on MRS agar and expressed as $\log_{10}$ CFU/ml.

Table 3.1 Organisms and their origin used for the acid and bile tolerance

Code	Bacteria	Origin
Lb1	*Lactobacillus acidophilus* (La5)	Pure culture from Chr. Hansen
Lb2	*Lactobacillus casei* (C431)	Pure culture from Chr. Hansen
Lb3	*Lactobacillus acidophilus* 701748	NCIMB
Lb4	*Lactobacillus casei* 11970	NCIMB
Lb5	*Lactobacillus paracasei* 700151	NCIMB
Lb6	*Lactobacillus johnsonii*	Isolated from goat yogurt
Lb7	*Lactobacillus acidophilus*	Isolated from cereal yogurt
Lb8	*Lactobacillus casei*	Isolated from drinking yogurt
Lb9	*Lactobacillus casei*	Isolated from drinking yogurt
Lb10	*Lactobacillus helveticus*	Isolated from kefir
Lb11	*Lactobacillus helveticus*	Isolated from fruit yogurt

3.2.2 *In vitro* assessment of bile tolerance

Bile salt tolerance of the isolates was studied in PBS (control) and PBS containing 0.5, 1, 1.5 and 2% w/v bile salt (B3883, Ox-bile, Sigma UK), which represented the physiological condition of small intestine (Collado and Sanz 2007). The tubes were incubated at 37 ^{o}C for 0, 0.5, 1 and 2 h to mimic the transit time in the small intestine as described by Prasad *et al.* (1998). All bacterial inocula were prepared as described in section 3.2.1 and inoculated

into control tube (free of bile) (PBS) and experimental tubes (with addition of bile salts). Viable cells were enumerated on MRS agar after 48 h incubation at 37 ^{o}C in an anaerobic cabinet and expressed as $\log_{10}$ CFU/ml.

3.2.3 Deconjugation of bile salts

Deconjugation of bile salt by tested strains was examined through the plate assay of Ahn *et al*. (2003). TCA–MRS agar and GCA-MRS plates were prepared by adding 1 mmol sodium salts of TCA (taurochenodeoxycholic acid) and GCA (glycochenodeoxycholic acid) (Sigma, UK) to MRS agar, respectively. All plates were streaked using an overnight culture, and were incubated in the anaerobic cabinet at 37 ^{o}C for 48 h. Subsequently, precipitate halos around colonies were considered to be deconjugation products of bile salts.

3.2.4 Effects of sequential treatment with acid, pepsin, pancreatin and bile salts on the viability of the tested bacteria

In order to study the resistance of the isolates in a condition similar to the upper GIT conditions, the tolerance of all strains 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 experiment, a batch fermentation system (Figures 3.1 and 3.2) was used. The system consisted of sterile, magnetically stirred, water jacketed batch fermentation vessels (300ml, Soham Scientific, Ely, UK). The temperature was maintained at 37 °C by means of a circulating water bath (type GD 120, Grant, Shepreth, UK), and pH was screened in each vessel using a pH meter (FerMac 260, Electrolab, Tewkesbury, UK). The system was set to maintain the required pH level in 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 throughout. Fermentations were run over a period of 9.25 h and samples were obtained at the start of the experiment and then at the end of the transit/incubation time through each section (Table 3.2).

The conditions in the vessels were set according to the method described by Gbassi *et al*. (2011). Four regions of the upper GIT with their specific pH and transit (incubation) time were studied. The conditions of GIT were simulated as shown in Table 3.2.

Figure 3.1 A single batch fermentation system used for studying the resistance to bile and acid

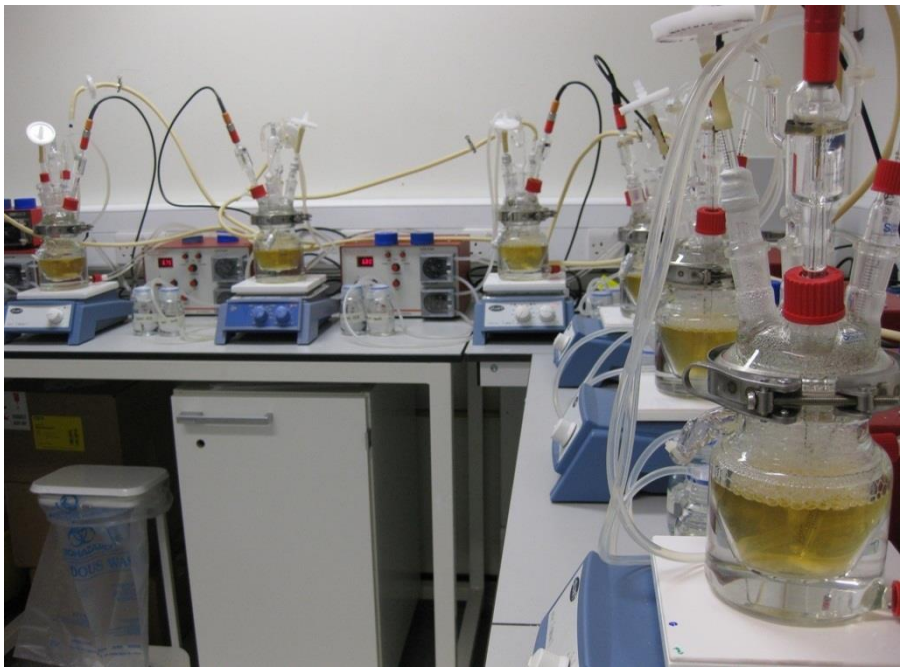


Figure 3.2 The series of batch fermentation system used for studying the resistance to bile and acid

Table 3.2 *In vitro* experimental condition in the GIT model

	Stomach	**Duodenum**	**Jejunum**	**Ileum**
Incubation/ transit time	2 h	0.25 h	3 h	4 h
pH	3.0	6.0	7.0	8.0

Source: 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 the control vessel, the pH was adjusted to the stomach pH values (3.0) using 2 M hydrochloric acid in all other vessels. The inoculum suspension (%1 v/v) and pepsin (3 g/l) were added to the gastric fluid at the beginning of the stomach transit/incubation time. After 2 h, the pH was slowly increased to the values of duodenum (6.0) with 2 M sodium hydroxide (Fisher Scientific, Loughborough, UK). Pancreatin (10 g/l)

(P7545, Sigma, UK) and bile salts (3 g/l) were also added at the beginning of the duodenum incubation. To evaluate any protective effect of pancreatin in the duodenum, these experiments were carried out with and without addition of 10 g/l of pancreatin to simulated gastric juice.

After 15 min duodenum incubation, pH values were changed to 7.0 to simulate the jejunum compartment and held for 3 h. These conditions were followed by those of the ileum (pH = 8.0) and the incubation continued for 4 h.

Sampling of gastric and intestinal fluids was carried out at the end of the average transit (incubation) time in each region of the artificial duodenum. One ml of each sample was suspended in 9 ml of MRD, the uniform suspension was decimally diluted. Twenty five µl of each dilution was spread on to quartered MRS plates in duplicate. The plates were incubated at 37 °C for 48 h anaerobically. The colony forming units (CFU) of the sample were calculated and $\log_{10}$ CFU/ml was reported.

3.2.5 Statistical analysis

Results are the mean±standard deviation. Data were analysed using the independent-samples t-test was performed using SPSS 21 software (Chicago, IL: SPSS Inc) to determine the statistical significance of differences. Data were considered significantly different when $p < 0.05$.

3.3 RESULTS

3.3.1 Resistance to acid

Different pH values in this study were selected to represent the pH range of the empty and full human stomach. The strains studied showed very poor survival at pH 2 with or without pepsin. At pH 2 (the lowest), the number of cells dropped from the inoculated level of 6-7 $\log_{10}$ CFU/ml to an undetectable level (<2.6 $\log_{10}$ CFU/g) after only 0.5 h for Lb5, Lb8, Lb9 and Lb10, 1 h for Lb3, Lb6 and Lb11 and 1.5 h for Lb1 and Lb4. Only isolate Lb7 showed good survival up to 1.5 h (Figure 3.3). Eight out of eleven tested strains lost their viability during the first hour of acid exposure and some degree of protective effects was seen in the presence of pepsin at pH 2 (Figure 3.4).

At pH 3, the survival of the isolates was generally greater than at pH 2 at all tested sampling intervals, with the exception of Lb5, Lb6 and Lb8 which were undetectable (<2.6 $\log_{10}$ CFU/g) after 1.5 h at this pH. Other tested strains showed some degree of resistance even after 3 h (Figure 3.5). The best resistance was observed with Lb1, Lb9 and Lb10 where the reduction was slightly more than one $\log_{10}$. An interesting observation was that the presence of 0.3% pepsin had a considerable effect on the survival at pH 3 (Figure 3.6), where all tested strains showed a steady population, very similar to their initial counts.

When the pH was increased to 4, a high survival rate was observed even in the absence of pepsin. As Figure 3.7 shows, the viability of strains Lb6, Lb7, Lb8, Lb9 and Lb10 were maintained the highest throughout 3 h of exposure to pH 4.

As expected in the presence of 0.3% pepsin, the survival rate was higher than the effect of pH alone (Figure 3.8).

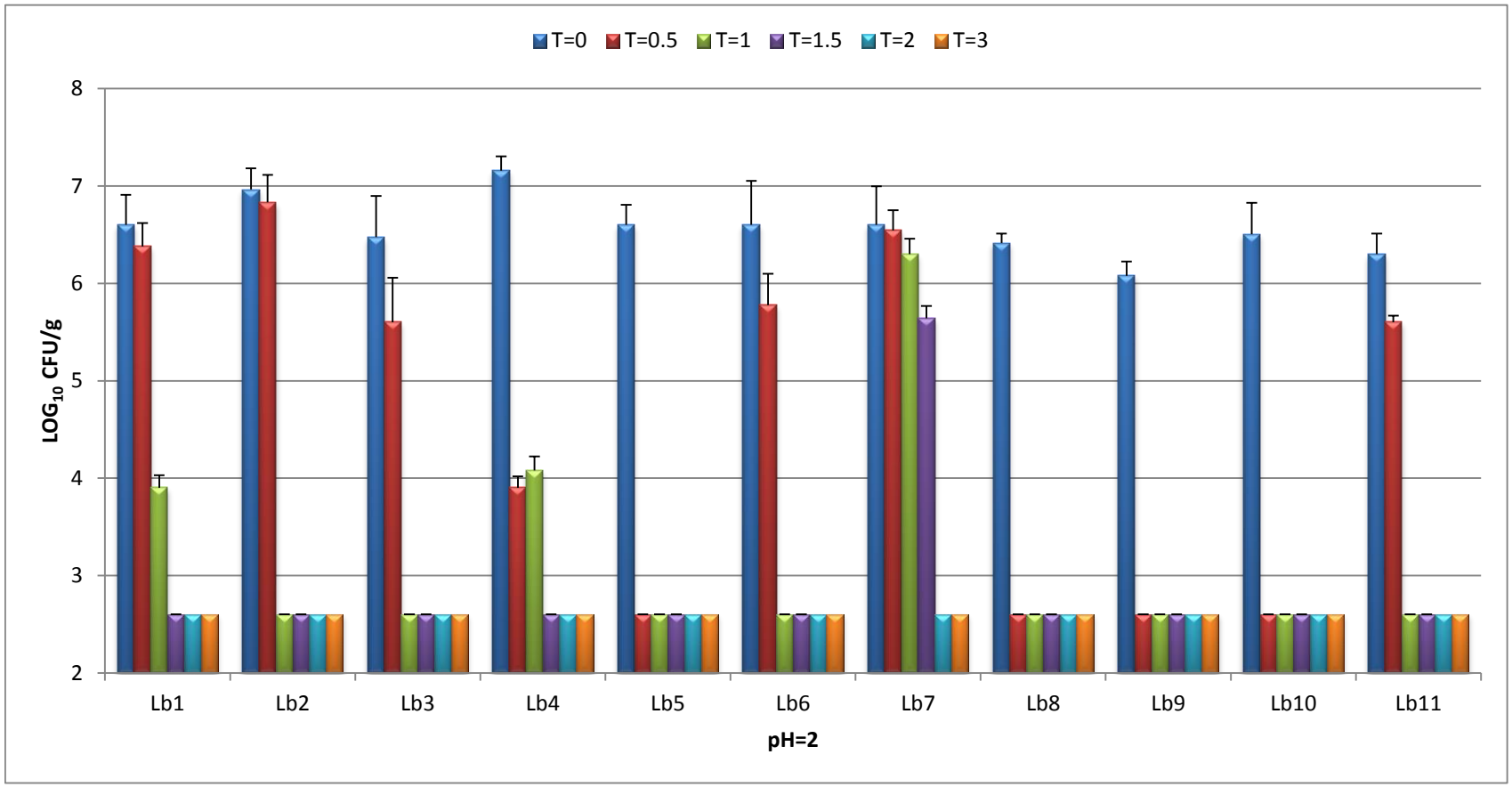


Figure 3.3 Survival of *Lactobacillus* species in PBS adjusted to pH 2 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 ºC under anaerobic condition (no added pepsin)

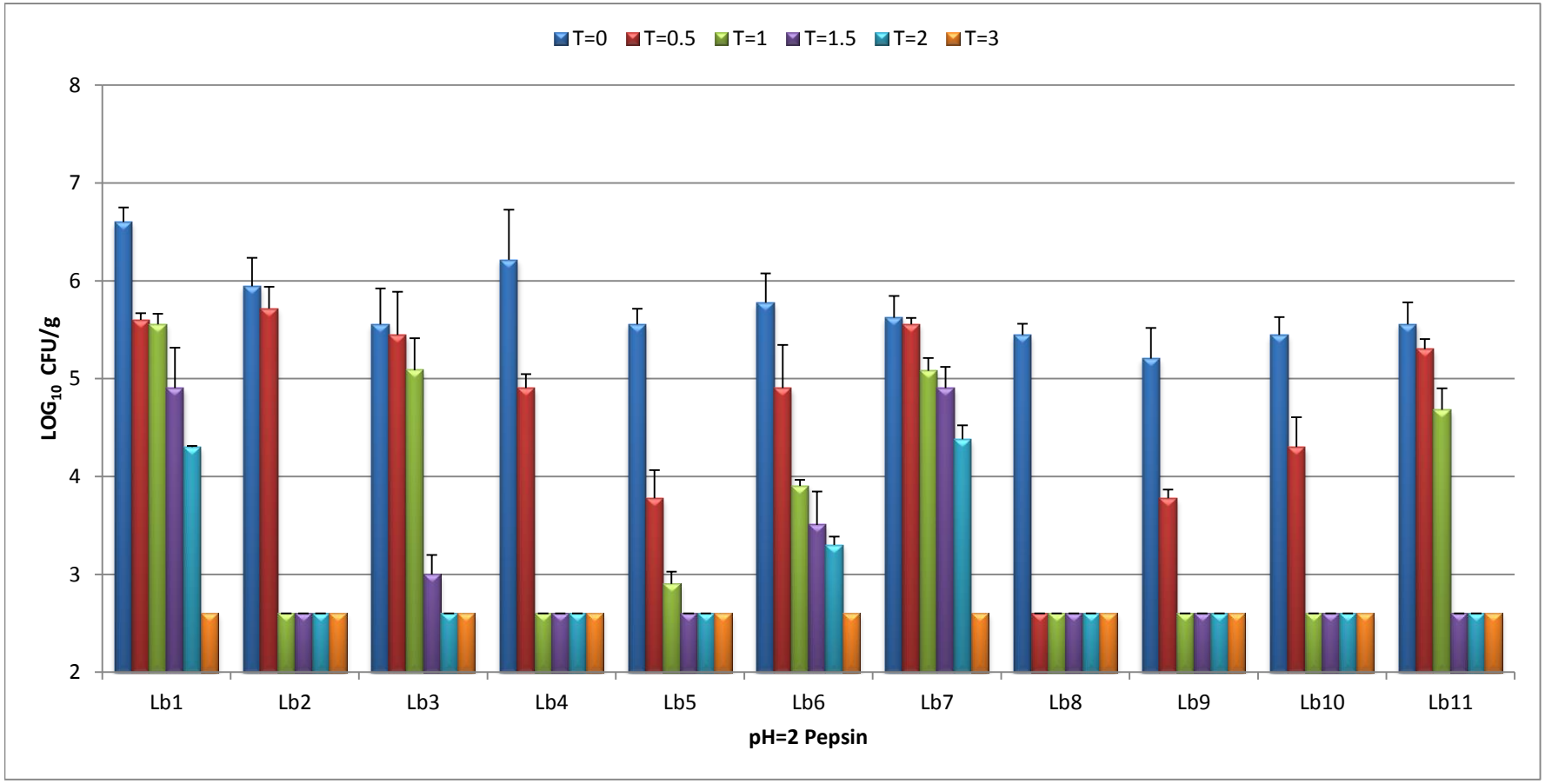


Figure 3.4 Survival of *Lactobacillus* species in PBS adjusted to pH 2 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition (with added pepsin)

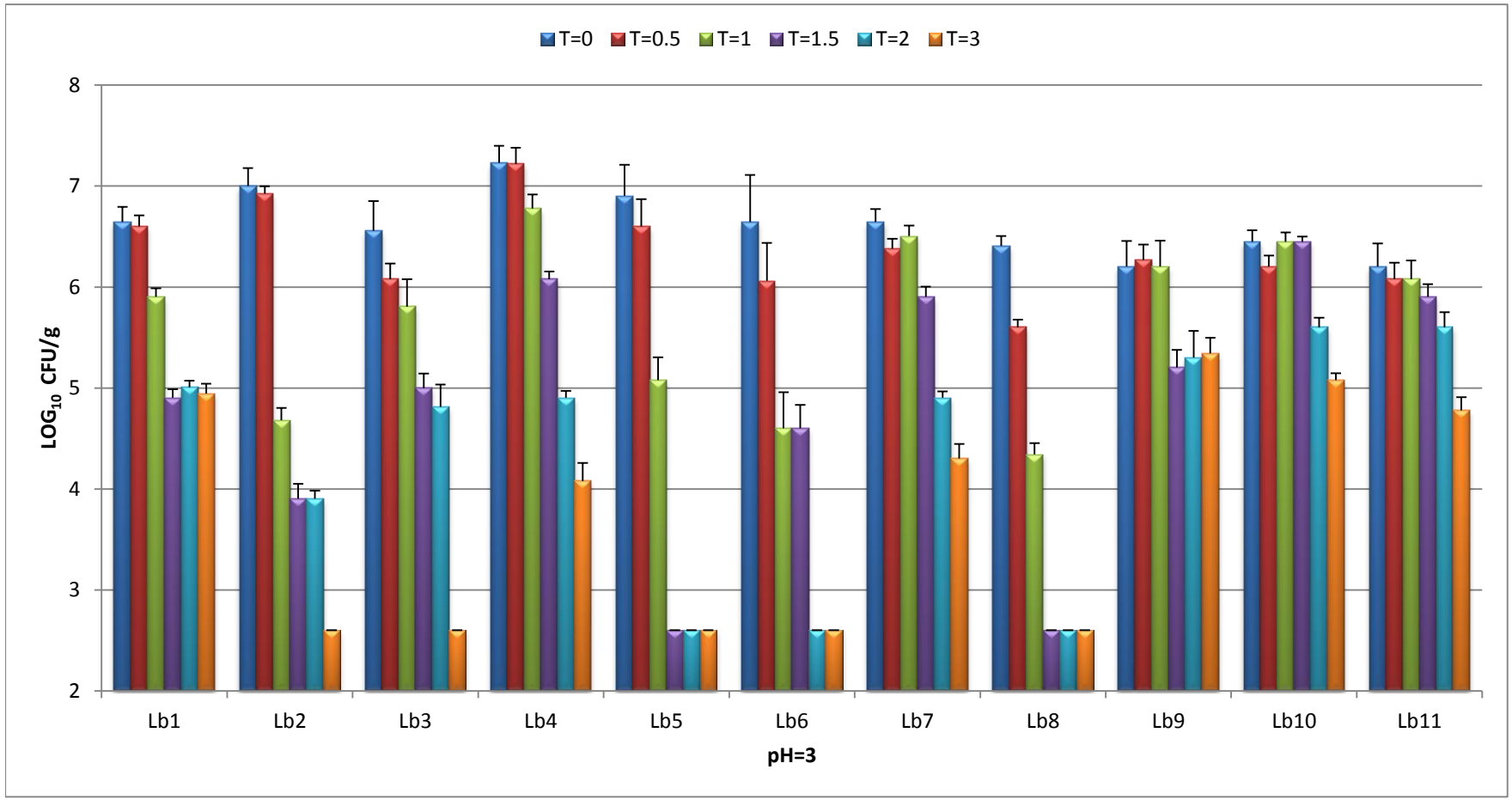


Figure 3.5 Survival of *Lactobacillus* species in PBS adjusted to pH 3 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition (no added pepsin)

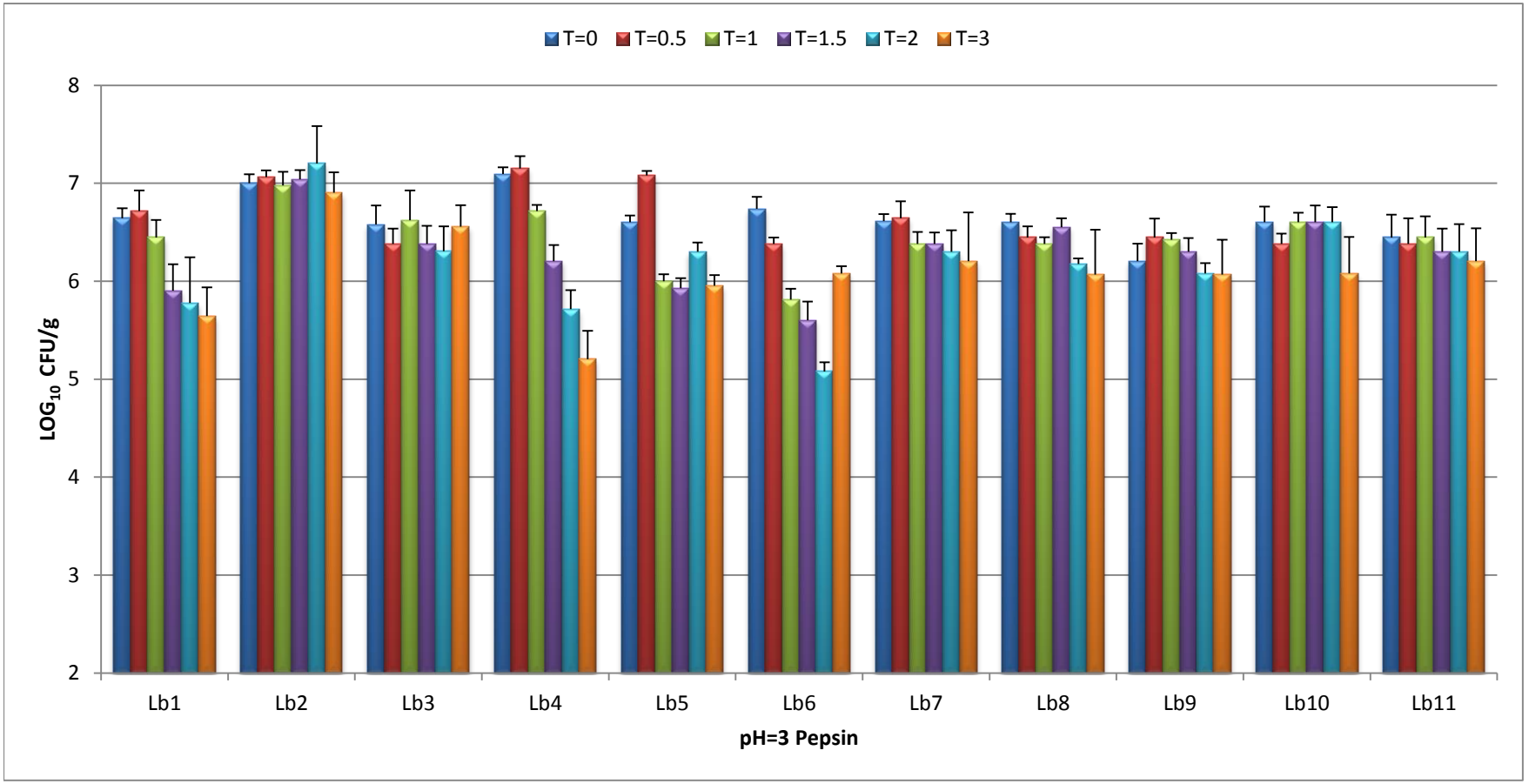


Figure 3.6 Survival of *Lactobacillus* species in PBS adjusted to pH 3 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 ºC under anaerobic condition (with added pepsin)

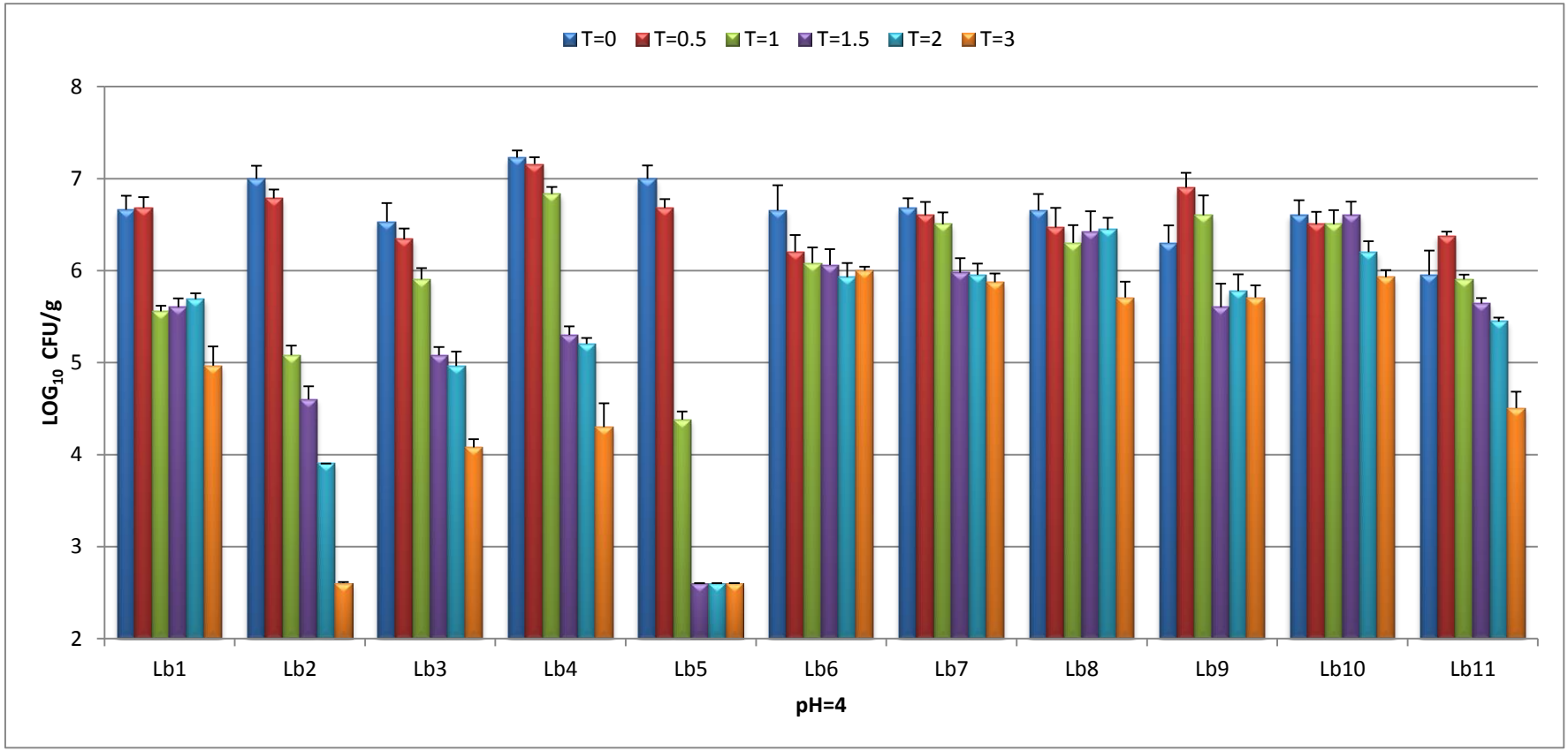


Figure 3.7 Survival of *Lactobacillus* species in PBS adjusted to pH 4 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition (no added pepsin)

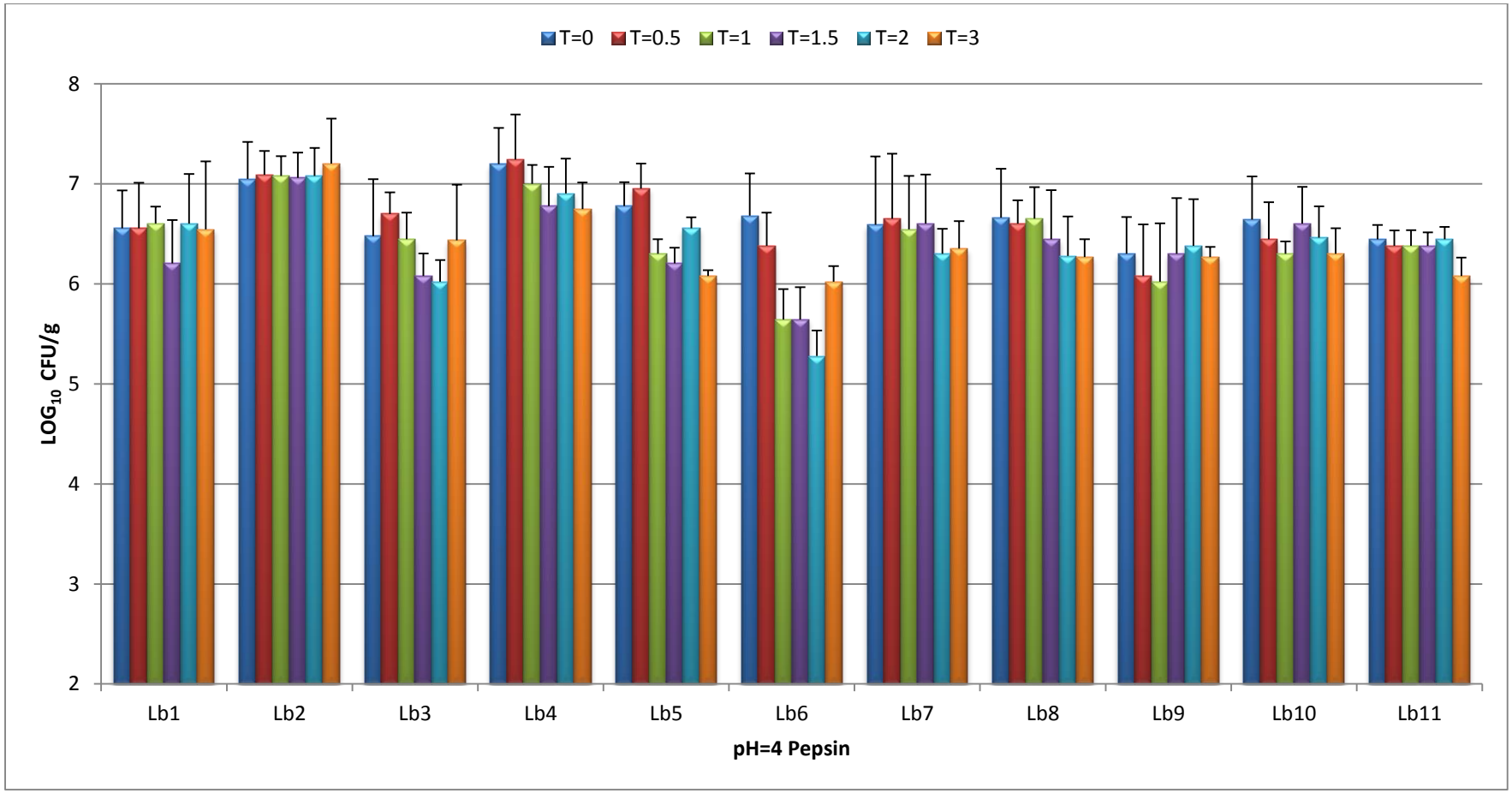


Figure 3.8 Survival of *Lactobacillus* species in PBS adjusted to pH 4 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 ºCX under anaerobic condition (with added pepsin)

3.3.2 Resistance to bile

The bile resistance of *Lactobacillus* spp. was evaluated by supplementing the PBS with bile. Figures 3.9, 3.10, 3.11 and 3.12 illustrate the tolerance of the tested *Lactobacillus* strains in the presence of 0.5, 1, 1.5, and 2% of bile salts. The survival rate of all tested *Lactobacillus* strains at four different concentrations of bile salts for a period of 2 h exposure, were very similar to the survival at the beginning of the experiment (t=0) with the exception of Lb3, Lb4 and Lb7 (Figures 3.9 – 3.12).

Tolerance to bile salts, allows bacteria to survive in the small intestine. At the beginning of the experiment, the initial cell population was in the range of 6-7 $\log_{10}$ CFU/ml, however, all tested isolates showed a very good resistance to bile salt even at 2% concentration and their viability was maintained with no or very little loss.

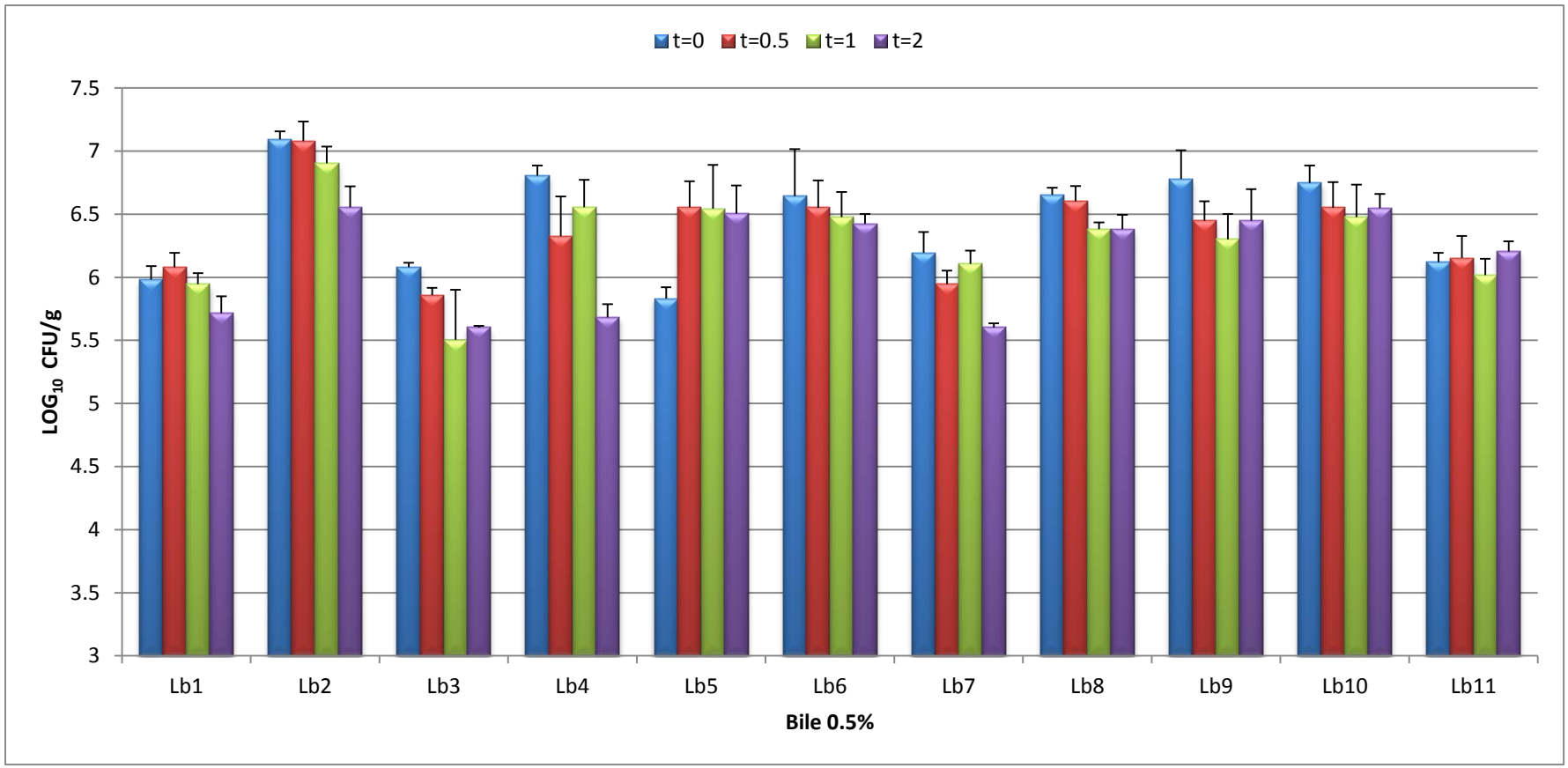


Figure 3.9 Survival of *Lactobacillus* species in PBS containing 0.5% of bile salt after 0, 0.5, 1 and 2 h incubation at 37 ^{o}C under anaerobic condition

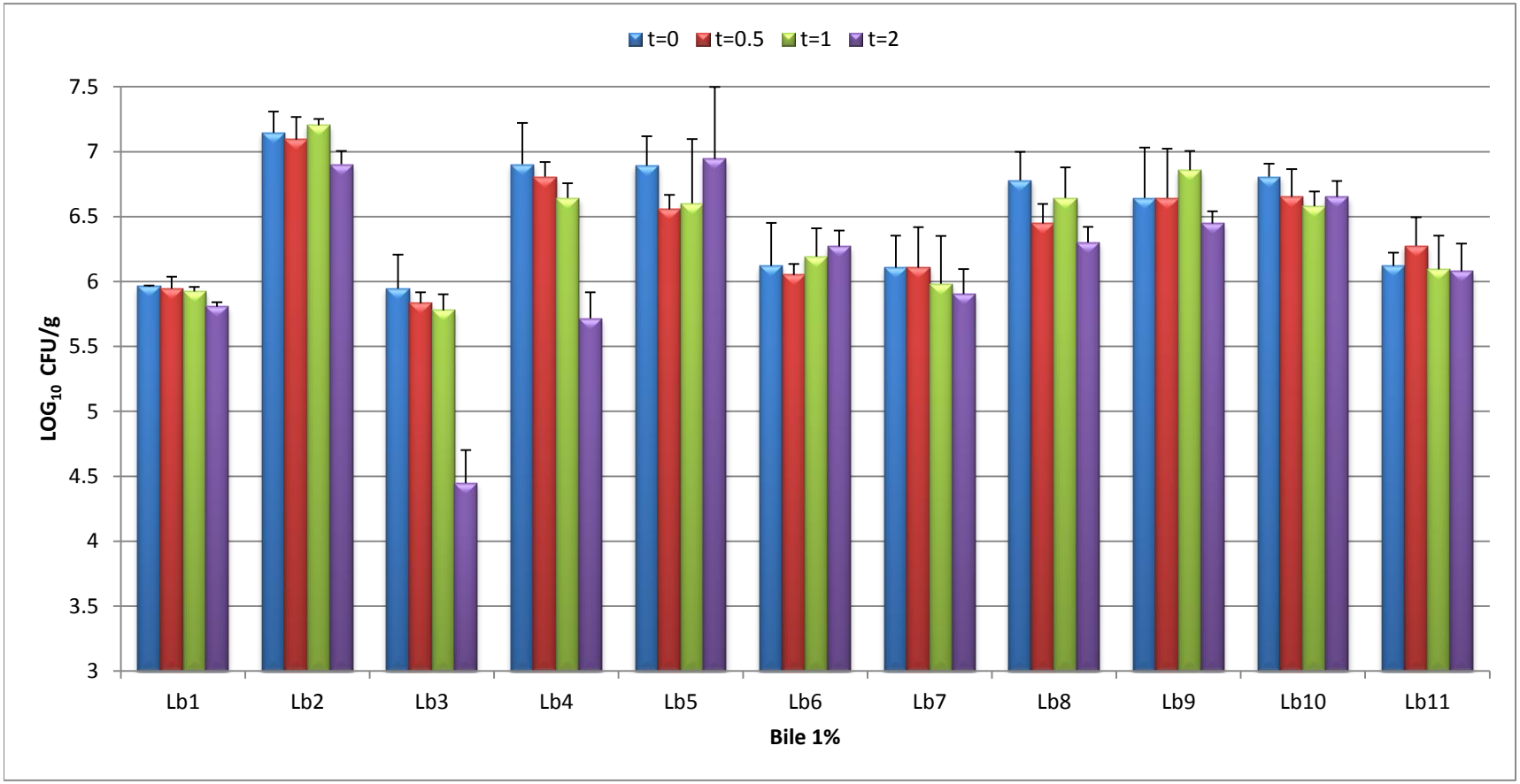


Figure 3.10 Survival of *Lactobacillus* species in PBS containing 1% of bile salt after 0, 0.5, 1 and 2 h incubation at 37 ºC under anaerobic condition

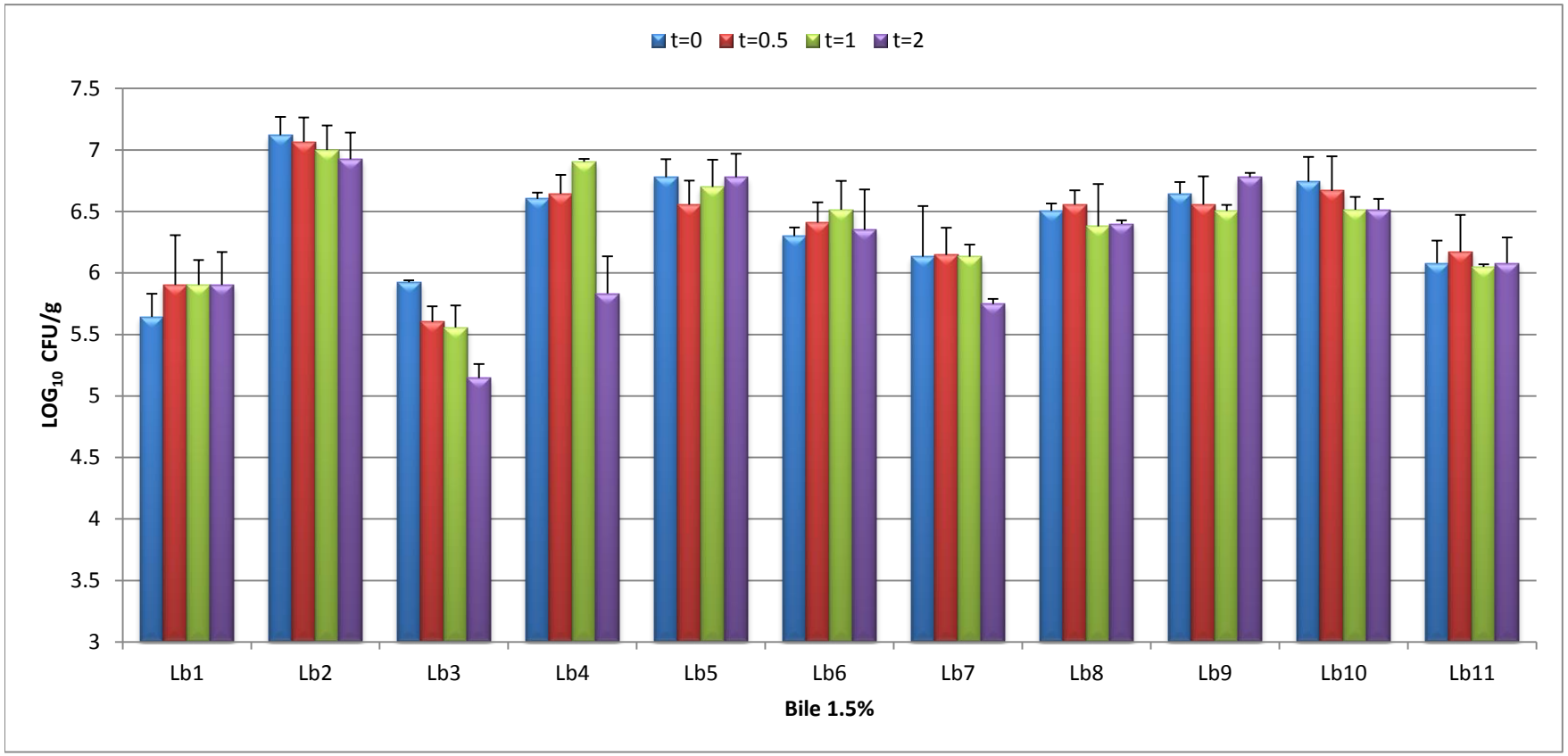


Figure 3.11 Survival of *Lactobacillus* species in PBS containing 1.5% of bile salt after 0, 0.5, 1 and 2 h incubation at 37 ºC under anaerobic condition

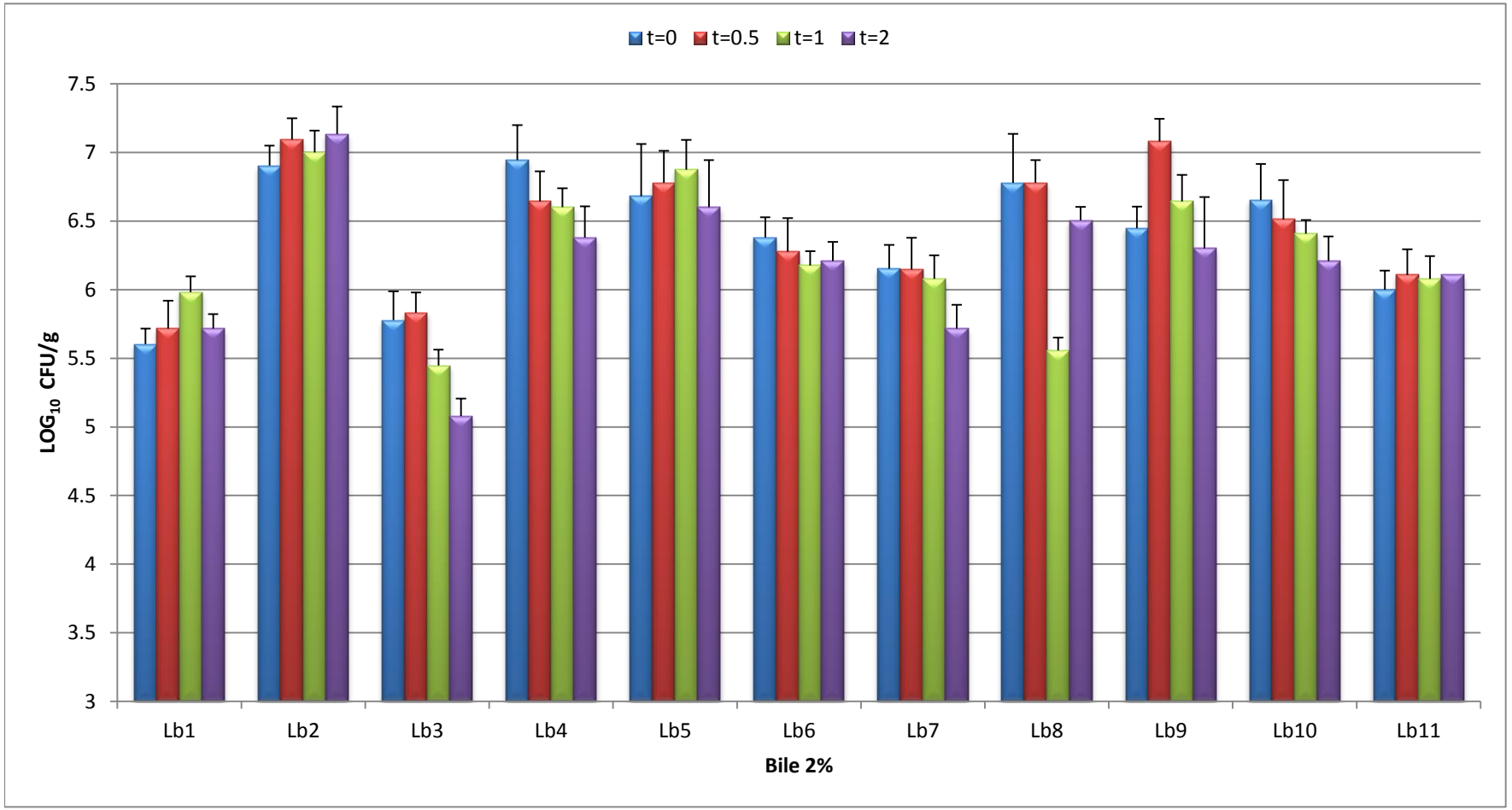


Figure 3.12 Survival of *Lactobacillus* species in PBS containing 2% of bile salt after 0, 0.5, 1 and 2 h incubation at 37 °C under anaerobic condition

3.3.3 Deconjugation of bile salts

All lactobacilli streaked on TCA and GCA–MRS agar plates were able to grow well and formed fine precipitate halos around the colonies, with appearance of a clear zone around the colonies (Figure 3.13 and 3.14). It was difficult to measure the diameter of the precipitated halo zone, but certainly, no inhibitory effects of TCA and GCA were seen on the growth of the tested isolates. All strains could deconjugate bile salts.

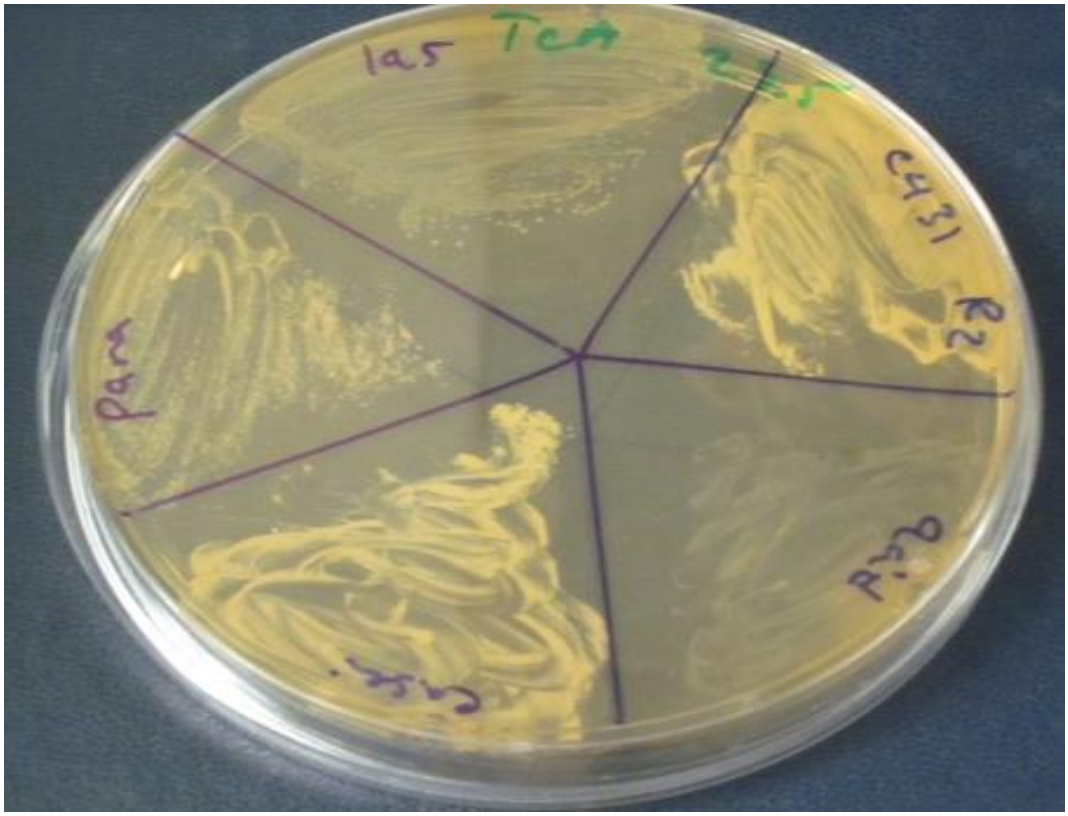


Figure 3.13 Representative of deconjugation of bile salt by Lb 1,2,3,4 and 5 on bile salt–MRS agar plates containing TCA.

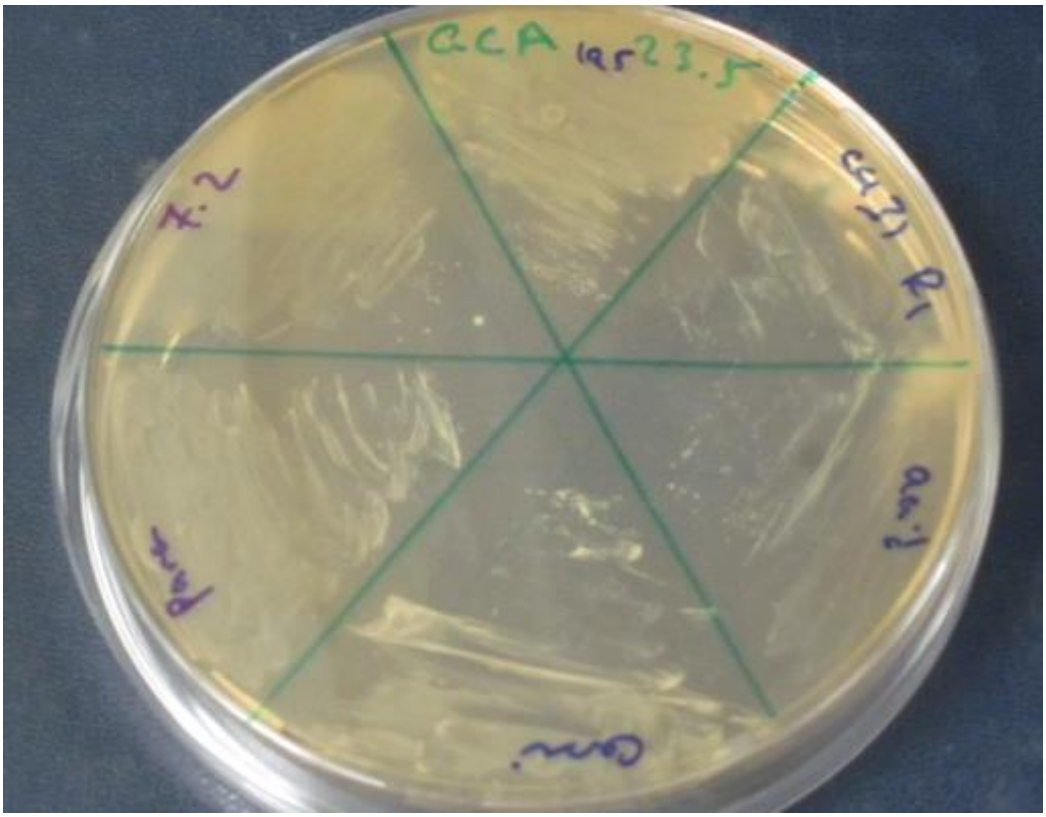


Figure 3.14 Representative of deconjugation of bile salt by Lb 1,2,3,4,5 and 6 on bile salt–MRS agar plates containing GCA.

3.3.4 Sequential effects of acid, pepsin, pancreatin and bile salts on viability of tested isolates

After initial screening of the isolates for their resistance to pH and bile salt and, due to complexity of the contents of the GIT, their behaviour was studied using an *in vitro* GIT model.

In this model the sequentially combined effects of pH, pepsin and bile salts and also pH, pepsin, bile salts and pancreatin on survival of testedstrains were studied.

As Figures 3.15 to 3.25 show apart from isolates Lb4, 7 and 9, a detrimental effect of bile salts was seen in all tested strains; as numbers were reduced by

more than 1 $\log_{10}$ CFU/ml. In the initial studies where the sole effect of acid and/or bile was examined, all strains showed tolerance to bile salts; but, after acid stress their tolerance to bile salts, was reduced.

The protective effect of enzyme pancreatin was observed for all tested strains. The number of recovered bacteria at the stage at which conditions mimiced leaving the stomach, duodenum, jejunum and ileum were more or less similar to the control except for isolates Lb 8 and 10. Numbers of these two isolates were drastically reduced after exposure to bile salts. No protective effect of pancreatin was noticed ($p < 0.05$).

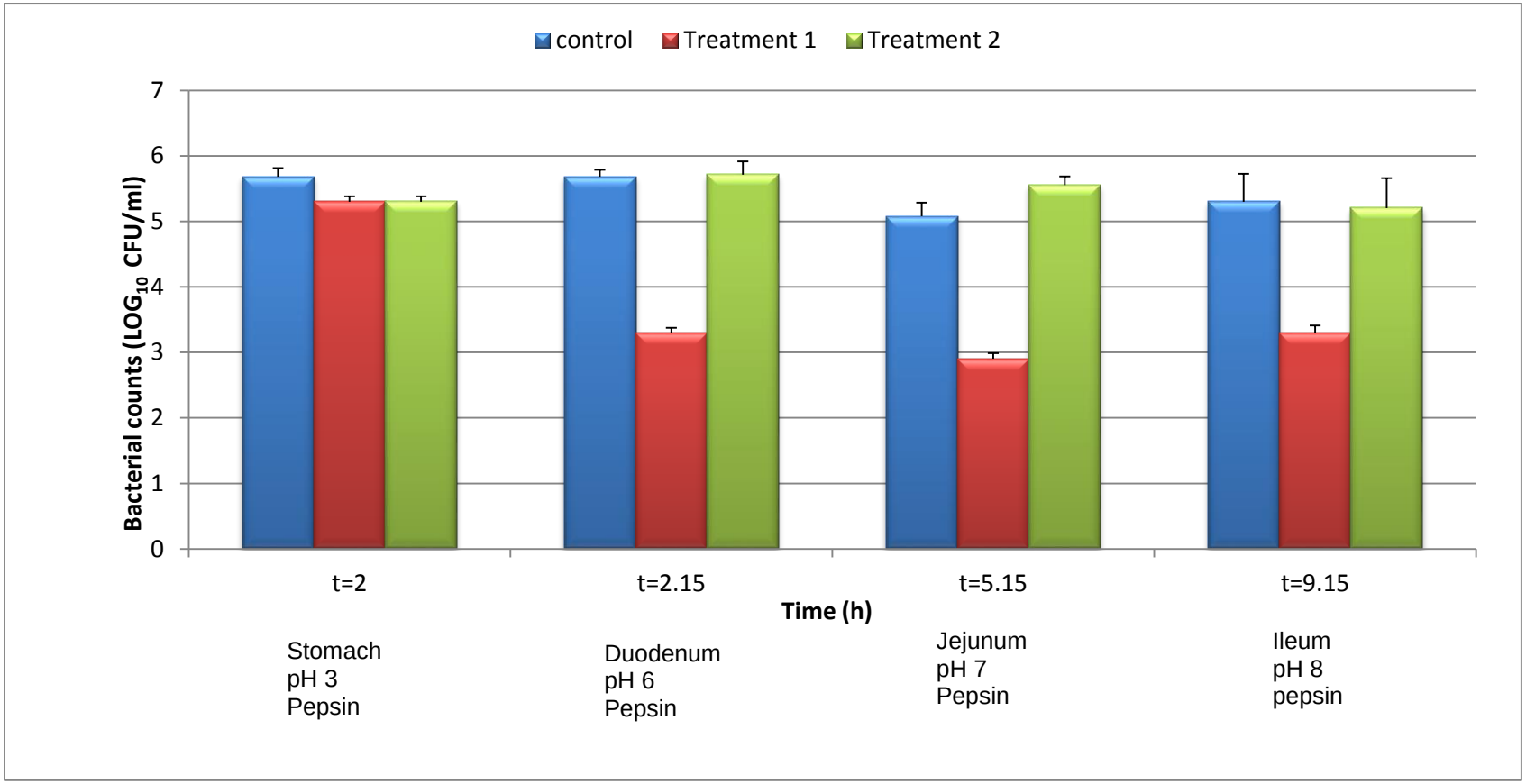


Figure 3.15 Survival of Lb1 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin (10g/l)

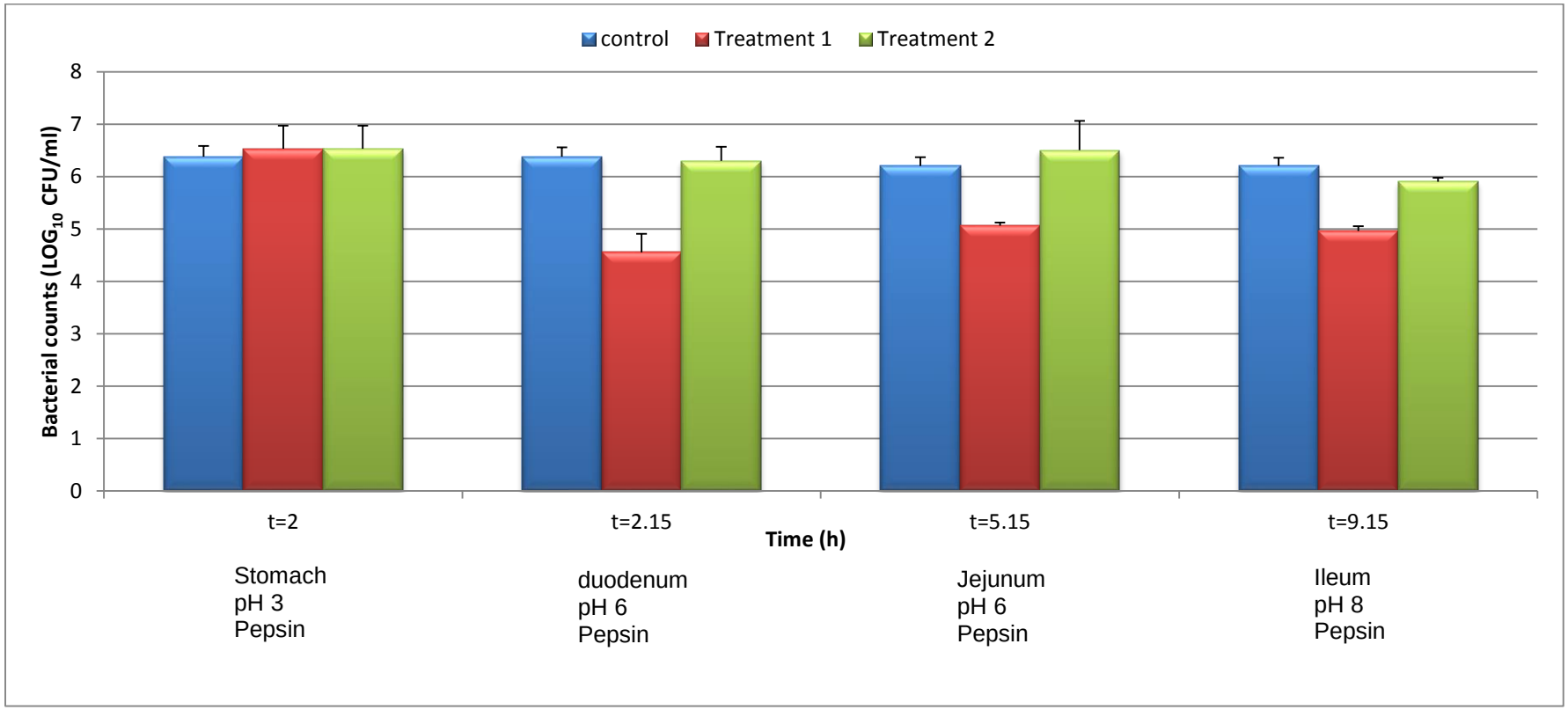


Figure 3.16 Survival of Lb2 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin (10g/l)

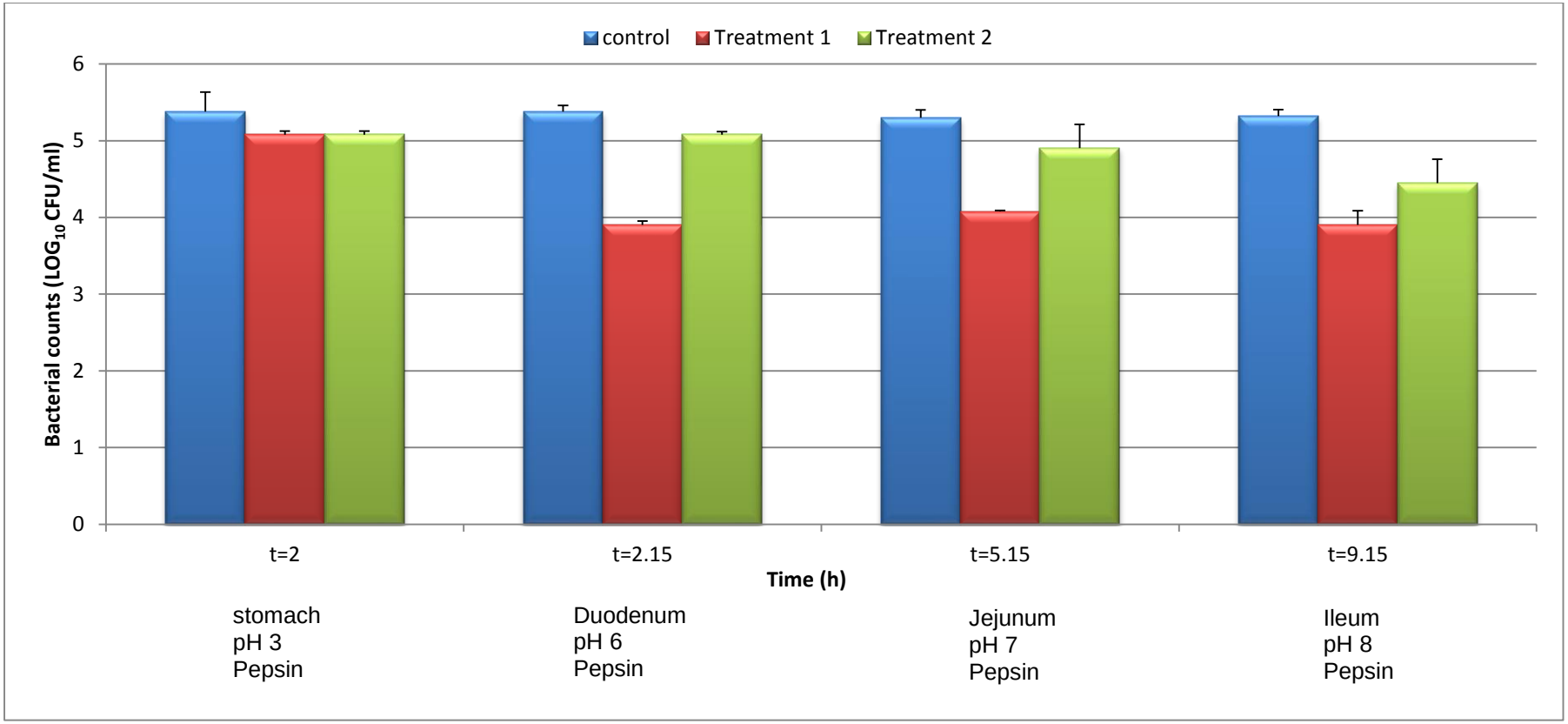


Figure 3.17 Survival of Lb3 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin (10g/l)

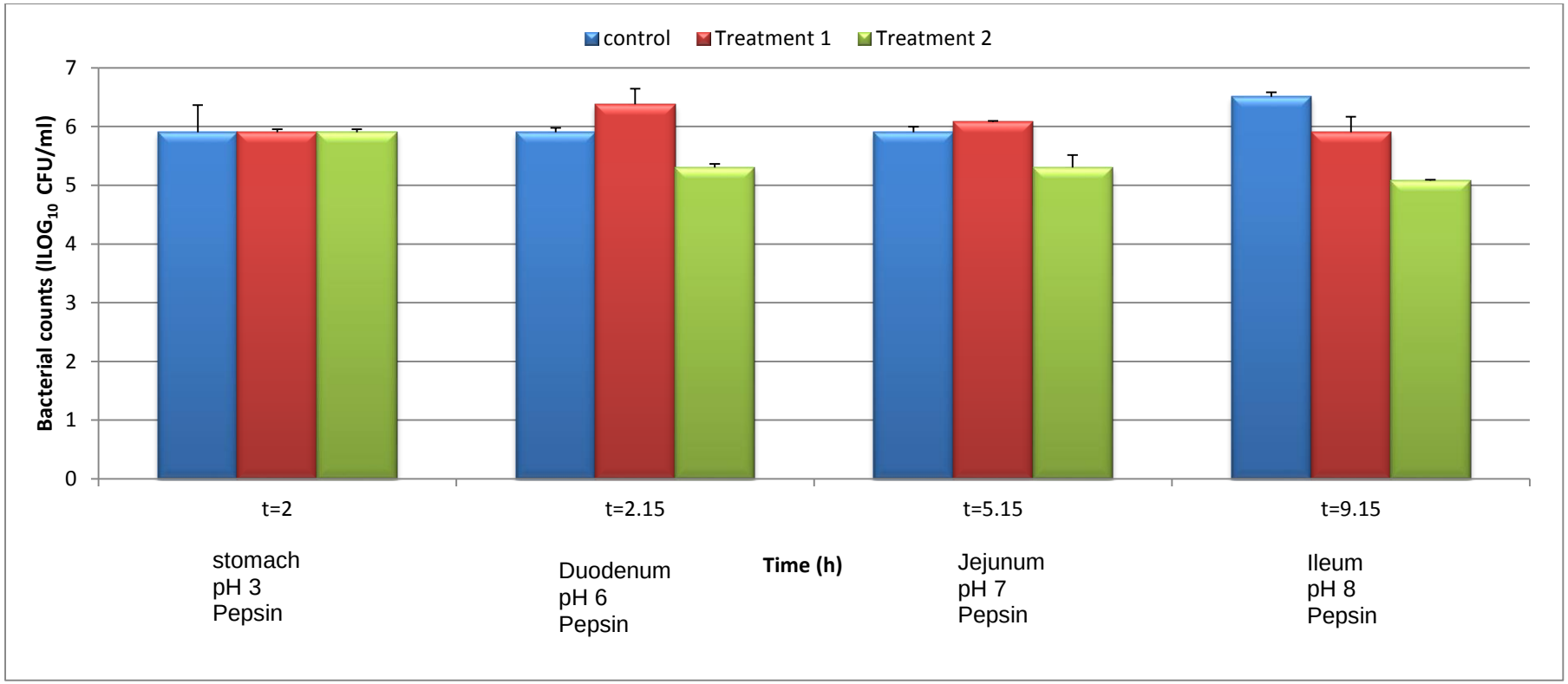


Figure 3.18 Survival of Lb4 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin(10g/l)

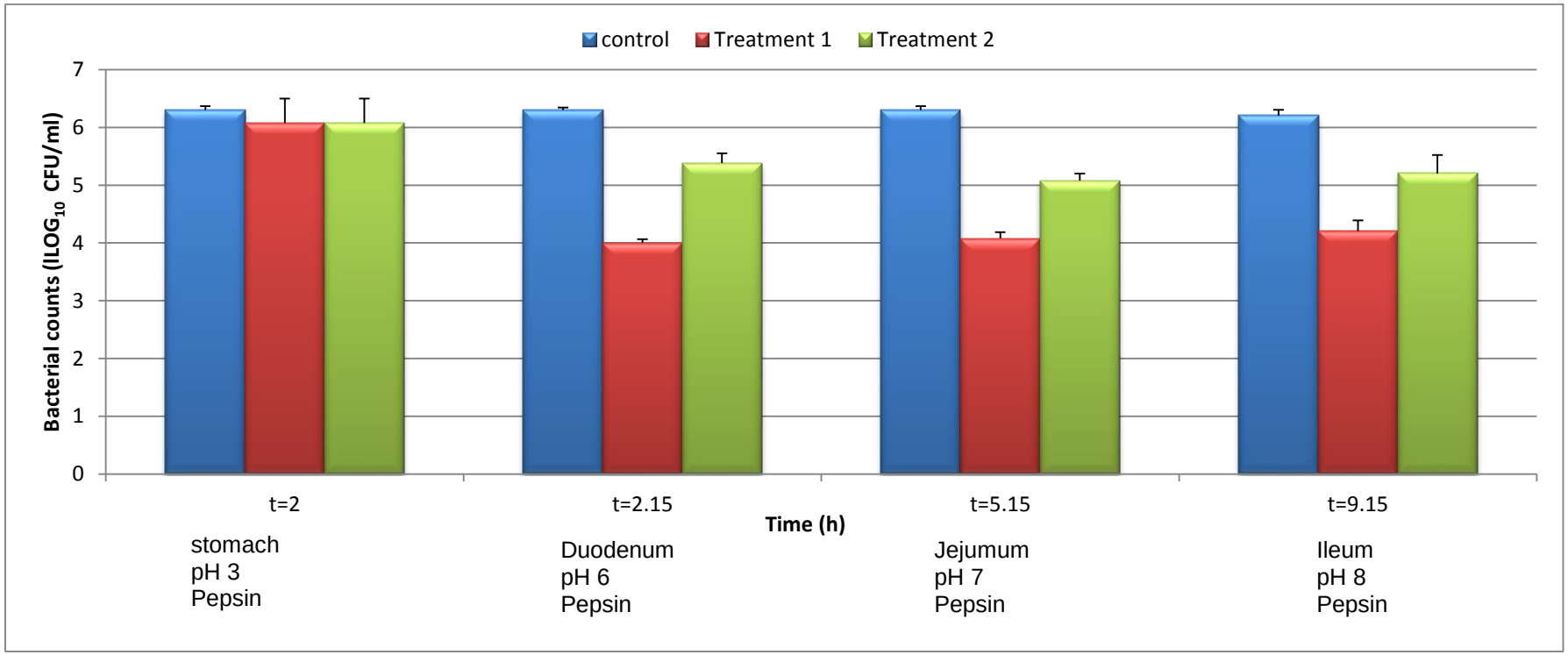


Figure 3.19 Survival of Lb5 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin(10g/l)

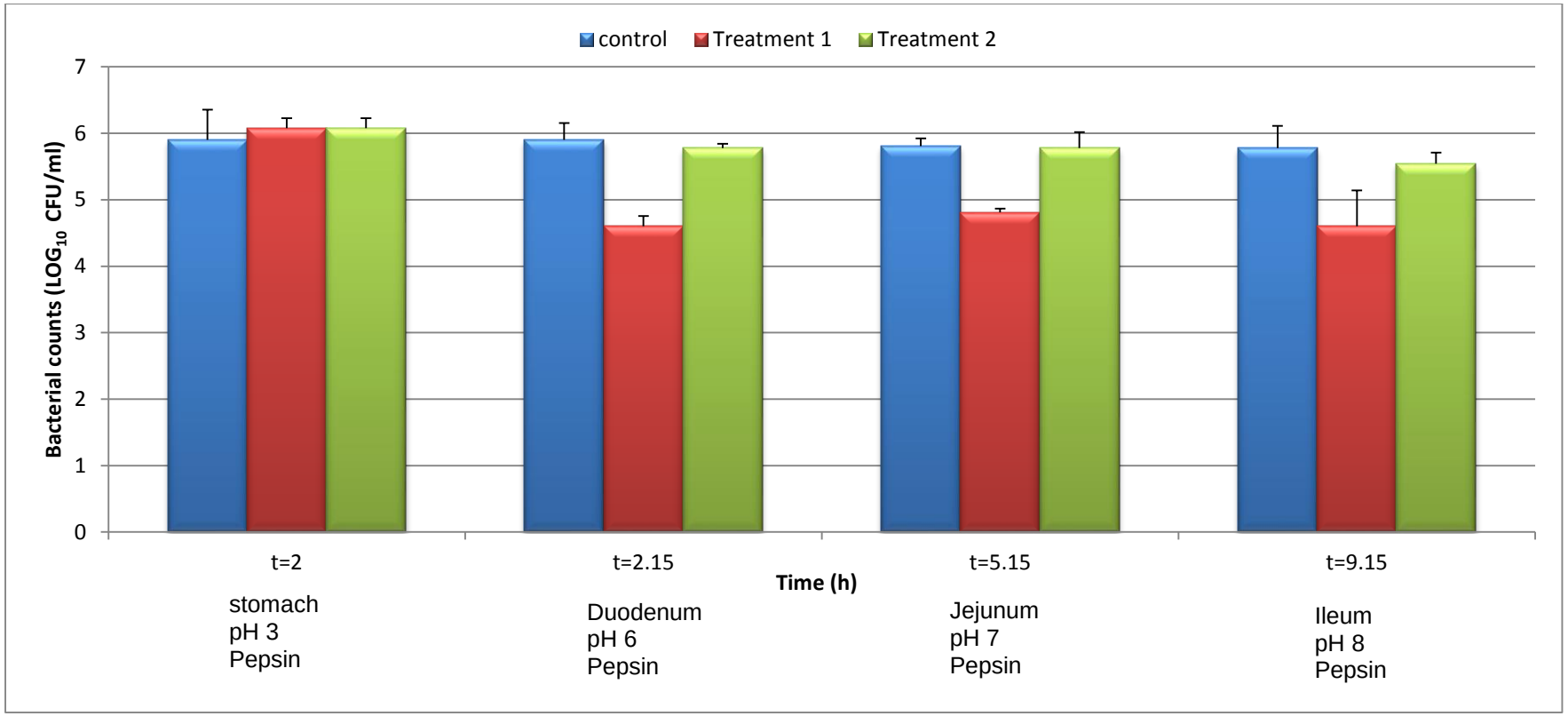


Figure 3.20 Survival of Lb6 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) +pancreatin (10g/l)

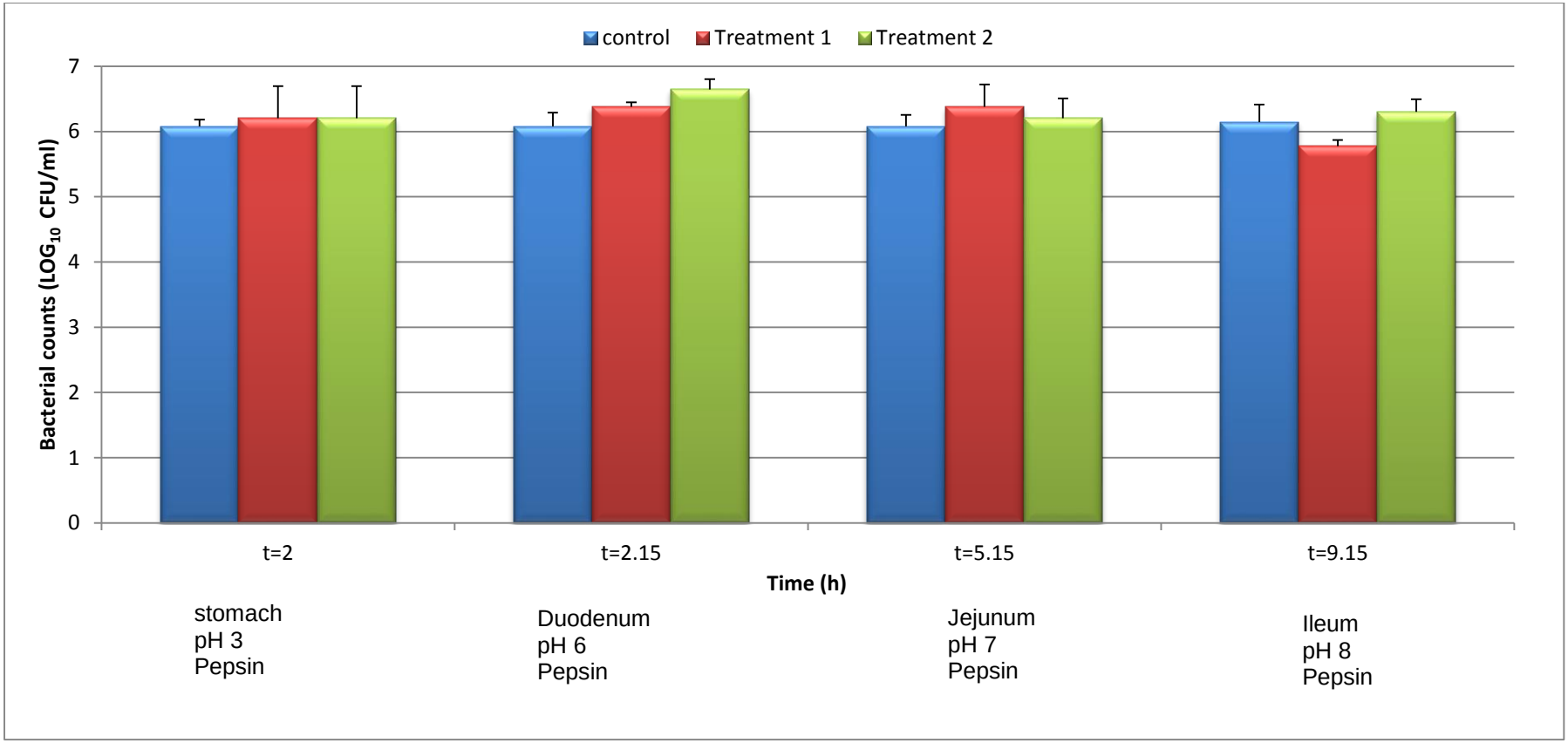


Figure 3.21 Survival of Lb7 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) +pancreatin (10g/l)

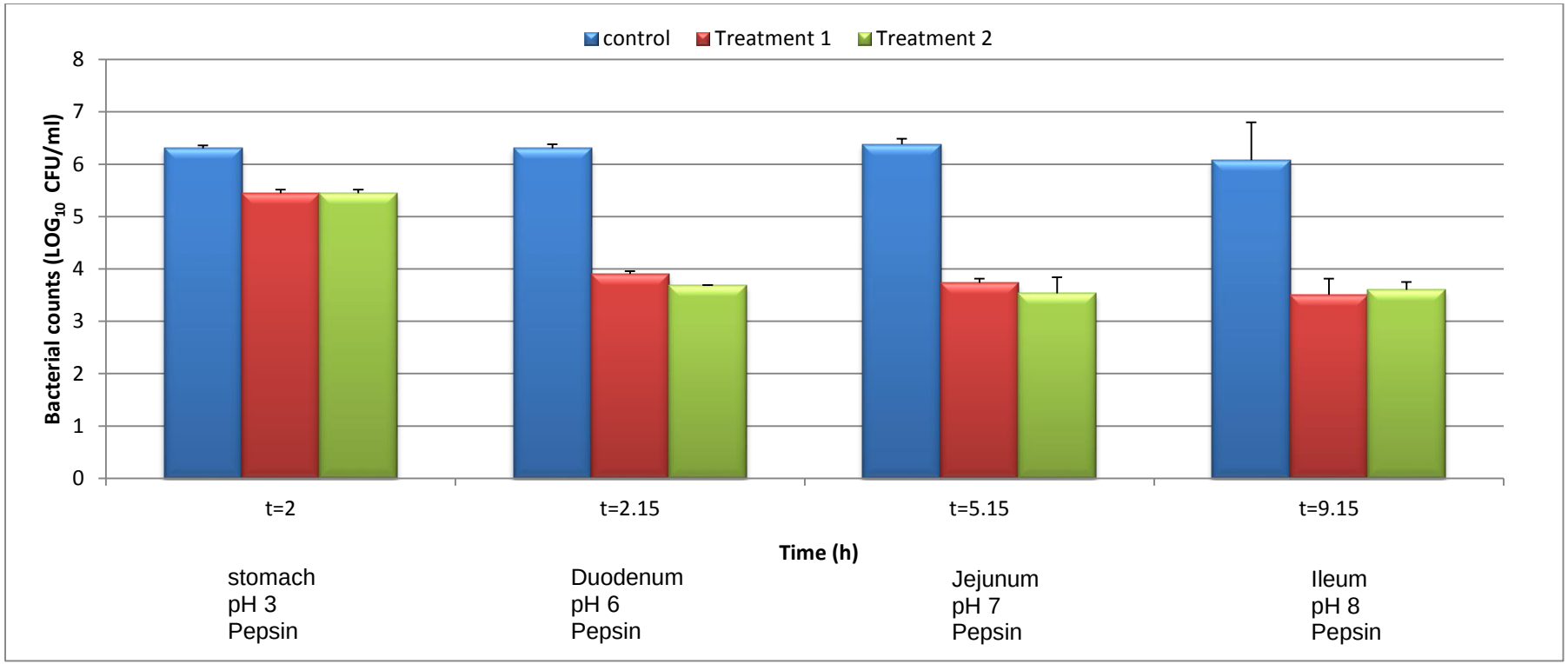


Figure 3.22 Survival of Lb8 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin (10g/l)

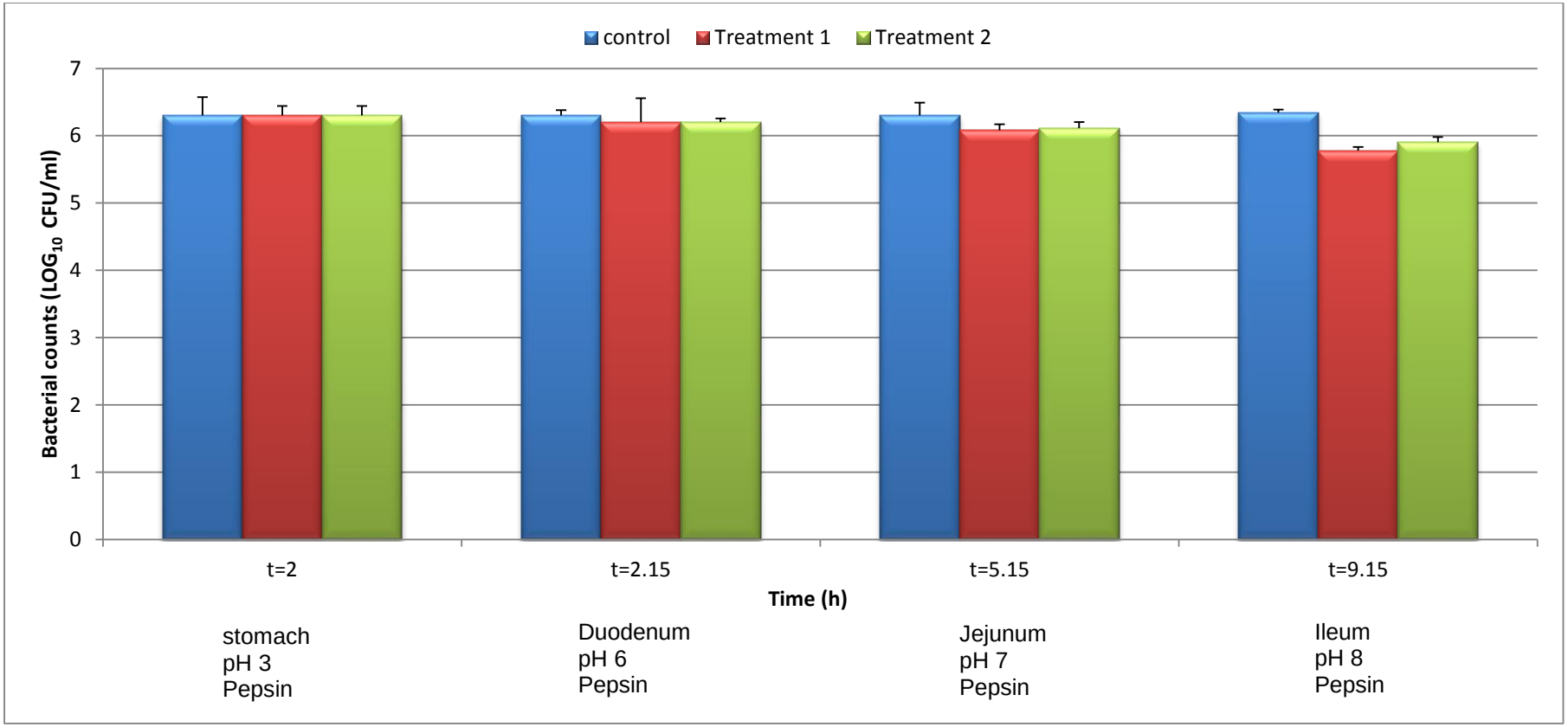


Figure 3.23 Survival of Lb9 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin (10g/l)

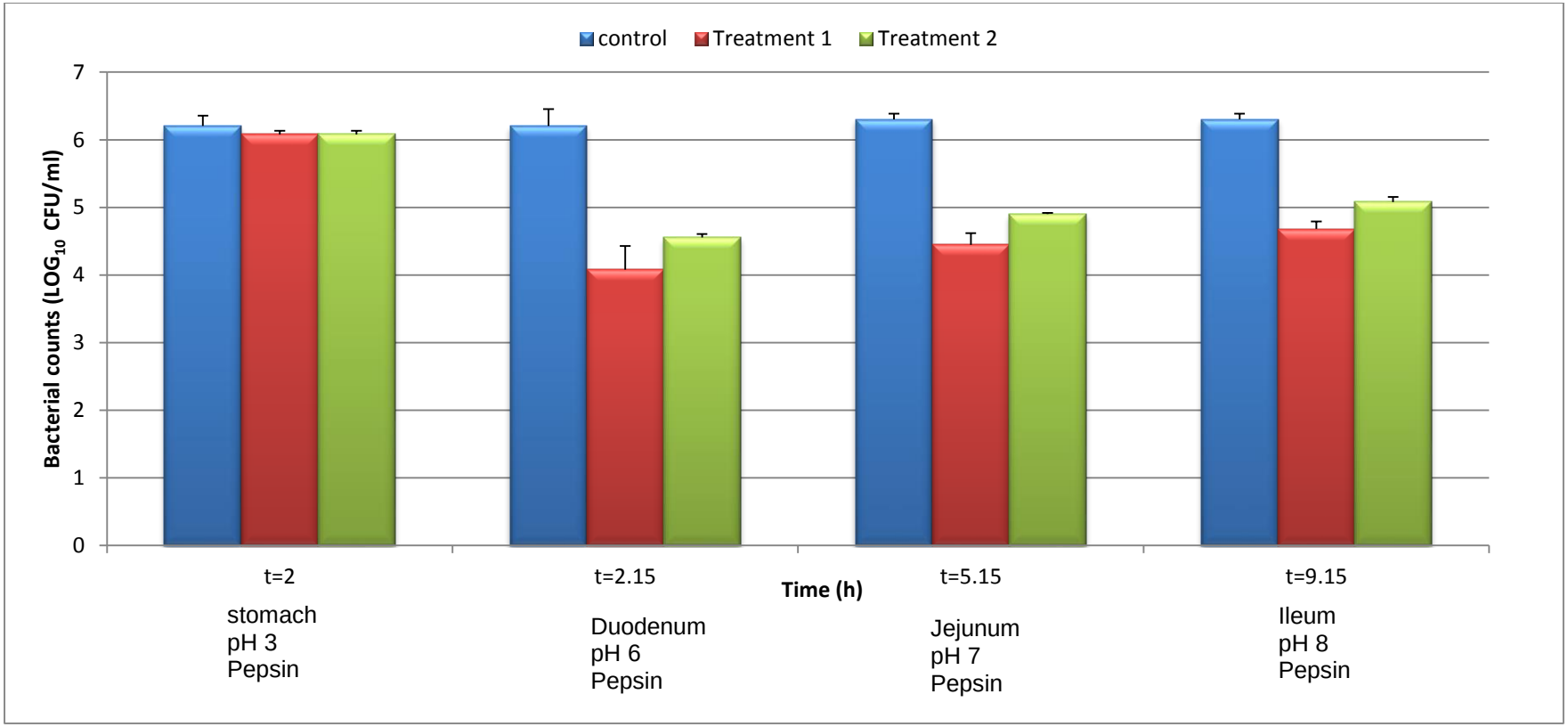


Figure 3.24 Survival of Lb10 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l) + pancreatin(10g/l)

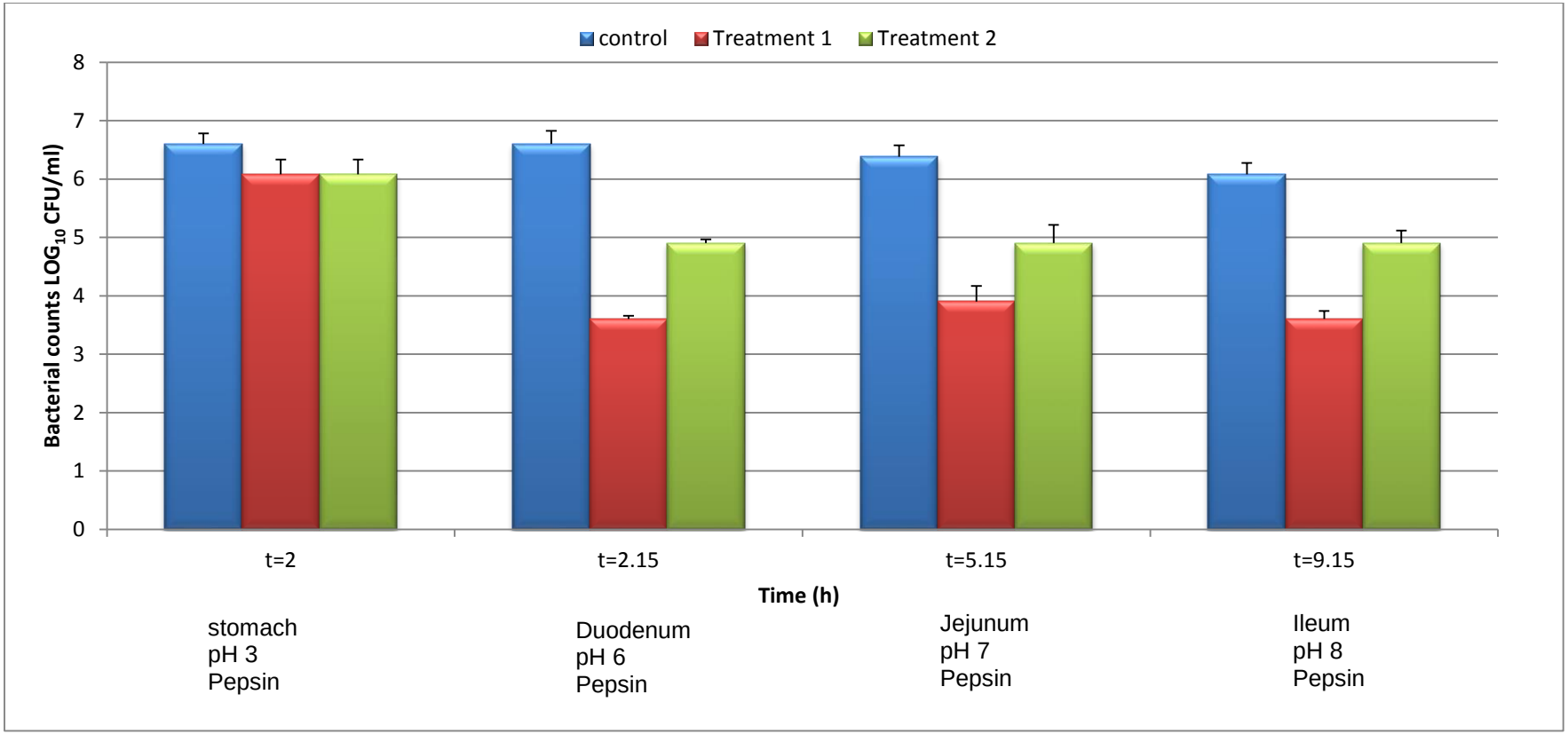


Figure 3.25 Survival of Lb11 (log_{10} CFU/ml) under different conditions of stomach and small intestine influenced by the presence of acid (pH), bile salt, pepsin, pancreatin and transit time (h)

Control: PBS (pH= 7)
Treatment 1: bile salt (3g/l)
Treatment 2: bile salt (3g/l)+pancreatin(10g/l)

3.4 DISCUSSION

The *in vitro* survival experiments of *Lactobacillus* spp. Mimicking transit from stomach to intestinal conditions are valuable for estimating the survival rate of strains. It is clear that strain behaviour in GIT would be different when the probiotic bacteria are protected by the food matrix or presented in microencapsulated forms.

However, the survival of strains in the harsh conditions of GIT needs to be assessed properly to determine changes in the total number of consumed probiotic bacteria. It would be useful to estimate the minimum requirement for oral administrations of probiotic.

Schillinger *et al*. (2005) reported that six *Lb. acidophilus* strains isolated from probiotic yogurts were able to survive for 90 min in a simulated gastric juice containing pepsin (3 mg/ml) at pH 2. The reduction of *Lb. acidophilus* strains varied from 0.1 to 2.5 $\log_{10}$ cycles during that period. They also reported that these *Lb. acidophilus* strains were more tolerant to low pH, compared to *Lb. paracasei* subsp. *Paracasei* and *Lb. rhamnosus*, which demonstrated complete loss of viability within 30 min of starting the experiment.

In the study of Madureira *et al*. (2005) no changes were seen in the viability of the *Lb. acidophilus* LAC-1 and *Lb. acidophilus* Ki strains in Requeijão cheese. These lactobacilli retained numbers above 7 and 8 $\log_{10}$ CFU/ml of simulated gastric content, respectively, when exposed at pH 2.5 and 3 with pepsin (1000 unit/ml) over 2 h.

The present study confirmed that all tested strains were able to survive in the neutral pH of PBS solution for 3 h. At pH values of 4 and 3, no considerable

reduction in the viable counts was observed except for a few strains. However, after one h, viable cells were undetectable at pH 2 for many of the tested isolates. Nevertheless, all tested strains are able to meet the requirements for probiotic properties in terms of acid tolerance in the stomach.

In addition, the fact that probiotic bacteria are carried in milk products and milk proteins support the survival of probiotic bacteria in the acidic condition of the stomach (Maragkoudakis *et al.* 2006). Lo Curto *et al*. (2011) investigated the survival of *Lb. casei* Shirota*, Lb. casei* Immunitas and *Lb. acidophilus* johnsonii in a gastric model of digestion followed by incubation under duodenal conditions. They incorporated those lactobacilli in water and milk as food matrices. According to their findings the survival in milk was higher than water. This can be related to the lower buffering capacity of the water in comparison to milk (Holzapfel *et al*. 2001).

Investigating the sensitivity of microorganisms to gastric juices, such as pepsin, is an important step for selection of potential probiotic lactobacilli. The combined influence of a pepsin-pH solution in simulated gastric juice is not clear. Pepsin introduces restrictive conditions for growth of lactobacilli (Radulovic *et al*. 2010).

Maragkoudakis *et al.* (2006) stated that actual gastric juice compared with low pH buffer, provides some degree of protection for bacteria. This study showed that survival of the tested strains during incubation at low pH in medium fortified with pepsin was higher than incubation without pepsin. These bacteria presented extreme sensitivity to acidic condition at pH 2 and the protective effect of pepsin at pH 2 was not as noticeable as at pH 3 and 4. The findings herein reported for protective effects of pepsin on tested isolates at low pH are in

agreement with the existing literature (Maragkoudakis *et al.* 2006). They indicated that all 29 strains of lactobacilli in their study maintained their viability after 3 h of exposure to pH 3. Among strains subjected to the pepsin solution at pH 2 for 1 h, highest survival was seen with *Lb. casei* Shirota ACA-DC 6002, *Lb. casei* Immunitas ACA-DC 6003 with lower than 1.0 $\log_{10}$ cycle reduction. After 3 h of exposure to pepsin, the best survival was observed with strains *Lb. rhamnosus* ACA-DC 112 and *Lb. paracasei* subsp. *paracasei* ACA-DC 130 with 2.0 $\log_{10}$ cycles reduction. All strains retained their viability after 4 h of exposure to pancreatin at pH 8.0 with below 1 log cycle reduction.

Ahn *et al*. (2003) have indicated that deconjugation of bile salt does not necessarily represent bile tolerance of *Lb. acidophilus*. Other researchers have reported similar results (Usman and Hosono 1999; Moser and Savage 2001). Contradictory reports, however, indicate that deconjugation of bile salt promoted bile tolerance of LAB (De Smet *et al*. 1995).

This study reported that no considerable retardation occurred of growth of the tested isolates in the presence of TCA and GCA, and all colonies appeared on the agar plates. Therefore, the precipitated halo around the colonies on the bile salt–MRS agar plates were believed to be free bile salts, produced by the deconjugation of the added bile salts.

All tested isolates in the current research showed good resistance to different levels of bile salts even after 2 h and were able to retain their viability, as well as deconjugating TCA and GCA into cholate. This might be due to presence of BSH activities in those isolates which deconjugate bile salts; therefore, the toxic effects of bile salts on bacteria were decreased. The deconjugation of bile salts

in human intestinal tract starts at the end of the ileum and is completed in the large intestine (Tanaka *et al*. 2000a). Noriega *et al.* (2006) reported that lactobacilli provide a protective system with BSH, which enables them to deconjugate the bile salts, and the results in this study are in line with them.

In spite of good tolerance of the tested strains to bile salts, most of them died in the sequential model experiment in which they were exposed to gastric conditions followed by bile salts. However, viable numbers were maintained when duodenal juice was complemented by pancreatin. It seems that all tested strains not only are intrinsically resistant to pancreatin, but also pancreatin helps them to recover strains from harsh conditions in the duodenum.

Frece *et al*. (2005) investigated the functional role of surface layer proteins in *Lb. acidophilus* M92. It was demonstrated that surface layer proteins of *Lb. acidophilus* M92 were resistant to pepsin and pancreatin and they have a protective role for this strain, but they were sensitive to enzymatic treatments with proteinase K.

In the study of Maragkoudakis *et al*. (2006), all tested *Lactobacillus* strains were resistant to pancreatin, and even after 4 h of exposure retained their viability with less than 1 $\log_{10}$ cycle reduction.

As mentioned earlier, probiotic bacteria are mostly delivered by fermented milk products. Lactobacilli grown in fermented milks are protected by protein and fat matrices. Due to this encapsulation, they might survive the critical passage through the GIT, much better than unprotected bacteria. Also, the proteins of milk contain some peptide segments, which may be released during digestion (Maubois and Leonil 1989). Their degradation products can be involved in the

physiological regulation of digestion (Yvon *et al*. 1994). Caseinomacropeptide is one of these bioregulators, which is produced by initial pepsin hydrolysis of κ-casein during milk digestion (Robitaille *et al*. 2012). It has a protective effect and improves the resistance of *Lb. rhamnosus* to acid stress.

Tolerance of intestinal lactobacilli to acid and bile has been described as strain dependent (Vinderola and Reinheimer 2003). The intrinsic characteristics of the *Lactobacillus* strains can be improved by the protective action of carrier foods and by the presence of nutrients (Charalampopoulos *et al*. 2003). Different food ingredients such as inulin-type fructans (Donkor *et al*. 2007), whey protein concentrate (Kos *et al*. 2000; Akalin *et al*. 2007), malt, wheat, and barley extracts (Patel *et al*. 2004) and pectin (Nazzaro *et al*. 2012) could exert a protective effect against the pH changes and the enzymes secreted during the passage through the GIT.

There are techniques to improve the survival of the lactobacilli, for example, when a microorganism is exposed to a stress such as cold, heat or acid beforehand, the surviving cells can better tolerate a subsequent unfavourable environment and adverse conditions of the GIT. This type of protection from one stress gained from prior exposure to another stress is named the cross-protective stress response (Saarela *et al*. 2004; Cruz *et al*. 2009a).

3.5 CONCLUSION

All tested strains were able to meet the requirements for probiotic properties in terms of acid tolerance in the stomach 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 *Lactobacillus* species was confirmed.

CHAPTER FOUR: *IN VITRO* ASSESSMENT OF *LACTOBACILLUS* SPP. FOR FUNCTIONAL PROPERTIES: BIOFILM FORMATION

4.1 INTRODUCTION

Adhesion and colonization ability of probiotic bacteria are important factors for enhancement of the epithelial barrier, immune modulation and pathogen exclusion (Perdigon *et al.* 2002; Kelly *et al*. 2005; Boekhorst *et al.* 2006). An intricate symbiotic interaction occurs between human intestinal flora and epithelial and immune cells of the GIT (Alander *et al*. 1999). The important role of the gut microbiota is to provide a primary defence line against pathogenic organisms and regulate the immune responses. The host supplies a secure and stable environment for the microbiota, including uniform temperature, pH, osmolarity and nutrient compounds. Part of the helpful activities of the gut microbiota is their capability to grow into microcolonies and biofilms (Perea *et al.* 2010).

Biofilms are defined as surface-associated microbial populations embedded in self-produced extracellular polymeric matrixes, which reduce their vulnerability to the host immunity systems and antimicrobial compounds (Donlan 2002).

It has been demonstrated that biofilms are involved in creating of an intimate relationship between the human GIT and its inhabitant microorganisms (Macfarlane and Dillon 2007).

Tuomola *et al*. (1999) stated that attachment to epithelial cells, potential colonisation power and better contact with the mucosa are regarded as reasons for some beneficial effects of probiotics including modulation of the immune system and combating the pathogens.

Two different growth modes are defined for bacteria. One is the single, free-floating cell form known as planktonic cells, and the second is a structured,

multi-cellular consortium described as a biofilm. Compared to planktonic cells, bacteria growing in biofilms present new behaviours, such as more resistance to stress, biocides, and host immunological defences (Branda *et al*. 2005; Kim *et al*. 2009).

Teitzel *et al.* (2003) stated that *Pseudomonas aeruginosa* biofilms were 2 to 600 times more resistant to heavy metal stress than the planktonic cells.

It has also been stated that biofilm of *Listeria monocytogenes* improved its resistance to heat treatment and also surfactant sanitisers (Simoes *et al*. 2010).

With regard to LAB, Kubota *et al.* (2008) demonstrated that the biofilms produced by *Lb. plantarum*, *Lb. brevis* and *Lactobacillus fructivorans,* could present greater protection to acetic acid and ethanol. They observed badly damaged surfaces of the treated planktonic cells with acetic acid and ethanol, but the surface of biofilm cells was only slightly damaged.

The potential for some LAB to form biofilm has been reported, and also genes associated with adhesion or biofilm formation have been described. For example, Lebeer *et al*. (2007) studied the role of *luxS* in biofilm development of *Lb. rhamnosus* GG, and, the *luxS* gene has been detected in streptococci (Stroeher *et al*. 2003), *Campylobacter* spp. (Plummer, 2012) and also *Staphylococcus* spp. (Xu *et al.* 2006). The *luxS* is involved for the synthesis of the autoinducer type 2 (AI-2), which is needed in quorum sensing (Cvitkovitch *et al.* 2003).

It is believed that biofilm formation in a synthetic medium comprises several stages (Figure 4.1) including:

- Movement of planktonic cells from the media toward the solid surface.
- Adsorption of cells at the surface (attachment is easier to coated and hydrophobic surfaces).
- Production of cell–cell signalling molecules.
- Transfer of substrates to and within the biofilm and also enhance substrate metabolism by the cells and transport of products out of the biofilm. During this stage, cell growth, replication, and extra-cellular polysaccharide (EPS) production take place.
- Biofilm disintegration by detachment of attached cells.

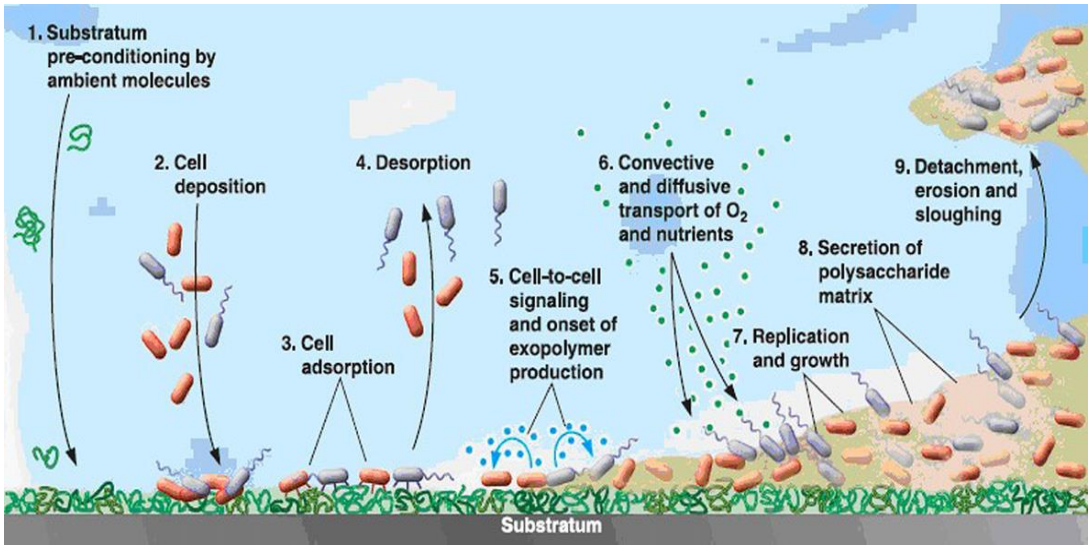


Figure 4.1 Processes governing biofilm formation (Source: Breyers and Ratner (2004) cited in Simoes *et al*. 2010)

4.1.1 Role of surface properties of the cell on adhesion

Microbial attachments depend on cell surface hydrophobicity and the presence of extracellular filamentous appendages (Donlan 2002).

Many types of bacteria have flagella, which are responsible for the motility of bacteria. A flagellum may create an adhesive bond with the adhesion surface.

Pili or fimbriae are fine, filamentous appendages, which are found on many Gram-negative bacteria. They are not involved in motility, but they also are able to make cells more adhesive to other bacterial cells and inorganic particles (Donlan 2002; Simoes *et al*. 2010).

4.1.2 Extracellular polymeric substances (ECPS)

Settled cells bind together (cohesion) and to a solid surface (adhesion) by ECPS. The main compositions of ECPS are polysaccharides and proteins, which account for 75–89%; however, nucleic acids, lipids, phospholipids, and humic substances are also involved in ECPS composition (Sutherland 2001; Tsuneda *et al*. 2003).

4.1.3 Cell–cell communication

The role of cell–cell signalling is important in bacterial attachment or detachment from biofilms (Donlan 2002; Daniels *et al*. 2004), and also where bacteria develop complex systems of interactions and communications to adapt to a new environment. For adaptation to a new situation, bacteria should sense and react to external changes and consequently modulate gene expression (Daniels *et al*. 2004). The process of quorum sensing is considered as auto-induction, which provides an environmental sensing system and enables bacteria to respond to their population densities (Beck von Bodman and Farrand 1995). A diffusible organic signal is generated by cells known as the auto-inducer (AI) molecules and it accumulates in the environment during growth. As a result of accumulation of such molecules, cell densities increase and expression of certain genes is induced (Simoes *et al*. 2010). The physiological response of the cell to a chemical signal depends on concentration of the signal molecule.

The most important AI molecules, which are involved in communication of Gram-positive and Gram-negative bacteria, are oligopeptides and N-acylhomoserine lactones (AHL), respectively. Also, boronated diester (AI-2) is the major molecule involved in communication among both Gram-positive and Gram-negative bacteria (Parsek and Greenberg 2005; Simoes *et al*. 2010). Quorum sensing systems are involved in some important microbial activities such as extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production and ECPS synthesis (Beck von Bodman and Farrand 1995; Daniels *et al*. 2004; Fux *et al*. 2005).

The composition of extracellular matrices in biofilms and the time for their formation are variable and the biofilm produced by different species are easily distinguishable (Simoes *et al*. 2010).

The microbial communities in biofilms might be single or multiple species which are able to form biofilms on biotic and abiotic surfaces. Single species biofilms are formed in many infections, however, multiple species biofilms are found in most environments (O'Toole *et al*. 2000).

Nutrient shortage might act as a particular environmental signal to initiate biofilm formation; however, environmental cues are different for bacteria. In most Gram negative bacteria, transition from planktonic cells to attached cells occurs when they are in nutrient rich medium. In the opposite case of nutrient shortage and in response to starvation, sessile cells turn to planktonic cells by detaching from the surface and searching for new sources of nutrients (Kolter *et al*. 1993). Biofilm of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* develop under good growth conditions. *Escherichia coli* O157:H7 is able to produce

biofilm in low nutrient conditions. However, *Escherichia coli* K-12 and *Vibrio cholera* cannot produce biofilm in minimal nutrient conditions unless amino acids are added to the medium (Dewanti and Wong 1995).

The signal complexity for forming or not forming biofilm might be due to the presence of multiple genetics pathways in the microorganisms, which control those behaviours.

Apart from nutritional signals, other environmental factors which might trigger biofilm development are pH, oxygen, temperature, osmolarity and iron (Lebeer *et al*. 2007).

Applications of probiotic microorganisms, as a remedial approach to target pathogens on the mucosal surface, are quite popular. Therefore, it would be valuable to understand the mechanisms, which might influence biofilm formation by probiotic bacteria. It would also give an insight into how gut microflora is sustained.

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, controversially, 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 would not be welcomed. The concern is that the illnesses caused

by the pathogens may become untreatable if the bacteria become antibiotic resistant (NIH 2002).

The effects of gastrointestinal stresses (e.g. acid and bile), nutritional stress and also source of energy on the potential probiotic lactobacilli may be carried out for assessing biofilm formation. Therefore, this chapter was aimed at examining the potential of the isolates for *in vitro* biofilm formation.

Invitro assessment of the isolates for biofilm formation on the different nutritional and environmental conditions, including:

a) Different media (MRS, NA and TSB).
b) Different media concentration (1, 1/2 and 1/20).
c) Different bile salt concentration (0.3% and 1.5%).
d) pH = 4.0.
e) Presence of inulin.

4.2 MATERIALS AND METHODS

4.2.1 Microorganisms

The name bacteria detailed in Table 3.1 were used in biofilm assay.

4.2.2 Media

The basic medium used in this study was de Man, Rogosa and Sharpe broth (MRS) (CM0359, Oxoid, Basingstoke, UK). Four modified versions of MRS broth were prepared as described below:

A) Acidified MRS: The final pH of MRS was adjusted to 4 by using 2 M HCl.

B) MRS with added bile salt: MRS broth was supplemented with 0.3 and 1.5% bile salt (B3883, Ox-bile, Sigma, Gillingham, UK). Bile salt was solubilized in water, and filter sterilized. Stock was added to autoclaved MRS broth.

C) MRS with added inulin: MRS broth was made by mixing of its basal ingredients but inulin (20g/ml) (Orafti GR, Beneo, Tienen, Belgium) was added instead of dextrose, and modified medium was autoclaved.

Tryptone Soya Broth (TSB) (CM0129, Oxoid, UK), Nutrient broth (NB) (CM0001, Oxoid, UK) and MRS broth, as well as, a diluted form of these media (1/2 and 1/20 strength) was examined. Hence, nine variants of the three media (MRS broth, TSB, NB and their 1/2 and 1/20 dilutions) were prepared and autoclaved at 121°C for 25 min. Anaerobic incubation was carried out using an anaerobic incubator (80% N_2, 10% CO_2, and 10% H_2; Don Whitley, Skipton, UK).

4.2.3 Quantitative biofilm assay

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

After two consecutive subcultures, a selection of single colonies on MRS plates were suspended in 1 ml of sterile MRD (this was used as a stock solution for preparing the final inocula). The working inoculum suspension was prepared by diluting the stock inoculum in 5 ml MRD in glass boiling tube and using a calibrated Sensititre nephelometer (Trek, Diagnostic Systems Ltd., East Grinstead, UK) to measure the turbidity. The final concentration of cells per tube was adjusted to 0.5 MacFarland standard (10^7-10^8 CFU/ml). Each medium (180 µl) was dispensed into the wells of a sterile 96 well flat bottomed polystyrene

micro plate (Sero-Well, Birmingham, 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. The micro plates were incubated anaerobically for 24, 48 and 72 h at 37 °C. At this stage the optical density (OD) was read as indicator of planktonic growth at 595 nm. 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) at 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, Gillingham, 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 dried and 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 recognised: (a) non-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).

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

OD: Mean sample OD_{595}

Strains were therefore classified as follows:

- OD ≤ ODc = non-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.

4.3 RESULTS

In this study the potential for 11 strains of *Lactobacillus* spp. to form biofilm was assessed by microtiter plate assay. Different media with normal and altered compositions were used and the potential for tested strains to grow in these media either in planktonic form or biofilm, were determined.

4.3.1 Influence of normal growth medium on biofilm formation

The general medium, MRS, was chosen as a nutritious laboratory medium that is frequently used for cultivation and enumeration of anaerobes. In this experiment, all tested strains were able to grow well (planktonic growth) in MRS broth after the incubation (Figure 4.2).

According to Figure 4.3 and Table 4.1, apart from three isolates (Lb5, Lb8 and Lb9) no considerable biofilm formation was formed within 24 to 72 h incubation ($p < 0.05$).

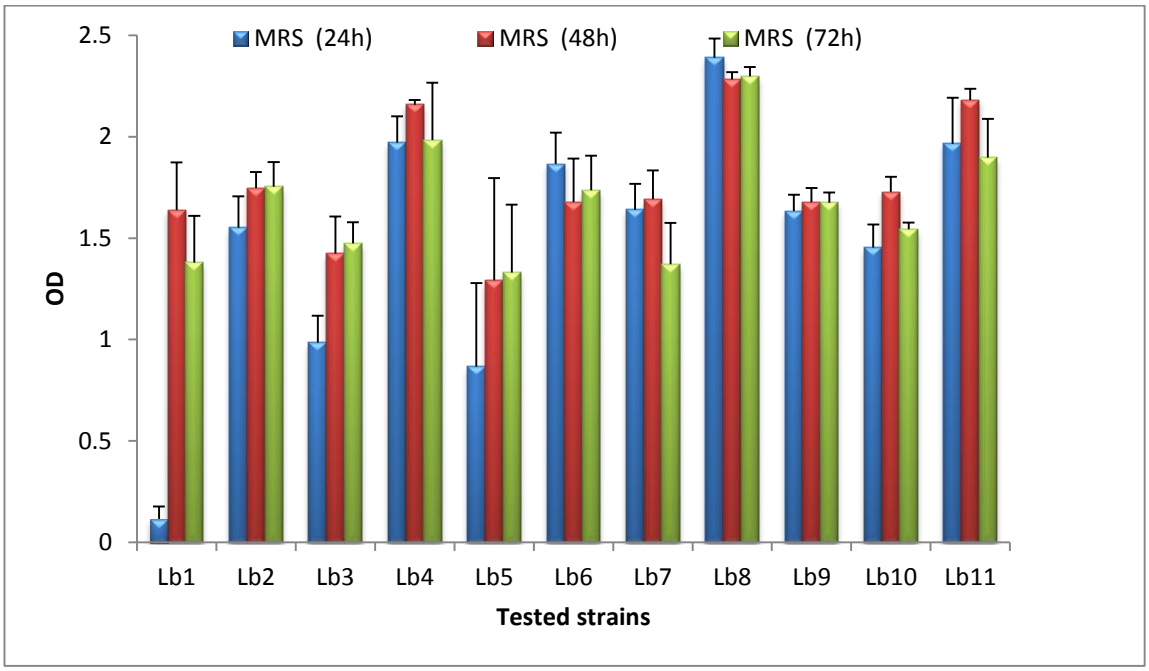


Figure 4.2 Planktonic growth of *Lactobacillus* isolates (expressed as OD) in original MRS broth after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n=9*)

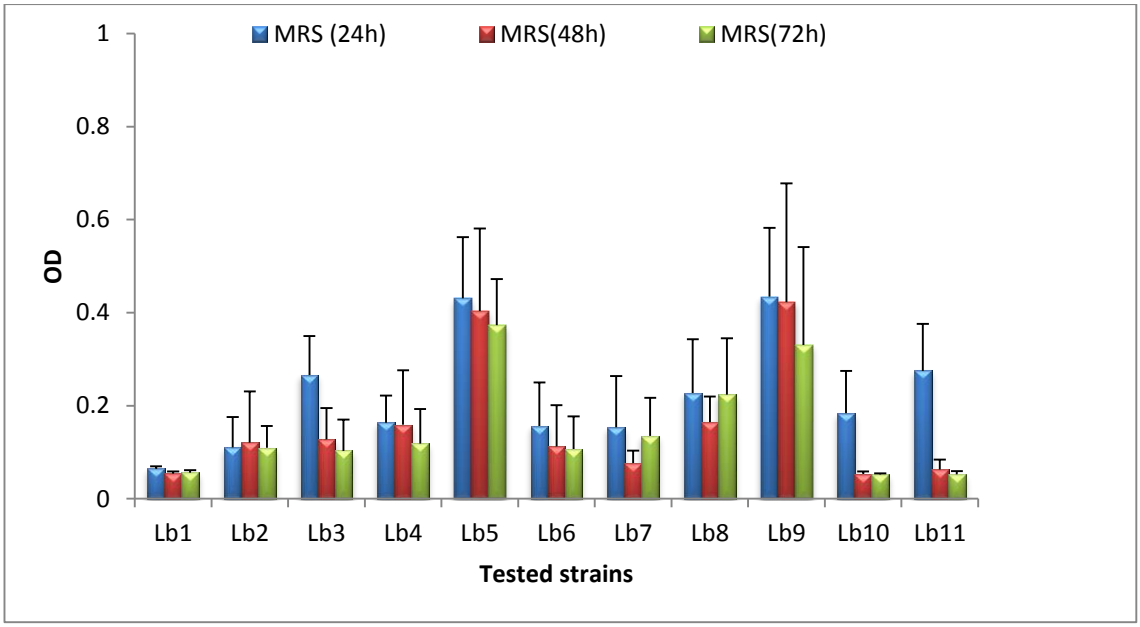


Figure 4.3 Biofilm formation by *Lactobacillus* isolates (expressed as OD) in original MRS broth after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n=9*)

4.3.2 Influence of growth medium adjusted to some gastrointestinal conditions on planktonic growth and biofilm formation.

Some gastrointestinal conditions, such as low pH and presence of bile, were able to affect the suspension growth yield of the tested strains and accordingly, the biofilm formation was influenced as a consequence of these conditions.

4.3.2.1 Low pH

Changing pH in gastrointestinal environment is considered as one of the most important stressful conditions, which might endanger the viability of intestinal bacteria. To test the pH impact on biofilm formation, conditions that probiotic bacteria might face in transition through the GIT.

In the MRS medium adjusted to pH 4, only few strains (Lb2, 4, 5, 8 and 11) were able to grow after 24, 48 and 72 h of incubation (Figure 4.4). However, the biofilm quantities formed by Lb5 and Lb9 were greater than moderate level (Figure 4.5 and Table 4.1). Other tested strains presented no or weak biofilm within 24 to 72 h of incubation. Table 4.1 summarises the extent of biofilm production by all isolates after 24, 48 and 72 h incubation.

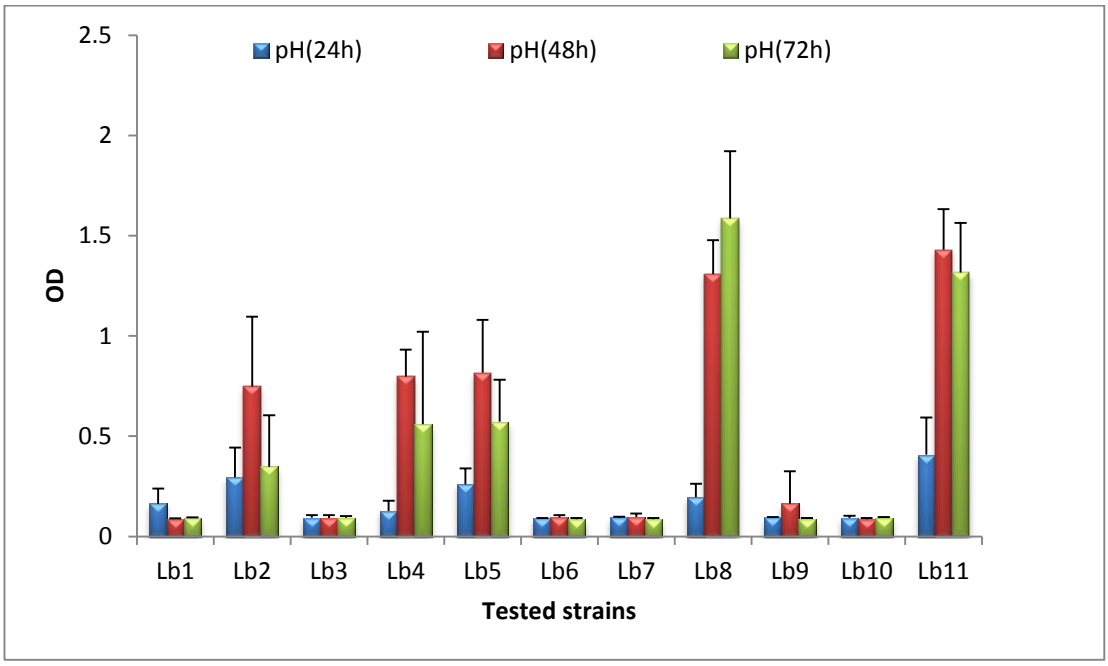


Figure 4.4 Planktonic growth of *Lactobacillus* isolates (expressed as OD) in reduced pH MRS (pH=4) broth after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n=9*)

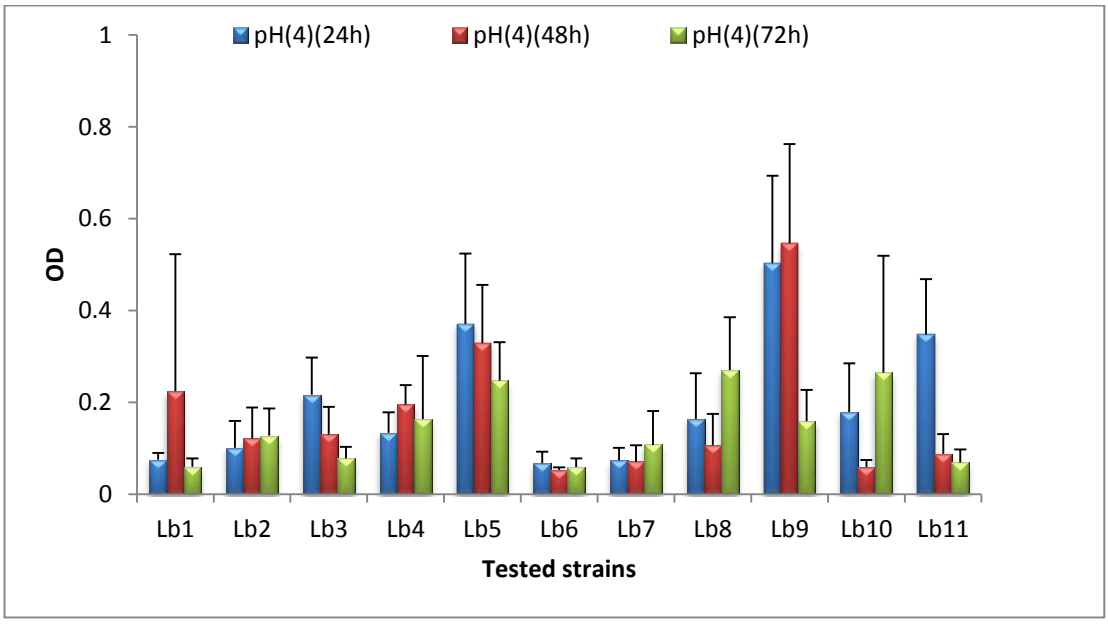


Figure 4.5 Biofilm formation by *Lactobacillus* isolates (expressed as OD) in reduced pH MRS (pH=4) broth after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n=9*)

4.3.2.2 Presence of bile

Bile acids are surface-active molecules, which show antimicrobial activities against enteric bacteria; however, several probiotic bacteria develop mechanisms to resist to bile. In the presence of 0.3% bile, apart from five strains (Lb1, Lb6, Lb7,Lb8, Lb9), the planktonic growth of other strains was limited, but the majority of the tested isolates presented extensive biofilm formation during incubation shown in Figure 4.6 and 4.7 and Table 4.1.

A higher concentration of bile salts (1.5% bile) reduced planktonic growth of some tested strains, but isolates Lb1, Lb3, Lb5, Lb6, Lb7 and Lb11 showed substantial biofilm formation, more than moderate levels, during different stages of incubation (Figure 4.8 and 4.9, Table 4.1).

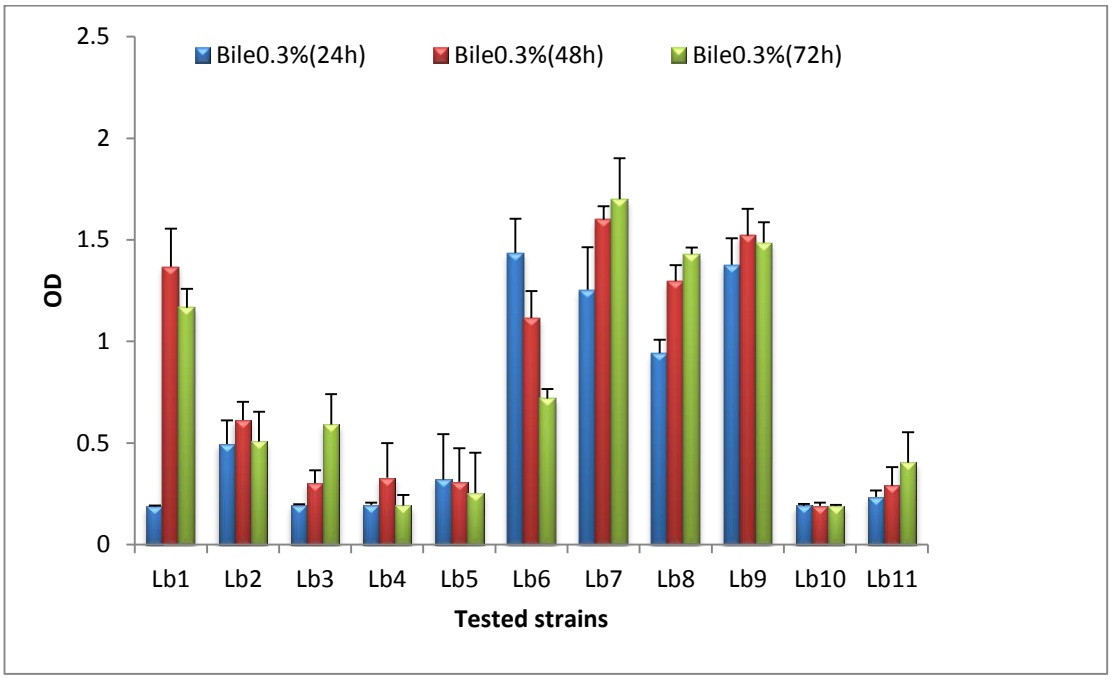


Figure 4.6 Planktonic growth of *Lactobacillus* isolates (expressed as OD) in MRS broth plus 0.3% bile after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n*=9)

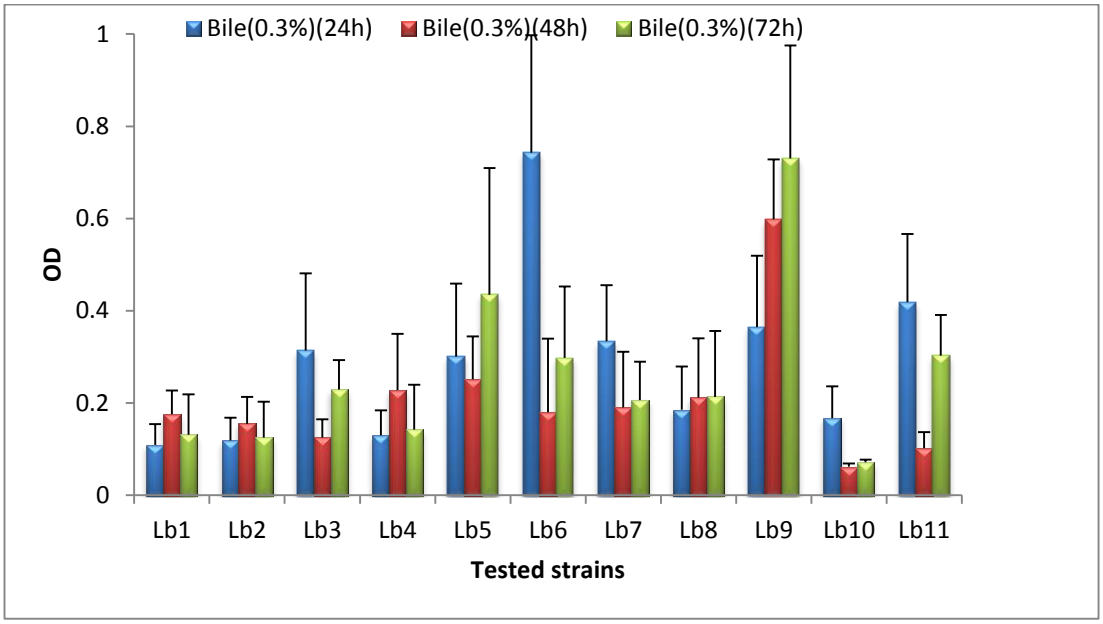


Figure 4.7 Biofilm formation by *Lactobacillus* isolates (expressed as OD) in MRS broth supplemented with 0.3% bile after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n*=9)

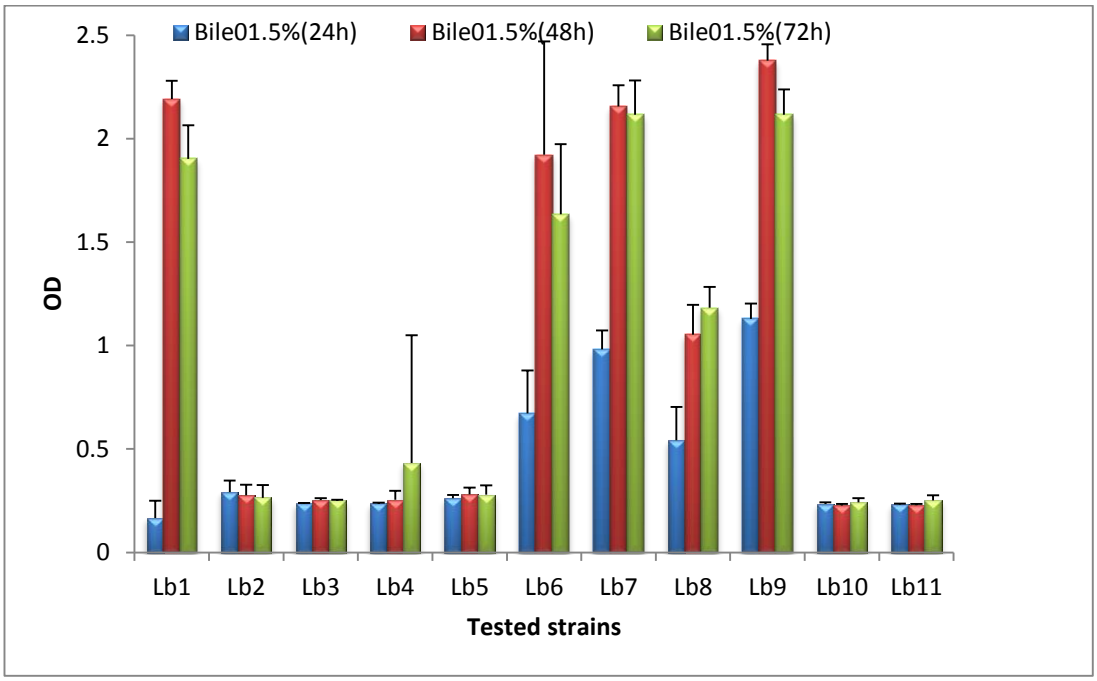


Figure 4.8 Planktonic growth of *Lactobacillus* isolates (expressed as OD) in MRS broth plus 1.5% bile after 24, 48 and 72 h incubation at 37°C

OD: optical density,
Data are means ± SD of three replications (*n=9*)

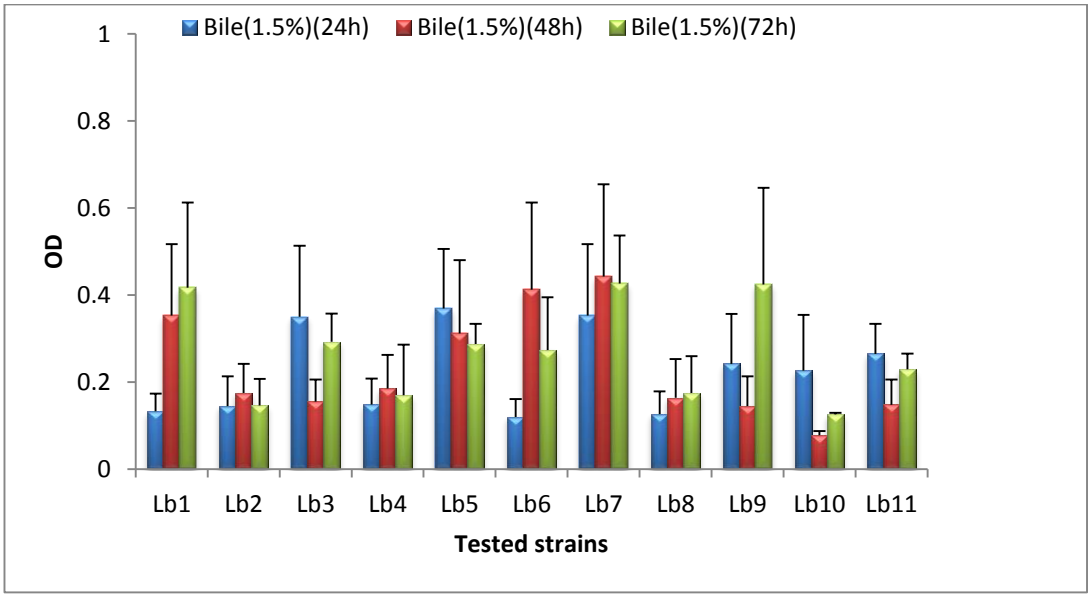


Figure 4.9 Biofilm formation by *Lactobacillus* isolates (expressed as OD) in MRS broth supplemented with 1.5% bile after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n=9*)

4.3.2.3 Presence of inulin

Prebiotics usually carry out their functions in the lower gastrointestinal environment. In the current experiment, the influence of inulin, a non-digestible complex carbohydrate, on potential formation biofilm was also studied. Glucose, the main carbon source for LAB was omitted from MRS medium and was replaced by inulin. The results showed that the planktonic growth of four strains was limited (Figure 4.10) and higher amounts of biofilm were observed only for Lb5 and Lb9 (Figure 4.11 and Table 4.1).

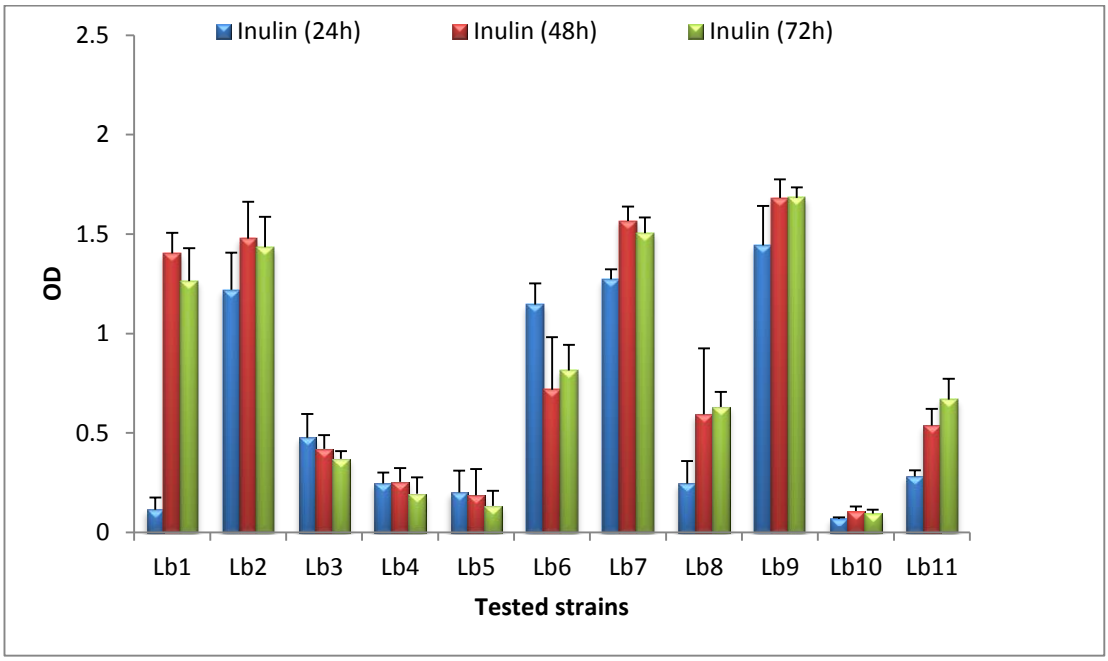


Figure 4.10 Planktonic growth of *Lactobacillus* isolates (expressed as OD) in replaced sugar MRS broth (with inulin) after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n*=*9*)

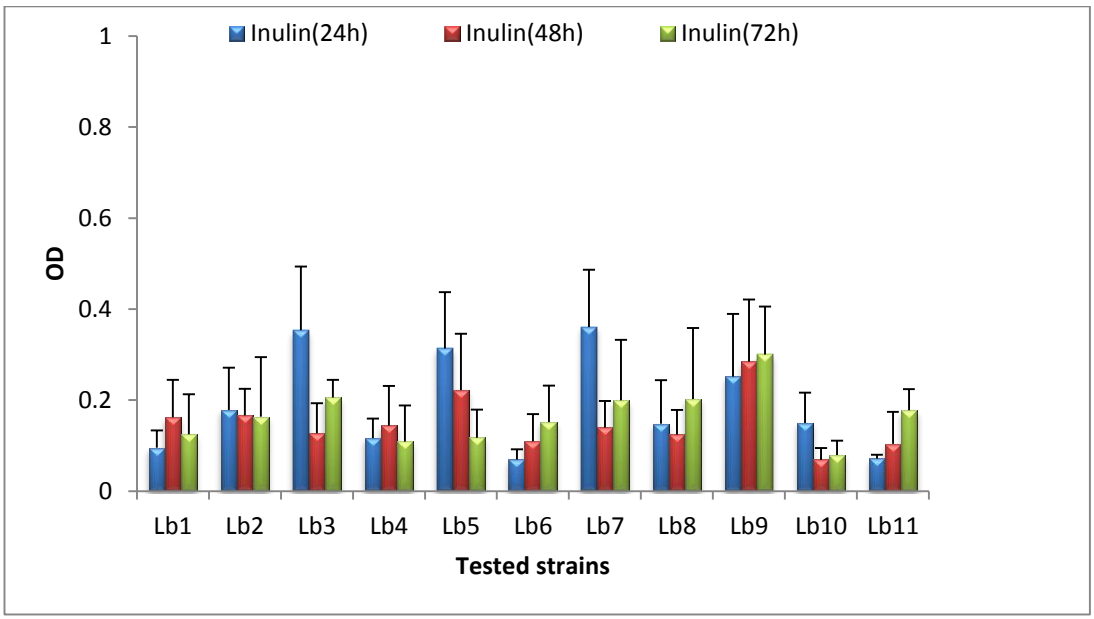


Figure 4.11 Biofilm formation by *Lactobacillus* isolates (expressed as OD) in replaced sugar MRS broth (with inulin) after 24, 48 and 72 h incubation at 37°C

OD: optical density
Data are means ± SD of three replications (*n*=*9*)

Table 4.1 Classification of the tested *Lactobacillus* for biofilm formation in different conditions

	Time	MRS	pH=4	0.3% Bile	1.5% Bile	Inulin
Lb1	24h	-	-	-	-	-
	48h	-	++	+	+++	+
	72h	-	-	-	+++	-
Lb2	24h	-	+	-	-	+
	48h	-	+	+	+	+++
	72h	+	-	-	+	++
Lb3	24h	++	+++	+++	++	++
	48h	+	+	+	-	-
	72h	+	-	++	++	+
Lb4	24h	+	+	-	-	-
	48h	+	++	+	+	+
	72h	++	+	-	+	+
Lb5	24h	++	++	++	++	++
	48h	++	+++	++	+	++
	72h	+++	++	++	++	++
Lb6	24h	++	-	++	-	-
	48h	-	-	+	+++	-
	72h	+	++	+	++	+
Lb7	24h	++	+	++	++	++
	48h	+	-	+	+++	-
	72h	+	-	+	+++	+
Lb8	24h	++	+	+	-	+
	48h	++	+	+	+	+
	72h	++	+	+	+	+
Lb9	24h	-	+++	++	+	++
	48h	++	+++	++	+++	++
	72h	++	++	++	+++	++
Lb10	24h	+	++	+	+	+
	48h	-	-	-	-	-
	72h	-	+++	-	+	-
Lb11	24h	++	+++	+++	+	++
	48h	-	-	-	-	-
	72h	-	-	++	+++	+++

- = No biofilm, **+** = Weak biofilm, **++** = Moderate biofilm, **+++** = Strong biofilm

4.3.3 Influence of growth media and dilutions on biofilm formation

The results of *Lactobacillus* spp. growth in three different media (MRS broth, NB and TSB) and their dilutions revealed that all tested strains were able to grow well in MRS broth, but growth in NB and TSB was less than in MRS broth. Diluting the medium reduced the available nutrients, which significantly influenced the cell growth of tested microorganisms (result not shown).

The results of biofilm formation on plastic surfaces by *Lactobacillus* spp. cultivated in different media and dilutions, thereof, revealed that almost all tested cultures produced biofilm in the original TSB medium. However, the quantities of biofilm produced by the different species varied. The nutrient content of the diluted medium influenced the quantity of biofilm produced by the tested isolates. Overall, TSB was the most effective medium in promoting biofilm production as three out of 11 tested strains were considered moderate biofilm producers in this medium (Table 4.2), followed by NB, in which eight out of 11 tested strains were considered biofilm producers. However, MRS was the least effective medium, in which as five of the tested isolates were not able to produce biofilm after 48 h. The results also illustrated that diluting MRS broth to 1/2 and 1/20 strength resulted in no biofilm formation.

Table 4.2 Classification of the tested *Lactobacillus* for biofilm formation in different nutritional conditions after 48 h

Isolates	MRS	MRS(1/2)	MRS(1/20)	NB	NB (1/2)	NB (1/20)	TSB	TSB (1/2)	TSB (1/20)
Lb1	-	-	-	+	-	+	+	+	+
Lb2	-	-	-	++	++	+	+	++	++
Lb3	+	-	-	+	-	+	+	-	-
Lb4	+	-	-	-	-	+	++	++	-
Lb5	++	-	-	+	+	+	+	++	++
Lb6	-	-	-	-	+	-	+	+	+
LB7	+	-	-	-	+	-	+	+	+
Lb8	++	+	-	+	+	-	++	+	+
Lb9	++	-	-	++	+	-	+	++	++
Lb10	-	+	-	+	-	-	++	++	+
Lb11	-	-	-	+	+	+	+	+	+

- = No biofilm **+**= Weak biofilm **++**= Moderate biofilm **+++** =Strong biofilm

4.4 DISCUSSION

Earlier research has indicated that biofilms are not just stacks of microorganisms with a slime matrix that bonds and safeguards them, instead they are a highly programmed and distinguished population with a greater capability to defy environmental stresses. However, there are very few studies, which elaborate on influences of different stresses on biofilm formation by probiotic cultures. This research focused on biofilm formation by *Lactobacillus* strains under different environmental and nutritional stresses for different incubation times.

The GIT is an important target for probiotics and its environment could influence the probiotic characteristics. Consequently, the effects of some parameters related to the GIT and also the influence of nutritional stresses on biofilm formation capabilities of *Lactobacillus* probiotic strains, were studied, *in vitro*.

The tested conditions including low pH (4.0), two concentrations of bile (0.3 and 1.5%) and also presence of inulin modulated biofilm formation of tested *Lactobacillus* strains and it can be concluded that the effect of each factor depends on the strain.

Numerous studies demonstrated that bile acids are able to increase adhesion and biofilm formation of enteric pathogens (Hung *et al*. 2006; Pumbwe *et al*. 2007). The physiological concentrations of bile ranges from 0.1 to 2.0% in the human intestine (Hung *et al*. 2006). Therefore, it seems reasonable to assume that the observed biofilm formation of some tested *Lactobacillus* strains in the presence of bile is due to stimulation of aggregation of cells, or bile may greatly

affect the cell surface of the tested strains and thereby influence biofilm development.

It has been stated that the presence of bile might be a signal for bacteria to form biofilms in the small intestine (Begley *et al*. 2005). However, the effect of bile does not appear to be dependent on its concentration, since the effect was similar when two different concentrations of bile (0.3 and 1.5%) were added to the medium. This might be due to good tolerance of bile by the tested isolates. The results obtained in this research are in line with those of Lebeer *et al*. (2007) who studied the influences of environmental stresses such as osmolarity, pH, oxygen and bile salts on biofilm production. They demonstrated that tested lactobacilli were able to form biofilms on abiotic surfaces.

This study also demonstrated that depending on the enviromental conditions, some isolates are more readily to form biofilm on plastic surfaces than others. Biofilm formation by these bacteria was considerably affected by the growth medium composition. Media, such as NB and TSB, are not suitable for *Lactobacillus* spp. and they cannot grow well in these media. However, it was decided to include these media in order to study biofilm formation under suboptimal nutritional composition. Biofilm formation in TSB was greater than NB and MRS, and this might be related to the nutritional composition of the media. However, the potential for biofilm formation by most isolates was reduced as the medium became nutrient limited by dilution.

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 enterica* serovar 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.

Lebeer *et al*. (2007) stated that composition of media could influence biofilm formation. They assessed the role of Tween 80, as surfactant active detergent present in MRS broth, on inhibition of biofilm formation. They stated that the effect of Tween 80 was medium specific.

This research found that the potential for tested strains to form biofilm in different media is strain dependent, as not all tested strains were able to produce considerable biofilm in different media. It is not possible to conclude which strain is the highest biofilm producer, as variations were observed according the tested isolate, the media used and the incubation time.

In general, the tested strains produced more biofilm in nutrient rich medium. It seems that there is not a direct relationship between biofilm formation and growth of planktonic cells in the media. Nutrient shortages and low availability of fermentable carbon sources (in diluted media) or the substituting glucose with inulin, led to limited or no growth. However, nutrient limitation alone is not enough to induce biofilm formation. Nevertheless, such a reaction might be different among several strains of the same microorganism. Stepanovic *et al*. (2004) investigated 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 extent of influence on all tested *Salmonella* spp. and *L. monocytogenes* strains.

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 responses of microorganisms to environmental conditions could be the results of mutations in genes that control biofilm formation (Romling *et al*. 1998).

Planktonic growth rates did not correlate with biofilm formation after 24, 48 and 72 h of incubation at 37°C, indicating that differences in biofilm formation were due to factors other than the ability to grow in MRS/modified MRS broth. Initiation, maturation, maintenance and dissolution are sequential steps in formation of biofilm matrix. In this study, biofilm formation was measured after 24, 48, and 72 h of incubation. Within such large time intervals (24 h), numerous changes may occur, and the cells may encounter some stressful conditions. This might explain irregularities in some of the recorded values and lack of any recognisable trend.

Therefore, further *in vivo* studies need to be carried out to examine the capabilities of *Lactobacillus* strains for biofilm formation in conditions mimicking different segments of the GIT, such as the small intestine and colon.

4.5 CONCLUSION

Tested isolates were able to produce biofilm in different media according to their nutritional/environmental stress. The data show some differences among the tested isolates, 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 FIVE: *IN VITRO* ASSESSMENT OF *LACTOBACILLUS* SPP. FOR FUNCTIONAL PROPERTIES: ANTIMICROBIAL ACTIVITIES

5.1 INTRODUCTION

Extensive prescriptions of antibiotics not only have led to an increase in antibiotic-resistant bacteria, but are also associated with the disruption of the protective flora, resulting in the tendency to infections. Hence, the control of infections through a non-antibiotic approach is always needed. Bacterial replacement therapy using non-pathogenic bacteria from the natural flora may introduce a promising alternative (Forestier *et al*. 2001). The most widely studied probiotics are *Lactobacillus* strains, which contribute to counteracting the influence of different infections, such as antibiotic-associated diarrhoea, *Helicobacter pylori* gastroenteritis and urovaginal infections (Reid *et al*. 2003; Canani *et al*. 2007). Lactobacilli can act as microbial barriers against gastrointestinal pathogens through competitive exclusion of pathogen binding, modulation of the host's immune system and production of inhibitory compounds (De Vuyst *et al*. 2004). *Lactobacillus* bacteria have been characterized as Gram-positive, non-sporeforming and non flagellated rods or cocci (Hammes and Vogel 1995), which have beneficial effects on the host by contributing to its intestinal microbial balance (Gomes and Malcata 1999).The distribution of lactobacilli is influenced by several environmental factors, including pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions (Salminen *et al*. 1996). Some of the potential health benefits of probiotic lactobacilli are control of antibiotic colitis by acidity, microbial inhibition and prevention of pathogen adhesion or pathogen activation. In some intestinal disease, altered microflora, impaired gut barrier and different types of intestinal inflammation are present (Salminen *et al*. 1996), thereby effective application of lactobacilli is necessary not only for treatment

but also for prevention of such changes through the production of antimicrobial substances (Gomes and Malcata 1999). There are versatile possible mechanisms responsible for positive clinical responses. It has been reported that lactobacilli promote the immunologic and non-immunologic defense barriers in the gut. They produce inhibitory metabolites, such as organic acids (lactic, acetic and propionic acid) (Makras and De Vuyst 2006), oxygen catabolites, such as hydrogen peroxide (Falagas *et al*. 2007), di-acetyl (De Vuyst *et al*. 2004), carbon dioxide, proteinaceous compounds such as bacteriocins (Zacharof and Lovitt 2012), antifungal peptides/proteins, other metabolites, such as short-chain fatty acids (Carr *et al*. 2002), phenyl lactic acid, and hydroxy-phenyl lactic acid, deconjugated bile salts (Gibson and Wang 1994; Huttunen *et al*. 1995; Magnusson *et al*. 2003; De Vuyst *et al*. 2004).

5.1.1 General antimicrobial properties

Different studies have explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders and infectious diseases (Coconnier *et al*. 2000), but little is known about lactobacilli underlying inhibitory mechanism toward adhesion/invasion action of pathogens. Two different hypotheses, which could explain these findings, are the production of antimicrobial substances and the competitive inhibition of entro-pathogen attachment to epithelial cells by lactobacilli (Santos *et al*. 2003). Ross *et al*. (2002) stated that the incorporation of bacteriocin-producing strains may have the disadvantage of the lack of compatibility with other starter cultures required for fermentation.

5.1.2 Antimicrobial substances produced by lactobacilli

5.1.2.1 Organic acids

Short-chain fatty acids, such as formic, acetic, propionic, butyric, and lactic acids, are produced during the anaerobic metabolism of carbohydrates and have an important role in decreasing pH (Gillor *et al*. 2008). It has been accepted that weak organic acids represent strong antibacterial activity (Ouwehand 1998; Alakomi *et al*. 2000).

The effectiveness of the organic acids as antimicrobial agents is associated with the level of completely un-dissociated acid. Organic acids are more effective antimicrobial agents at lower pH because more undissociated acid is present when pH falls. The effectiveness of dissociated acids is lower because their hydro-phobicity is lower (Yang 2000). The undissociated acid, being lipophilic, can diffuse passively across the membrane (Kashket 1987). The un-dissociated acids collapse the electrochemical proton gradient or alter the cell membrane permeability, which leads to disruption of substrate transport systems (Earnshaw 1992).

Organic acids, in particular acetic acid and lactic acid, have a strong inhibitory effect against Gram-negative bacteria (Makras and De Vuyst 2006). *Lactobacillus* species produce mainly lactic acid, resulting in reduced pH that may suppress pathogenic bacterial growth and proliferation (Spinler *et al*. 2008). The undissociated form of the organic acid enters the bacterial cell and dissociates inside the cytoplasm. Eventually, the decreasing of intracellular pH, accumulation of the ionized organic acid and collapsing in the electrochemical proton gradient have a bacteriostatic or bactericidal effect and result in the death of the pathogen (Kashket 1987; Russell and Diez-Gonzalez 1998). Lactic acid

is detected in low concentrations in the human colon, where it is produced as an intermediary product of carbohydrate fermentation (Topping and Clifton 2001; Macfarlane and Macfarlane 2003; Duncan *et al*. 2004).

Weak acids dissociate in the cytoplasm, resulting in the lowering of isoelectric pH and the accumulation of anions (Salmond *et al.* 1984). This anions accumulation can make an osmotic problem for the cell if it leads to an enhanced in turgor pressure of the cell (Roe *et al.* 1998).

Some reports have suggested that the production of organic acids with concomitant lowering of the pH of the medium is the major factor in the activity of lactobacilli against Gram-negative bacteria (Ogawa *et al*. 2001; Fooks and Gibson 2002; Fayol-Messaoudi *et al*. 2005; De Keersmaecker *et al*. 2006) Acidic pH has been recognized as an important factor tending to limit the populations of certain gut pathogens (Diez-Gonzalez 2007). In the study of Makras *et al*. (2006) on antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium, all *Lactobacillus* tested followed a similar lactic acid production patterns. Moreover, *Lb. johnsonii* La1 produced succinic acid and phenyl lactic acid. *Lactobacillus acidophilus* IBB 801 produced high amounts of succinic acid in addition to phenyl lactic acid and hydroxy phenyl lactic acid. Organic acids, especially succinic, not only fulfill a barrier effect on pathogenic bacteria, but also play an important role in maintenance of the colon health (Cook and Sellin 1998). It has been indicated that inhibitory compounds such as aromatic and heterocyclic molecules e.g. mevalonolactone produced by *Lb. plantarum* E 76, are active against Gram-negative bacteria only at low pH values and in the presence of lactic acid (Niku-Paavola *et al.* 1999).

5.1.2.2 Bacteriocins

Lactic acid bacteria (LAB) are a promising group of bacteriocin producing microorganisms due to their GRAS (Generally Recognised As Safe) status, which reveals their safe application in food (Nishie *et al*. 2012). Among the LAB, lactobacilli have gained particular attention, due to the production of bacteriocins (Zacharof and Lovitt 2012). Bacteriocins produced by lactobacilli are antibacterial proteinaceous compounds that exhibit antagonism mainly against closely related Gram-positive bacteria (De Vuyst and Vandamme 1994; Cotter *et al*. 2005). Bacteriocins are ribosomally synthesised, extracellularly released, low-molecular-mass peptides or proteins, which have a bactericidal or bacteriostatic influence on other bacteria, food-borne pathogen microorganisms and food spoilage bacteria (Klaenhammer 1988). The bacteriocin family includes a diversity of proteins in terms of size, mode of action, microbial target, release, and immunity mechanisms. They are sensitive to proteolytic enzymes such as proteinase K, pronase (Melancon and Grenier 2003).

In a classification system, which is based primarily on the genetics and biochemistry of bacteriocins (Klaenhammer 1993), four classes of LAB bacteriocins have been described: Class I includes of modified bacteriocins, known as lantibiotics, such as nisin (Twomey *et al*. 2002); class II including heat stable, minimally modified bacteriocins, such as lactacin F (Eijsink *et al*. 2002; Drider *et al*. 2006); class III includes larger, heat-labile bacteriocins, such as helveticin J; and class IV encompasses complex bacteriocins carrying other elements, such as lipid or carbohydrate moieties (Heng *et al*. 2007).

Most probiotic researches have focused on classes I and II (Gillor *et al*. 2008). Some of the bacteriocins namely; lactacin F, lactocin 705, lactoccin G and

plantaricin EF are the most important ones, which are produced by *Lb. johnsonii, Lb. casei, Lactobacillus delbrueckii* subsp*. lactis and Lb. plantarum* spp., respectively (Zacharof and Lovitt 2012).

Some other lactobacilli used as starter cultures can produce many different bacteriocins. *Lactobacillus helveticus* produces helveticin J and lacticin LP27 (Joerger and Klaenhammer 1986); *Lb. casei* produces caseicin 80 (Rammelsberg *et al*. 1990); *Lb. acidophilus* produces lacticins B and F and acidocinJ1229 (Tahara and Kanatani 1996) and *Lb. plantarum* produces pediocin ACH (Ennahar *et al*. 1996). The cell target of these agents is the cytoplasmic membrane.

It should be noted that determination of the cytotoxicity is a considerable factor in the characterisation of bacteriocins in order to recommend their application either as a food biopreservative or as an alternative to antibiotics in medical practice.

5.1.2.3 Bacteriocin-like inhibitory substance

In addition to bacteriocins, there are bacteriocin-like inhibitory substance (BLIS) with inhibitory activities against a wide range of Gram-positive and Gram-negative bacteria (Batdorj *et al*. 2006; Cheikhyoussef *et al*. 2009). Bacteriocin like inhibitory substance is small cationic peptide, which are produced by gram-positive bacteria (Chen and Hoover 2003).

Bacteriocin-like peptides are different in molecular mass, antagonistic spectrum and heat resistance (He *et al*. 2006). Because molecular characterization of the compounds has not yet been done, they will be referred to as bacteriocin-like substances (Nemade and Musaddiq 2012). Bacteriocin like inhibitory substance

have molecular masses between 12 to 14 and 30 kDa (Melancon and Grenier 2003), but bacteriocins molecular masses have been reported in the 30 to 50 kDa range (Thompson *et al.* 1996; Van de Guchte *et al*. 2001).

These peptide antibiotics are secondary metabolites, and insensitive to various proteolytic enzymes (Froyshov 1975). Some of the bacteriocin-like peptides are lichenin (Chang *et al*. 2001), bacillocin (Martirani *et al*. 2002) and coagulin (Hyronimus *et al*. 1998).

In some studies, bacteriocin-like substances and other low molecular mass compounds with antifungal activity have been produced by *Lb. pentosus* and *Lb. coryniformis* (Magnusson *et al*. 2003). Other bacteriocin-like inhibitory substances with anti- *H. pylori* activity has been identified in probiotic *Lb. johnsonii* LA1 (Gotteland *et al*. 2008) and *Lb. acidophilus* LB (Coconnier *et al*. 1998).

Some theories have been proposed to explain the mechanism by which antimicrobial peptides kill bacteria. The antimicrobial activity of these peptides has been described as a carpet-like mechanism, where the antimicrobial peptides are proposed to bind to the surface of the membrane and cover it in a carpet-like manner and disturb its barrier function (Jensen *et al*. 1997; Papo *et al*. 2002).

5.1.2.4 Other antimicrobial substances

Common LAB metabolites including acetaldehyde, diacetyl, acetoin and 2, 3-butanediol are active towards Gram-negative bacteria only at high concentrations (De Vuyst and Vandamme 1994). Some substances, such as phenyl lactic acid and hydroxy phenyl lactic acid, are produced by lactobacilli

(Valerio *et al*. 2004), and represent a wide antifungal spectrum in the concentration range of 100–400 µM (Lavermicocca *et al*. 2003). It is relevant to mention that phenyl lactic acid, in concentrations ranging from 20 to 80 mM, is also inhibitory towards Gram-positive bacteria, as well as against Gram-negative bacteria (Dieuleveux *et al*. 1998). *Lactobacillus casei*, *Lb. pentosus*, *Lb. coryniformis*, *Lb. sakei* and *Lb. curvatus* have been reported to harbour antifungal activities. The production of antifungal proteinaceous substances by LAB isolated from Feta cheese has also been reported (Voulgari *et al*. 2010). Antifungal activities of LAB have been attributed to organic acids, cyclic peptides and bacteriocin-like substances (Lavermicocca *et al*. 2000; Magnusson *et al*. 2003). High percentages of H_2O_2-producing lactobacilli from vaginal sources have been found by some researchers (Eschenbach *et al*. 1989; Song *et al*. 1999). Peroxide-producing has been described as a strain-specific property (Annuk *et al*. 2003). On the other hand, H_2O_2 production has been associated with certain species like *Lb. gasseri* and *Lb. crispatus* (Song *et al*. 1999). Huttunen *et al*. (1995) studied production of antimicrobial substances by *Lb. casei* subsp. *casei* LC-10 and *Lb. casei* subsp. *pseudoplantarum* LB1931. In addition to lactic acid predominantly produced in the MRS broth, 2-pyrrolidone-5-carboxylic acid (pyroglutamic acid) was formed and it was separated and found to contribute to antimicrobial activity of the noted lactobacilli. The present study aimed to assess the potential antagonistic activities of *Lactobacillus* spp. against pathogen organisms. The specific objectives were:

a) Assessment of the isolates for production of inhibitory compounds in contact with three pathogen bacteria (spot test).

b) Determining the activity of metabolites obtained from tested *Lactobacillus* cultivated in broth medium.

5.2 MATERIALS AND METHODS

5.2.1 Media

The following media were used: MRS, TSB and NA, all from Oxoid, UK were prepared as recommended by the manufacturer.

5.2.2 Microorganisms

The antimicrobial activities of six *Lactobacillus* spp., which were isolated from yogurt and other fermented milk products (Lb6 - Lb11), two commercial cultures of *Lactobacillus* spp. kindly provided by starter culture supplier, Chr. Hansen (Lb1 and Lb2) and three different type strains purchased from NCIMB (National Collection of Industrial Food and Marine Bacteria, UK) (Lb3, Lb4 and Lb5) were assessed (Table 3.1).

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

- *Salmonella enterica* serovar Typhimurium DT124
- *Salmonella enterica* serovar Enteritidis P125582
- *Escherichia coli* NCIMB 555

Further experiments were carried out by *Lb*. *delbrueckii* subsp. *bulgaricus* to assess any inhibition effects of tested bacteria against Gram-positive bacteria.

5.2.3 Screening lactobacilli isolates for antimicrobial activities

5.2.3.1 Preparation of *Lactobacillus* spp. inoculum

A suspension in MRD (final concentration of 10^7 CFU/ml) was made from overnight culture of isolates grown on MRS agar. For this purpose, after two consecutive subcultures of lactobacilli on MRS agar, 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 reached to 0.5 MacFarland standard (10^7 CFU/ml) using a calibrated sensititre nephelometer (Trek, Dignostic Systems Ltd., East Grinstead, UK). This suspension was used as inoculum for further experiments, described in this chapter.

5.2.3.2 Preparation of indicator organisms

The active overnight culture of the indicator bacteria was prepared by growing each organism on NA. The harvested colonies were added to MRD solution and adjusted with 0.5 MacFarland standard to make a standard suspension (final concentration of ~10^7 CFU/ml) using a calibrated sensititre nephelometer (Trek, Diagnostic Systems Ltd., UK).

5.2.3.3 Assessment of antimicrobial activity of isolates

5.2.3.3.1 Conventional spot 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 MRS agar, and plates were left for half an hour to dry at room temperature and then incubated at 37 °C for 24 and 48 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 24 h aerobically.

5.2.3.3.2 Concurrent spot test

For assessment of competition 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 MRS plates, and then were immediately overlaid with 10 ml of soft TSB, which already was seeded with indicator bacteria and incubated for 24 and 48 h at 37 °C.

5.2.3.3.3 Buffered spot test

In order to exclude the effect of acid on indicator organisms, a buffer was added to the medium to neutralize the produced acid in the medium. MRS containing 2 g/l sodium bicarbonate was prepared for this purpose and spot test was carried out again using overlaying technique after 24 h incubation of isolates.

5.2.3.3.4 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.

5.2.3.3.4.1 Well diffusion assay with un-buffered supernatant

An overnight culture of isolates in MRS broth was centrifuged at 4 °C and 3000 × *g* for 10 minutes and the supernatant was immediately 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 supernatant of the test organisms. All plates were kept 3-5 h in a fridge to allow diffusion of the supernatant (test organism) and then incubated at 37 °C for 24 h aerobically.

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

5.2.3.3.4.2 Well diffusion assay with concentrated, buffered/unbuffered supernatant

An overnight culture of isolates in MRS broth was centrifuged at 4 °C and 3000 × g for 10 minutes. The cell free supernatant was prepared by filtration of the supernatant through 0.2 µm syringe filter.

The cell free supernatant with original pH of 4-4.4 was concentrated to 1/10 and 1/20 of initial volumes by vaccum evaporation at 50 °C (Buchi, Switzerland). These concentrated supernatants were utilized in well diffusion assay. Also, in order to elucidate the mechanism of inhibition in the concentrated filtered supernatant the investigation was continued by neutralising the supernatant to pH 7 using 2 M NaOH. The aim was to exclude any inhibitory effect from organic acids. All plates were kept 3-5 h in a fridge and then incubated at 37 °C for 24 h aerobically.

5.2.3.3.4.3 Sensitivity of produced inhibitory compounds to heat, proteolytic enzymes and catalase, where the inhibition was observed even after neutralisation

The supernatant was tested for heat stability (100 °C for 5 min) using heating block (Techne, Stone, UK) and treated with proteinase K, pronase E (both from Sigma), each at a final concentration of 0.1 mg/ml according to Karaoglu *et al*.

(2002). The samples with proteases were incubated at 30 °C for 1 h and residual activity was determined.

In order to examine whether the inhibition is a result of H_2O_2 production by the isolate, 20 µl of catalase enzyme at a final concentration of 1 mg/ml was added into 180 µl of supernatant. One well having no catalase enzyme was used as the control. The presence or absence of an inhibition zone around wells was determined to be the effect of H_2O_2.

5.3 RESULTS

5.3.1 Spot test

All 11 isolates were studied for their antimicrobial potential against three indicator bacteria using the spot test. In this test, all isolates were first spotted on the appropriate medium and then either straightaway (concurrent overlaying), or after 24 and 48 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.

As Table 5.1 illustrates all isolates showed clear inhibition zone with overlaying after 24 and 48 h and, therefore, were able to inhibit all three used indicator bacteria. Interestingly, inhibition effect of all strains was considerably higher after 48 h of incubation. However, the spot test with concurrent overlaying did not result in inhibition zone for any of the tested isolates (results not shown).

Table 5.1 Antibacterial activity of *Lactobacillus* isolates (Lb6-Lb11), commercial cultures (Lb1-Lb2) and type strains (Lb3-Lb5) against indicator bacteria using spot test.

	Indicator strains					
	MRS agar					
Microorganisms	***Salmonella enterica* serovar Typhimurium DT124**		***Salmonella enterica* serovar Enteritidis P125582**		***Escherichia coli* NCIMB 555**	
	24h	**48h**	**24h**	**48h**	**24h**	**48h**
Lb1	++	+++	++	+++	++	+++
Lb2	+++	+++	++	+++	++	+++
Lb3	++	+++	++	+++	++	++
Lb4	++	+++	++	+++	++	+++
Lb5	++	+++	++	+++	++	+++
Lb6	++	++	++	++	+	++
Lb7	++	+++	++	++	++	++
Lb8	+++	+++	++	+++	++	+++
Lb9	++	+++	++	+++	++	+++
Lb10	+	+++	+	++	+	+++
Lb11	++	+++	++	+++	++	+++

The different scores represent different degrees of growth inhibition expressed in mm
(-) no inhibition
(+) zone of inhibition <10 mm
(++) zone of inhibition between 10-20 mm
(+++) zone of inhibition >20 mm

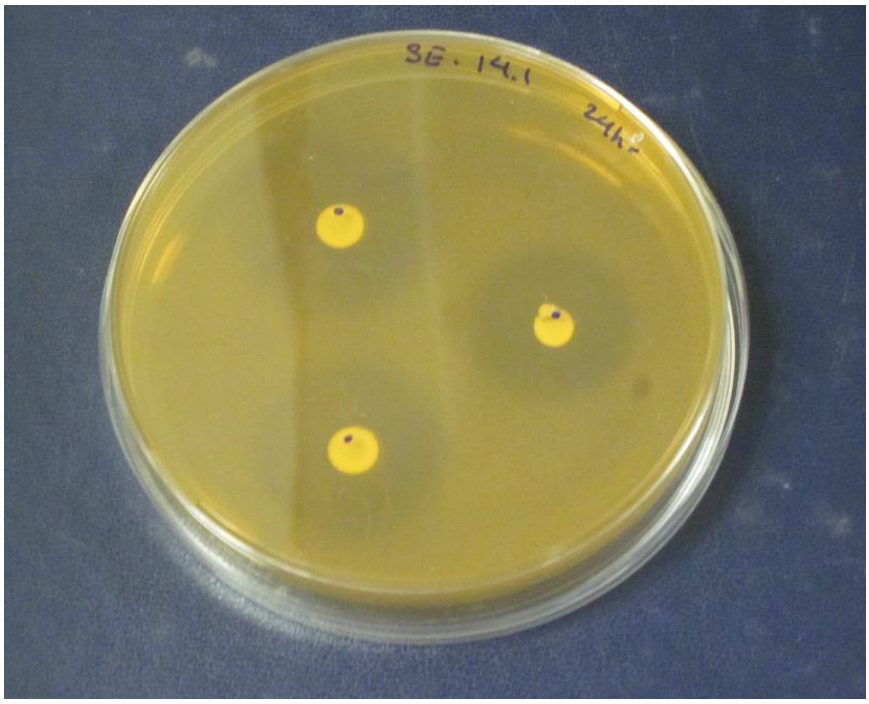


Figure 5.1 Inhibition zone produced by *Lactobacillus* spp. against *Salmonella enterica* serovar Enteritidis using spot test

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

As Table 5.2 shows, when medium was buffered by 0.2% w/v sodium bicarbonate, antagonistic property of two strains (Lb3 and Lb10) disappeared after 24 h. After a longer incubation time (48 h), Lb3 showed some inhibitory effect, but no inhibition was seen for Lb10. All other tested strains displayed a clear inhibition zone, which were far less than that of the un-buffered medium.

Table 5.2 Antibacterial activity of *Lactobacillus* isolates (Lb6-Lb11), commercial cultures (Lb1-Lb2) and type strains (Lb3-Lb5) against indicator bacteria using the medium buffered with sodium bicarbonate in spot test

	Indicator strains					
	Buffered MRS agar					
Microorganisms	***Salmonella enterica* serovar Typhimurium DT124**		***Salmonella enterica* serovar Enteritidis P125582**		***Escherichia coli* NCIMB 555**	
	24h	**48h**	**24h**	**48h**	**24h**	**48h**
Lb1	+	++	+	++	+	++
Lb2	+	++	+	++	+	++
Lb3	-	+	-	+	-	+
Lb4	+	+++	+	++	+	++
Lb5	+	++	+	+	+	+
Lb6	+	+	+	+	+	+
Lb7	+	++	+	++	+	++
Lb8	+	+++	+	++	+	++
Lb9	+	++	+	++	+	++
Lb10	-	-	-	-	-	-
Lb11	+	+++	+	++	+	++

The different scores represent different degrees of growth inhibition expressed in mm
(-) no inhibition
(+) zone of inhibition <10 mm
(++) zone of inhibition between 10-20 mm
(+++) zone of inhibition >20 mm

5.3.2 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 (Table 5.3).

Since none of the cell free supernatants was able to show any antagonistic activity against indicator bacteria (neither un-buffered nor buffered cell free supernatant), it was thought that it could be because of low concentration of antibacterial compounds in the filtered supernatant. Therefore, the cell free supernatant was concentrated to1/10 and 1/20 of initial volumes.

As can be seen in Table 5.3, unbuffered concentrated supernatant showed inhibition zone against indicator bacteria; however, the inhibition strength of the isolates were not consistent amongst tested isolates as they presented larger clear inhibition zone against *S. enterica* serovar Typhimurium DT124.

Table 5.3 Antibacterial activity of cell free concentrated supernatant (1/10 and 1/20) against indicator bacteria using the well diffusion method

	***Salmonella enterica* serovar Typhimurium DT124**					***Salmonella enterica* serovar Enteritidis P125582**					***Escherichia coli* NCIMB 555**				
	NC		**1/10**		**1/20**	**NC**		**1/10**		**1/20**	**NC**		**1/10**		**1/20**
	U	**B**	**U**	**B**	**B**	**U**	**B**	**U**	**B**	**B**	**U**	**B**	**U**	**B**	**B**
Lb1	-	-	+++	+P	++P	-	-	++	++P	++P	-	-	++	-	-
Lb2	-	-	+++	+P	+P	-	-	++	-	-	-	-	++	+P	+P
Lb3	-	-	+++	++P	++P	-	-	++	+P	+P	-	-	++	+P	+P
Lb4	-	-	+++	+P	+P	-	-	+++	-	-	-	-	++	+P	+P
Lb5	-	-	+++	+P	++P	-	-	+++	-	-	-	-	++	+P	+P
Lb6	-	-	+++	+P	+P	-	-	++	-	-	-	-	++	+P	+P
Lb7	-	-	++	++P	++P	-	-	++	-	-	-	-	+	+P	+P
Lb8	-	-	+++	+P	+P	-	-	+++	-	-	-	-	+++	-	+P
Lb9	-	-	+++	+P	++P	-	-	+++	-	-	-	-	++	-	-
Lb10	-	-	+++	+P	++P	-	-	++	-	-	-	-	++	-	-
Lb11	-	-	+++	+P	+P	-	-	++	-	-	-	-	++	-	-

The different scores represent different degrees of growth inhibition expressed in mm

(-) no inhibition
(+) zone of inhibition <10 mm
(++) zone of inhibition between 10-20 mm
(+++) zone of inhibition >20 mm
NC not concentrated (original)
U un-buffered
B buffered
P Partial

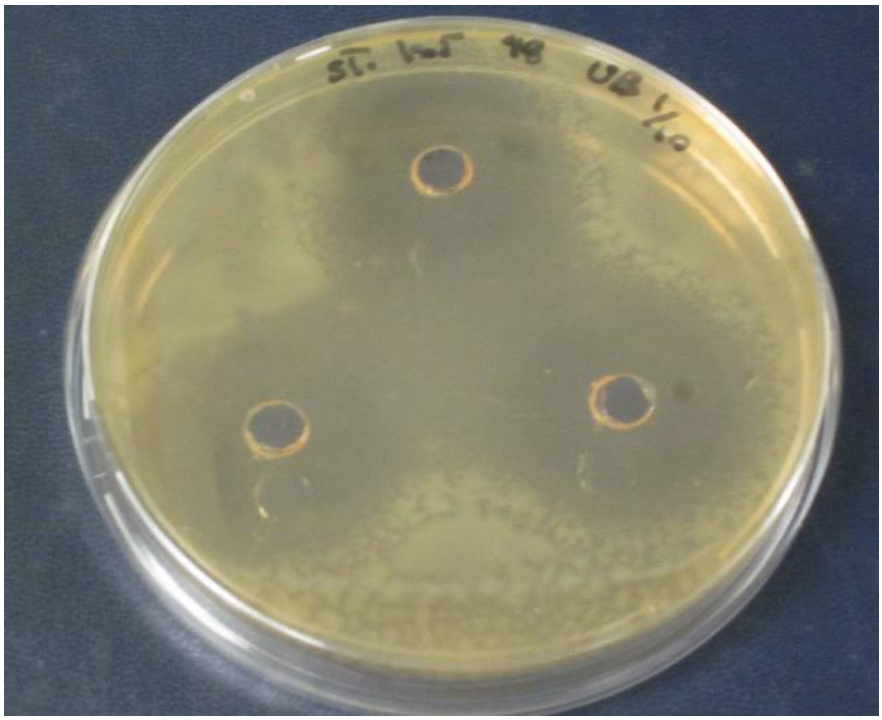


Figure 5.2 Inhibition zone produced by *Lactobacillus* spp. against *Salmonella enterica* serovar Typhimurium DT124 using well diffusion test

Also, when concentrated filtered supernatant (1/10 and 1/20) was buffered by neutralising to pH 7, opaque (not clear) inhibition zone was detected around the wells of all tested strains against *S. enterica* serovar Typhimurium DT124 (it was interpreted as partial inhibition). However, the antimicrobial effects of buffered concentrated filtered supernatant were reduced or disappeared against *S. enterica* serovar Enteritidis P125582 and *E. coli* NCIMB 555.

The antagonistic activity exhibited by different *Lactobacillus* strains was further evaluated against a Gram positive bacterium, *Lb. delbrueckii* subsp. *bulgaricus* 11778. When the neutralized supernatants of tested strains were concentrated to 1/20 of the original volume they all produced inhibition zones (Table 5.4).

The inhibitory action of concentrated buffered supernatants of the tested strains did not disappear by heating at 100 °C for 5 min (except for Lb2, Lb4 and Lb8).

However, the treatment of concentrated buffered supernatants by pronase-E and proteinase-K at final concentration of 1 mg/ml resulted in complete elimination of their inhibitory potency except for Lb5, Lb6 and Lb10.

Table 5.4 Antibacterial activity of cell free concentrated supernatant (20 fold) against *Lb. bulgaricus* using the well diffusion method

	***Lactobacillus delbrueckii subsp. bulgaricus* 11778**					
	20 fold(1/20)					
	UB	**B**	**BH**	**Catalase**	**BP**	**BPN**
Lb1	++	++	++	++	-	-
Lb2	++	+P	-	++	-	-
Lb3	++	++	++	+++	-	-
Lb4	++	+	-	++	-	-
Lb5	+	+	+	++	+	+
Lb6	++	+	+	++	+P	+P
Lb7	++	+	+	++	-	-
Lb8	++	+	-	++	-	-
Lb9	+	+P	+P	++	-	-
Lb10	++	++	+	++	++	++
Lb11	++	+	+	++	-	-

The different scores represent different degrees of growth inhibition expressed in mm

(-) no inhibition
(+) zone of inhibition <10 mm
(++) zone of inhibition between 10-20 mm
UB un-buffered
B buffered
BH heated-buffered
BP Buffered + Protease
BPN Buffered + Proteinase

5.4 Discussion

Several studies have undertaken about the potential of lactobacilli to produce antimicrobial compounds, but still the antimicrobial effects of lactobacilli have not been characterized to a great extent and the antagonistic property of lactobacilli has not been described in as much detail.

Therefore, this study was conducted to assess *Lactobacillus* isolates capabilities for producing antimicrobial compounds.

Antimicrobial production is a strain-dependent property (Annuk *et al*. 2003). As mentioned earlier the production of organic acids and lowering of the pH of the medium is the main factor in the activity of lactobacilli against Gram-negative bacteria (Ogawa *et al*. 2001; Fooks and Gibson 2002; Fayol-Messaoudi *et al*. 2005; De Keersmaecker *et al*. 2006).

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). In this study, apart from organic acids, all tested strains appeared to produce unknown antimicrobial compounds against indicator bacteria. The antibacterial compounds produced by tested strains against *Lb. delbrueckii* subsp. *bulgaricus*, were heat-stable proteinaceous, and lost their activities in presence of proteinase K and pronase E (except Lb5, Lb6 and Lb10). This indicates that the nature of antimicrobial substances produced by tested strains vary and depends on the bacteria that compete with *Lactobacillus* spp.

In this study, all tested *Lactobacillus* spp. showed a good degree of antimicrobial activity against three different indicator organisms using the spot test. Such activity was more noticeable after 48 h of incubation, which may indicate that higher concentration of metabolites, were accumulated through the time. Neutralisation of MRS with sodium bicarbonate, in spot test, diminished antimicrobial activity of all tested isolates, where inhibition zone was observed around the isolates but to a much lesser degree compared to contol (MRS with

original pH). It indicates that production of organic acids might be considered as the main inhibition substances.

Neutralisation of MRS agar in the spot test provides a localised and ongoing neutralisation. In other words, the acids produced by *Lactobacillus* isolates are neutralised as they are produced and, as a result, no reduction in pH occurs.

Zhang *et al*. (2011) stated that the nature of antimicrobial substances are dependent on the production of organic acids, in particular the lactic acid, while bacteriocins are not involved in the antimicrobial activity of the lactobacilli. Also, they concluded that *Lb. johnsonii* F0421 was unable to produce antibacterial substances other than organic acids, but it has a great potential as a gastrointestinal probiotic.

Apart from lactic acid, some of the *Lactobacillus* strains have different organic acid production patterns. *Lactobacillus johnsonii* La1 and *Lb. acidophilus* IBB 801 can produce succinic acid, phenyl lactic acid, and hydroxy phenyl lactic acid (Makras *et al*. 2006).

In some studies it has been hypothesized that bacteriocin is not produced by *Lactobacillus* spp. In a study conducted by Huttunen *et al*. (1995), *Lb. casei* subsp. *casei* LC-10 and *Lb. casei* subsp. *pseudoplantarum* LB1931 did not produce bacteriocin. In addition of lactic acid, which is predominantly produced by these strains, 2-pyrrolidone-5-carboxylic acid (pyroglutamic acid) was formed and contributed to the antimicrobial activity against *Bacillus subtilis* 1205, *B. subtilis* MCM-I, *E. cloacae* 1575 and *Pseudomonas putida* 1560-2.

As previously mentioned, production of short-chain fatty acids has been considered to be the main factor allowing LAB to dominate mucosal ecosystems, but other reports suggest that H_2O_2 production by *Lactobacillus* spp. may be more relevant than acid production (Aslim and Kilic 2006; Kaewsrichan *et al*. 2006). However, addition of catalase to the supernatant in our research did confirm lack of H_2O_2 production and therefore resulted in the inhibition effect of *Lactobacillus* strains via the hydrogen peroxide mechanism.

Other compounds may also contribute towards antimicrobial activity. It has been indicated that aromatic and hetero-cyclic molecules, including mevalonolactone, benzoic acid, methyl hydantoin produced by *Lb. plantarum* E 76, are active against Gram-negative bacteria only at low pH values and in the presence of lactic acid (Niku-Paavola *et al*. 1999).

This study found that unconcentrated culture supernatants of all tested strains did not exhibit inhibitory or suppressive activity against indicator bacteria when tested by the well diffusion method. The inability to detect suppression by well diffusion does not necessarily imply the absence of suppressive activity, but might be due to a lower concentration of such antimicrobial compounds. Confirming this hypothesis the antibacterial activity of tested strains was amplified (noticeable only) after concentrating their filtered supernatant.

According to Toure *et al.* (2003) study, the failure of unconcentrated supernatant to inhibit the same target strain as in the spot test might be due to absence of cell-to-cell interaction between test and indicator bacteria. The presence of *Lactobaillus* spp. along with indicator bacteria in the same medium might induce the production of antimicrobial compounds.

In this research, production of organic acid besides other unknown substances was detected to suppress the growth of indicator bacteria, as when media in spot test or supernatant in well diffusion was buffered by NaOH, this property was reduced or even disappeared in some of tested strains.

Formic, acetic, propionic, butyric, and lactic acids are the main short-chain fatty acids which decrease the pH (Gillor *et al*. 2008). Among these organic acids the suppressive effect on pathogenic bacterial growth due to the reduced pH is mainly related to the lactic acid (Spinler *et al*. 2008). Lactic acid acts as a permeabilizer of the outer membrane of Gram-negative pathogens, allowing other antimicrobial compounds to penetrate the bacteria and thus may trigger the antibacterial activity of other inhibitory substances (Alakomi *et al*. 2000). The suppressive effects of unbuffered concentrated supernatant disappeared when they were buffered in this study. 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). This could explain why the buffered supernatant lost their antimicrobial activity.

Makras *et al*. (2006) distinguished between the influence of lactic acid and other inhibitory compounds produced through investigation of production kinetics of antibacterial activity and applying the appropriate acid and pH control samples. They concluded that the antimicrobial activity of *Lb. acidophilus* IBB 801, *Lactobacillus amylovorus* DCE 471, *Lb. casei* subsp. *casei* Shirota and *Lb. rhamnosus* GG was solely due to the production of lactic acid, while the antibacterial activity of *Lb. johnsonii* La1 and *Lb. plantarum* ACA-DC 287 was due to the production of lactic acid and unknown inhibitory substances.

5.5 CONCLUSION

Our research reported various unknown inhibitory substances and variable mechanisms by which *Lactobacillus* strains can inhibit both Gram-positive and Gram-negative bacteria. It is more likely that the inhibition of indicator organisms by tested strains is the result of the combined effect of organic acids and bacteriocin. However, no further characterisation of these compounds was carried out and such presumption needs further investigation. The use of *Lactobacillus* strains capable of challenging with pathogenic bacteria, provide a useful alternative for inhibiting intestinal pathogens and also improving the intestinal flora ecosystem, especially with the current increase in antibiotic resistance among pathogens. Further characterisation of the inhibitory substances produced by isolated strains and their effects on intestinal flora is necessary in future studies.

CHAPTER SIX: *IN VITRO* ASSESSMENT OF *LACTOBACILLUS* SPP. FOR FUNCTIONAL PROPERTIES: PRODUCTION OF CONJUGATED LINOLEIC ACID

6.1 INTRODUCTION

Conjugated linoleic acid (CLA) contains a heterogeneous group of positional and geometric isomers of linoleic acid (LA) (C18:2). Conjugated linoleic acid is considered as an intermediate product, which is formed by bacteria. Therefore, ruminant meat and also dairy products contain a mixture of CLA isomers. Various isomers of CLA have different structure and present more than one biochemical mechanism in their specific effects (Sieber *et al.* 2004). The biological activities of CLA mainly are attributed mainly to the presence of *cis9-trans11* CLA (known as rumenic acid) and *trans10- cis12* CLA. Among all different CLA isomers which have been identified in natural foods, predominantly in ruminant meat and dairy products, 75%–90% is accounted to *cis9- trans11* CLA (Kelley *et al*. 2007).

The positive benefits of CLA have been welldocumented. Some important health-promoting properties, such as antiatherosclerotic, anticarcinogenic, antiadipogenic, immunoenhancing, antioxidative, hypotensive, and anti-inflammatory effects, are attributed to CLA (Nagao and Yanagita 2005; Bhattacharya *et al*. 2006; Kelley *et al*. 2007; Benjamin and Spener 2009).

6.1.1 Structure of CLA

Conjugated linoleic acid structure contains 18 carbon chains, which are connected to a carboxyl group at the end, and conjugated double bonds present along this chain (Figure 6.1). Double bonds in CLA isomers are adjacent to each other (i.e. –C=C-C=C–). Due to several possible locations for double bonds (C7 and C9; C8 and C10; C9 and C11; C10 and C12; C11 and C13 or C12 and C14) and also *cis* or *trans* configuration, which depends on whether they are located

on the same side or the opposite sides of the double bond, respectively, a wide variety of CLA isomers could be generated (Jang *et al*. 2005). Twenty eight possible positional and geometrical isomers of CLA were reported by Liu *et al*. (2011). However, it does not necessarily mean that all CLA isomers occur in significant amount.

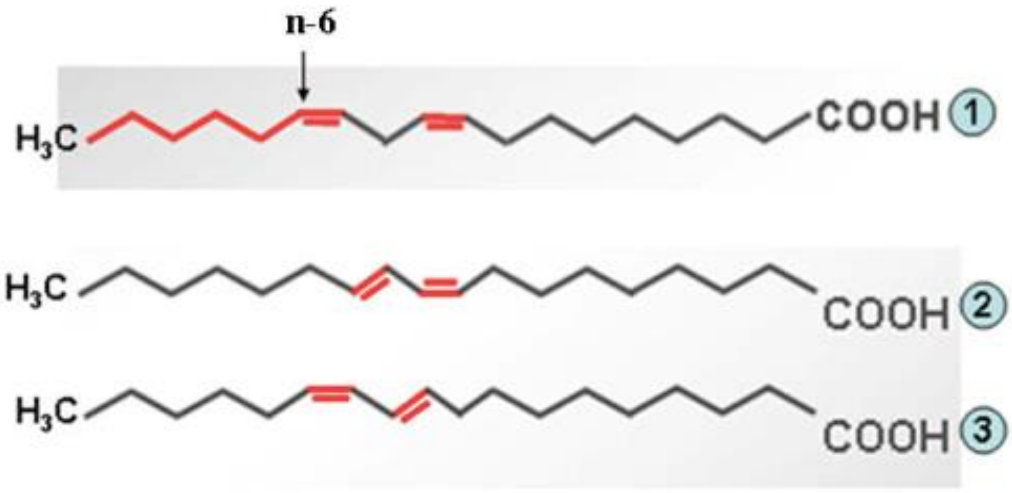


Figure 6.1 Structures of linoleic acid (typical n-6 polyunsaturated fatty acid) (1), *cis9- trans11* octadecadienoic acid (in fact an n-7 fatty acid) (2) and *trans10-cis12* octadecadienoic acid (3) (Source: Benjamin and Spener 2009).

6.1.2 Sources of CLA

6.1.2.1 Ruminant

Compared to non-ruminant animals, ruminant's products (meat and milk) contain higher levels of CLA, and such a difference is solely the result of LA biohydrogenation in the rumen (Coakley *et al*. 2003). The main dietary sources of CLA for human consumption are ruminant fats and dairy products. *Butyrivibrio fibrisolvens* plays an important role in the conversion by biohydrogenization of LA to CLA, which naturally takes place in rumen such as cows. It is interesting

to know that the concentration of CLA in some dairy products are even more than the fat and meat of ruminants (Table 6.1).

Table 6.1 The mean positional /geometric isomer composition (% of total isomers) and the CLA content of milk, butter, cheese, and beef fat (Source: Khanal, 2004).

CLA isomer	Milk	Butter	Cheese	Beef
***cis, trans*- isomers**				
7,9	5.5	6.7	3.6	7.0
8,10	1.5	0.3	1.0	2.6
9,11	72.6	76.5	83.5	72.0
10,12	0.4	1.1	-	2.6
11,13	7.0	0.4	4.7	1.1
12,14	0.7	0.8	0.4	0.7
Total *cis, trans*	87.7	85.8	93.2	86.0
***trans, trans* isomers**				
6,8	-	-	0.1	0.7
7,9	2.4	-	0.6	1.5
8,10	0.4	-	0.3	0.7
9,11	2.0	-	1.5	3.7
10,12	0.6	-	0.5	1.9
11,13	4.2	-	2.3	1.9
12,14	2.8	-	0.9	1.9
13,15	-	-	0.1	-
Total *trans, trans*	12.3	9.4	6.3	12.3
***cis, cis* isomers**				
8,10	-	-	0.1	-
9,11	-	-	0.3	-
10,12	-	-	0.3	-
11,13	-	-	0.3	-
Total *cis, cis*	-	4.8	0.7	-
Total CLA (% of fat)	-	0.5	0.93	0.27

It has been stated that CLA content in milk depends on cattle feed pattern (fresh pasture versus regular corn diet), the presence of poly unsaturated fatty acids

(PUFA) in their diet, breed of cow and also season. As a result for example, the CLA content of milk is considerably higher in May, June and July (Sieber *et al*. 2004; Nieuwenhove *et al*. 2007).

CLA formation in ruminants (meat and milk) occurs by transformation of LA by anaerobic ruminal bacteria and also by conversion of vaccenic acid in the mammary gland.

Butyrivibrio fibrisolvens, a strict anaerobic bacterium, found in human faeces and in rumen fluid of sheep (Sieber *et al*. 2004), is able to hydrogenate the PUFA in consumed food. It has been reported that the biohydrogenation of LA to stearic or oleic acids in rumen includes several steps (Kepler *et al*. 1966). At the first step of bioconversion, in presence of LA isomerise enzyme, *cis9-trans11* CLA are formed. In the next step, by the partial hydrogenation of *cis9-trans11* CLA, trans-vaccenic acid (*trans11* C18:1) is produced and finally this substrate is reduced to stearic acid at the last step (Figure 6.2). Conjugated linoleic acid may also be produced from the vaccenic acid (*trans11* -C18:1) by the action of Δ9-desaturase in the mammary gland (Parodi 1994).

The toxicity of CLA to rumen bacteria is less than LA, and the toxicity of saturated fatty acids is much less than polyunsaturated fatty acids; therefore, biohydrogenation is a detoxification activity to reduce the harmful effect of free fatty acids (Jenkins and Courtney 2003).

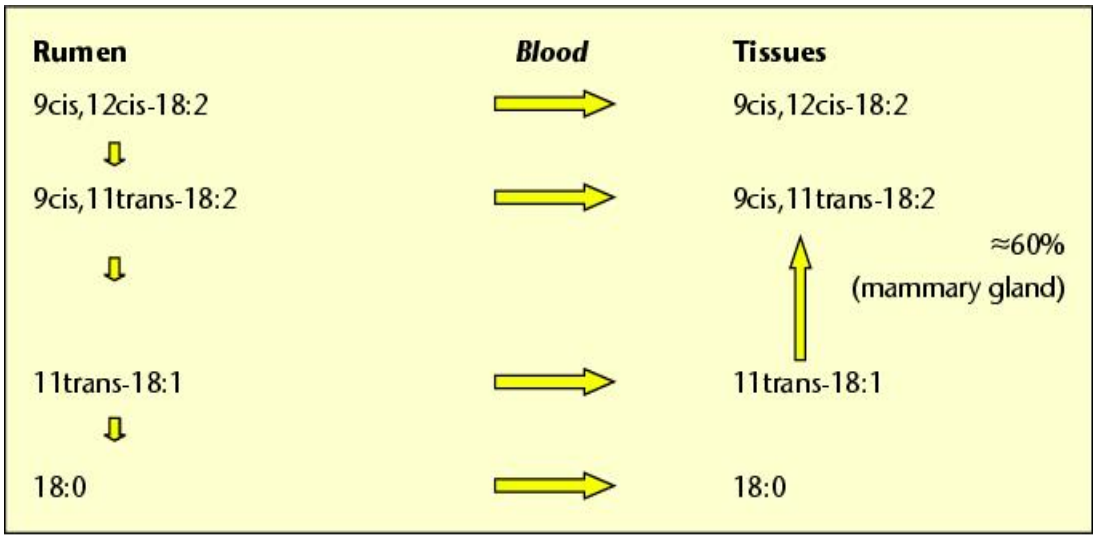


Figure 6.2 Metabolic pathways involved in the biosynthesis of rumenic acid in ruminants (Source: Chardigny *et al.* 2005).

6.1.2.2 Non-ruminant

A variety of animal and plant derived foods contain CLA. According to the nature of product, the amount and distribution of CLA isomers may vary.

Meat from non-ruminal animals such as pigs contains low quantities CLA which might originate from their feed that contain CLA (Fritsche *et al*. 1999).

Conjugated linoleic acid was reported in turkey meat which contains 2.5 mg/g fat. This amount of CLA is significant compared with other non-ruminant animal meat (Jang *et al.* 2005). The content of CLA in egg yolk is minimal. Some plant oils such as olive, sunflower and canola contain small amount of CLA. Vegetable oils and partially hydrogenated oils such as shortenings and margarines are considered as main sources of *trans10- cis12* CLA (Sieber *et al*. 2004).

6.1.2.3 Chemical isomerisation

Chemical isomerisation is a commercial technique for production of *cis9-trans11* and *trans10- cis12* CLA from LA, but they are not pure and are contaminated with different other isomers and toxic materials. Therefore, such synthesized CLAs are not reliable and recommendable (Pariza *et al*. 2001).

6.1.3 CLA in dairy products

Conjugated linoleic acids are extensively found in numerous foods, mainly in dairy products and meat from ruminants.

Shantha *et al*. (1995) reported 5.25 mg CLA/g fat in yogurt with 0.05% fat compared to 4.4 mg CLA/g fat in the skim milk before fermentation. However, others reported no significant difference between CLA content of milk and its fermented products (Boylston and Beitz 2002).

Lactic acid bacteria (LAB) and propionibacteria are the main starter cultures used for producing Emmental cheese. Some strains of propionibacteria show good potential for formation of CLA from free LA. Therefore, an increased level of CLA is expected in this Swiss cheese (Sieber *et al.* 2004). As a consequence of lipolytic strain activities during cheese ripening, LA is released continuously and could then be converted to CLA.

6.1.4 Health benefits

Several animal models and cell culture studies reported that a daily CLA consumption delivers positive effects on health. Some of their potential beneficial effects are discussed below.

6.1.4.1 Anti-obesity

Animal studies showed that CLA improve feed efficiency and induce greater growth rate. Conjugated linoleic acid changes body composition, reduces whole body fat and significantly increases body protein and ash (Khanal 2004). Conjugated linoleic acid might also increase lipolysis and energy expenditure and decrease lipogenesis (Salas-Salvado *et al*. 2006). *Trans10- cis12* CLA is responsible for lipid metabolism (Sieber *et al.* 2004).

6.1.4.2 Anti carcinogenesis

Conjugated linoleic acid may suppress cancer by inhibiting the growth and spread of tumours. It has been claimed that the action of CLA to inhibit the tumours is fast. However, anticarcinogenic activities of CLA are quite complex. It is also reported that cell transformation through signal transduction might be inhibited by CLA (Benjamin and Spener 2009). Conjugated linoleic acid also presents antioxidant properties and quenches free radicals. The mixture of both isomers (*cis9- trans11* and *trans10- cis12*) is more effective than either isomer alone. The *cis9- trans11* CLA isomer has the most biological activities, and presents an anticarcinogenic effect (Sieber *et al.* 2004).

6.1.4.3 Anti atherosclerosis

Atherosclerosis is known as hardening of the arteries. When fat and cholesterol precipitate in the artery walls, hard structures called plaques are formed and might block the arteries.

Conjugated linoleic acid, as dietary fatty acid has presented some anti-atherogenic activities in animal models of atherosclerosis. Conjugated linoleic acid is capable of diminishing the level of atherogenic low density lipoprotein

cholesterol (LDL) in plasma and also increase the level of anti atherogenic high density lipoprotein cholesterol (HDL) (Weldon *et al.* 2004).

6.1.4.4 Bone formation

Ostheosynthesis also is affected by different isomers of CLA. Animal studies show that CLA increases calcium absorption from diet and reduces the rate of bone resorption (Kelly and Cashman 2004). Also CLA has been suggested as a preventive supplement for rheumatoid arthritis (Hur and Park 2007). Platt *et al.* (2007) reported that number and size of mineralized bone nodules were increased by *cis9- trans11* CLA; however, *trans10- cis12* did not show such effect and it is an evidence of isomer-specific effects of CLA on health benefits.

6.1.5 Safety of CLA

Although many positive health benefits of CLA have been stated, there are some negative impacts of concern. Some negative results were obtained in some animal studies. There were indications of increased liver and spleen weight and as well as insulin resistance (Clement *et al*. 2002; Syvertsen *et al*. 2007). One should also bear in mind that different animal or human studies use different CLA (mixed or individual isomer), and due to isomer-specific effects of CLA isomers, results from such studies might considerably vary and sometimes report conflicting and less convincing for health benefits of the components (Benjamin and Spener 2009).

The adverse effects of CLA, such as insulin resistance and non-alcoholic fatty liver disease were attributed to the shortage of n-3 PUFA in the diet, and by inclusion of n-3 PUFA (such as α- linolenic acid), the reported adverse effects would be avoided (Kelley *et al*. 2009). Also it was concluded that most of the

reported adverse effects are related to the short-term studies in humans, and in long term adverse effect rate was decreased (Gaullier *et al*. 2005).

6.1.6 CLA intake

Conjugated linoleic acid level is estimated at 0.1% (w/v) of the total fatty acid content in the human body (Zlatanos *et al*. 2008), but CLA level in foodstuffs does not seem to be sufficient for any significant therapeutic effect (Chung *et al*. 2008). Conjugated linoleic acid intake can be improved by manipulations of food products. Fortification of the ruminant diet with vegetable oils is an important practice for increasing of CLA level (Sieber *et al.* 2004).

Desirable consumption levels of CLA on human health is not well documented, However, based on different studies, It has been suggested that for obtaining positive biological effects, CLA should be taken about 2-3 g per day for 6 to 12 months (Sieber *et al*. 2004; Whigham *et al*. 2007), and CLA in form of triacylglycerol is the best form for consumption, as *cis9- trans11* and *trans10-cis12* are absorbed similarly into chylomicrons (Fernie *et al.* 2004). However, a mixture of n-3, n-6 (CLA) and n-9 fatty acids in human diet can be suggested to ameliorate any possible adverse effect of CLA consumption (Kelley *et al*. 2009).

6.1.7 Bioconversion of LA by bacteria

Apart from rumen-derived bacteria, certain bacterial strains used in fermented dairy foods are able to biosynthesise the CLA isomers (Sieber *et al*. 2004). Therefore, these bacteria could offer an opportunity for manufacturing enriched CLA products. It is noticeable that environmental parameters affect the production of CLA by these strains (Jang *et al.* 2005).

The present study aimed to assess the capabilities of the isolates for converting LA to free CLA. The specific objectives were:

a) *In vitro* assessment of the isolates for the conversion of different concentration of LA to CLA.
b) *In vitro* assessment of the isolates forfor the conversion of LA to CLA at different incubation time (24 and 48 h).

6.2 MATERIALS AND METHODS

6.2.1 Microorganism and culture conditions

In this study, a total of 11 lactobacilli were screened, including six isolates from yogurt and other fermented milk products, 2 commercial cultures of *Lb. acidophilus* (La5) and *Lb. casei* (C431) kindly provided by Chr. Hansen as well as three different type strain lactobacilli purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, UK) (*Lb. casei* subsp. *casei*, *Lb. paracasei* subsp. *paracasei* and *Lb. acidophilus)* (Table 3.1). The cultures were prepared and kept on beads as described earlier (section 2.2.4).

6.2.2 Inoculum preparation

Three consecutive subcultures (anaerobically at 37 °C for 24 h on MRS agar) were carried out on all bacterial cells kept at - 20 °C on beads in cryovials before use. The active inoculum's concentration was adjusted to ~10^7-10^8 CFU/g by comparing the turbidity of the bacterial suspension in Maximum Recovery Diluent (MRD) with 0.5 MacFarland standard using sensititre nephelometer (Trek, Diagnostic Systems Ltd., East Grinstead, UK).

6.2.3 The extraction of CLA

Quantification of CLA formation was performed according to the method of Alonso *et al*. (2003) with some modifications. The extraction of CLA involved the following main steps:

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

6.2.3.1 Preparation of stock solution of LA

A stock solution of LA (30 mg/ml) was prepared by suspending 30 mg LA/ml in 2% v/v Tween 80 (polyoxyethylene sorbitan monooleate) solution. Tween 80 was needed to dissolve LA in the medium. The resulting solution was filter sterilized through a 0.45 µm syringe filter (Nalgene, Fisher Scientific, Loghborough, UK) and stored in the dark at – 20 °C until use.

6.2.3.2 Measuring microbial production of CLA

In order to assess *in vitro* production of CLA by the isolates, the reaction medium was prepared. The stock solution of LA was added to MRS broth to a final concentration of 0.2 and 0.5 mg LA/ml.

One percent inoculum (v/v) of each strain was added to 10 ml reaction medium, and incubated anaerobically at 37 °C for 24 and 48 h. The CLA content in the cultures was expressed as percentage of the added LA, using the following equation:

$$\% \text{ CLA} = \text{CLA} / (\text{CLA} + \text{LA}) \times 100.$$

6.2.3.2.1 pH value

The pH of the reaction medium was measured with a pH meter instrument (Whatman PHA 2000), which was calibrated using pH buffer at pH 4·0 and 7·0 according to the operating manual.

6.2.3.2.2 Viable count

The viable count of the reaction medium was determined by plating serial dilutions of the bacterial suspensions on MRS agar (Oxoid) followed by incubation under anaerobic conditions at 37 °C.

6.2.3.3 Lipid extraction from bacterial supernatant fluids

Samples (500 µl) were taken after 24 and 48 h incubation and centrifuged at 4 °C at 7000 x *g* for 10 min. The supernatant was mixed with 15 ml hexhane/isopropanol (HIP) (3:2 v/v) (Fisher Scientific, Loughborough, UK) in a universal bottle and 0.01% v/v butylated hydroxytoluene (BHT) (Fisher Scientific, UK) was added to the solvent to prevent potential oxidation. All samples were flushed for about 1 min with nitrogen and stored overnight at 4 °C.

6.2.3.3.1 Partitioning

The HIP mixture (15.5 ml) was fully evaporated under nitrogen flush for 1 h and 3 ml of chloroform/methanol (2:1 v/v) (Fisher Scientific, UK), BHT (0.01% v/v) and 1 ml of distilled water was added to each tube. The tubes were vortexed and the mixture centrifuged at 2500 x *g* for 5 min. After centrifugation, there was a clear separation of the organic phase (bottom layer) from the aqueous phase (top layer). The lipid enriched organic phase was collected and transferred to

new tubes, and the solvent was fully evaporated under nitrogen flush as described above.

6.2.3.3.2 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 for few second, sealed and left in the oven at 70 ^{o}C for 3 h. Afterwards, 4 ml of 5% w/v NaCl and 2 ml of petroleum ether (Fisher Scientific, UK) 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 and the tubes were shaken again and the upper petroleum ether was transferred to the tube containing 2% (w/v) potassium bicarbonate. The tubes were then vortexed and the upper layer was transferred to a new tube containing 100-200 mg of dried granular sodium sulphate. This solution containing fatty acid methyl esters (FAME) in petroleum ether was transferred to a 3 ml glass vial. The petroleum ether was removed under nitrogen and the samples were dissolved in 1 ml heptane (Fisher Scientific, Loughborough, UK) and BHT (0.01% v/v). The samples were flushed under nitrogen and stored at -20 ^{o}C until use.

6.2.3.3.3 Gas chromatography

Fatty acid methyl ester (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 4 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).

6.2.4 Statistical analysis

Results are the mean±standard deviation. Data were analysed using the univariate analysis of variance was performed using SPSS 21 software (Chicago, IL: SPSS Inc) to determine the statistical significance of differences. Data were considered significantly different when $p < 0.05$.

6.3 RESULTS

Eleven *Lactobacillus* isolates were studied for their ability to convert free LA to CLA. These bacteria were selected from different origin (dairy products, commercial cultures and type strains).

6.3.1 Effect of the incubation time and LA concentration on the growth of tested isolates

The effect of free LA on cell counts of the tested bacteria was determined by enumeration of viable cells after 24 and 48 h of incubation. As shown in Figure 6.3, the growth of all isolates was not affected by the presence of 0.2 mg LA/ml. However, the growth of some of the isolates was rather inhibited in the presence

of 0.5 mg LA/ml and, in most isolates the reduction was not considerable after 24 h (Figure 6.4).

Enumeration after 48 h in MRS+0.2 mg LA/ml showed higher counts compared to the enumeration after 24 h. Also in MRS+0.5 mg LA/ml, except few isolates (Lb3, Lb7, Lb8), all others presented higher number after 48 h ($p < 0.05$).

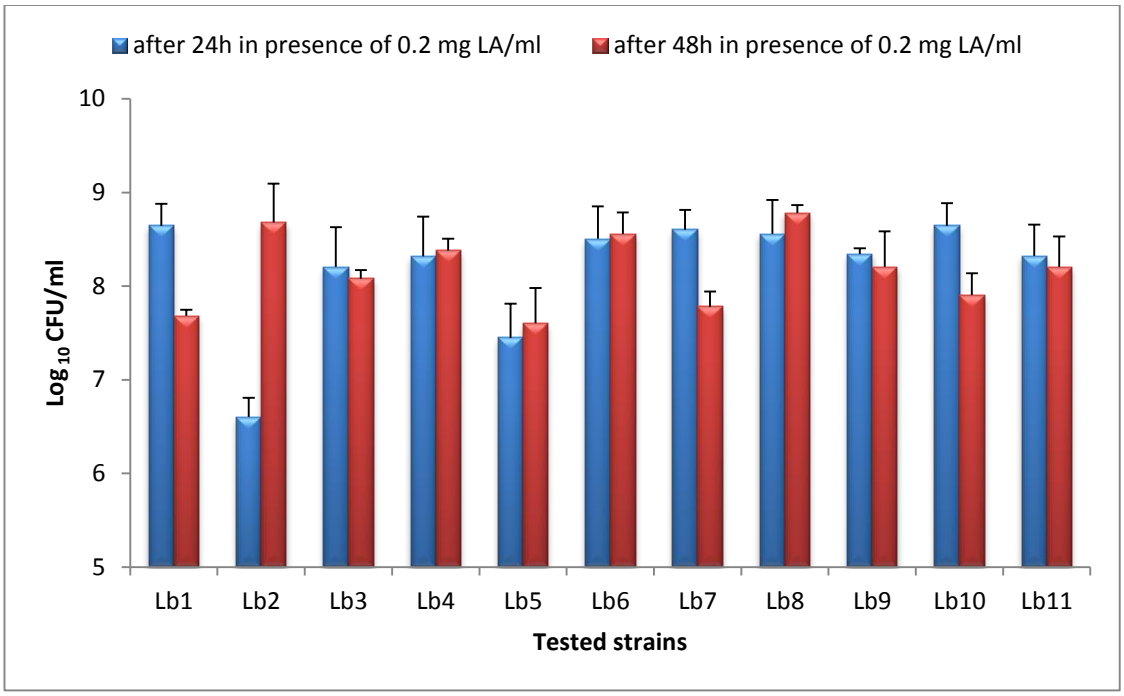


Figure 6.3 Number of *Lactobacillus* spp. in MRS broth with LA (0.2 mg/ml) after 24 and 48 h anaerobic incubation at 37 °C (n=3)

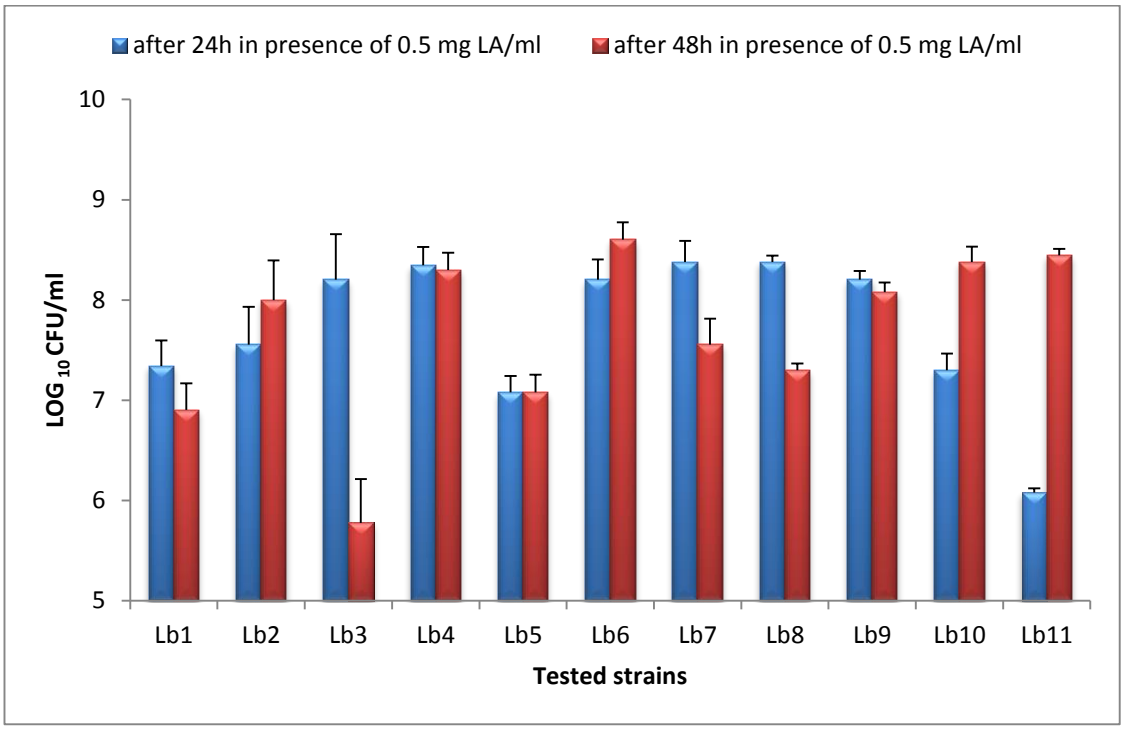


Figure 6.4 Number of *Lactobacillus* spp. in MRS broth with LA (0.5 mg/ml) after 24 and 48 h anaerobic incubation at 37 °C (n=3)

6.3.2 Effect of incubation time and LA concentration on pH changes in growth media

When pH changes were measured during cultivation, all isolates showed a similar pH profile after 24 and 48 h incubation. However, the inhibitory effect of high level LA (0.5 mg/ml) on the growth of isolates was demonstrated by their higher pH compared to 0.2 mg/ml LA ($p < 0.05$). The pH of the medium gradually decreased as the cells grew, reaching values of 3.71- 4.37 in MRS broth containing 0.2 mg LA/ml after 24 and also 3.52 – 4.01 after 48 h of incubation. Additionally, the final pH for MRS broth treated with 0.5 mg LA/ml varied from 3.77- 4.78 after 24h and 3.51- 4.38 at the end of the incubation time (Figure 6.5 and 6.6).

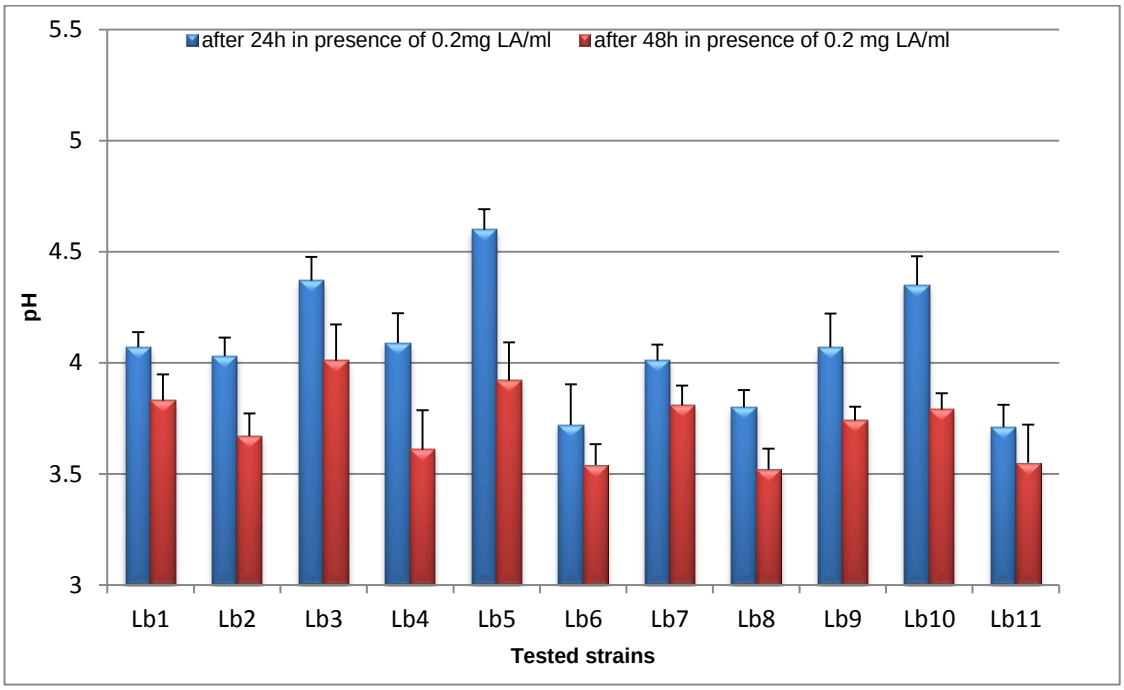


Figure 6.5 Effect of LA concentration (0.2 mg/ml) on the pH of MRS broth inoculated with the tested *Lactobacillus* spp. and anaerobically incubated for 24 and 48 h at 37 ºC (n=3)

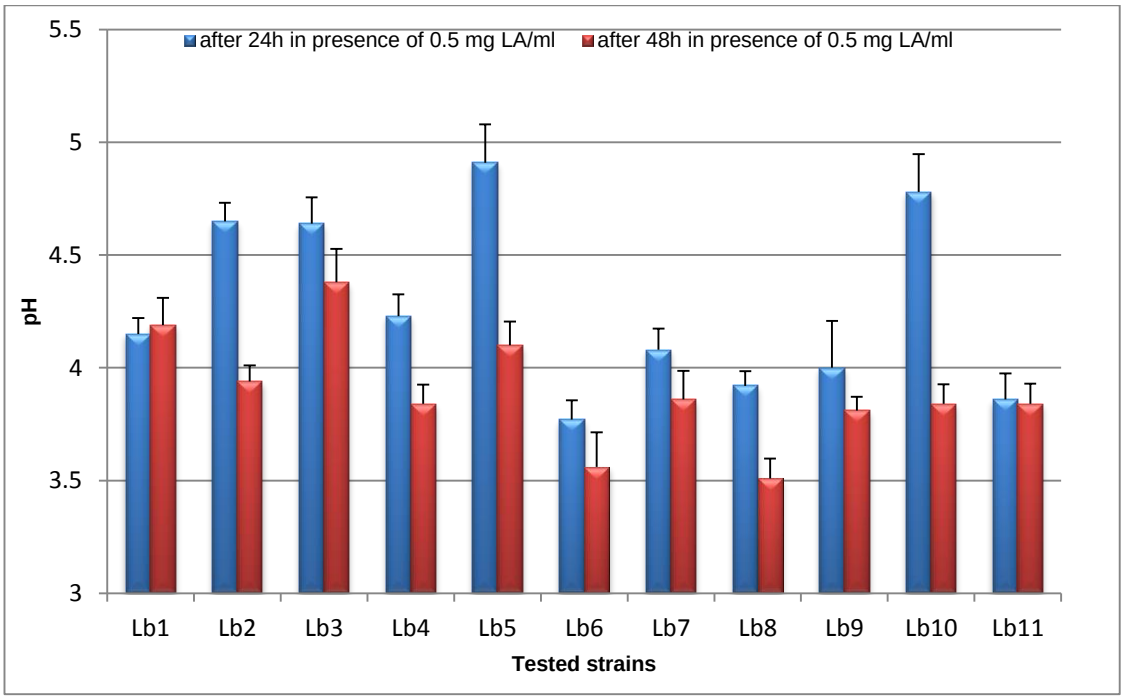


Figure 6.6 Effect of LA concentration (0.5 mg/ml) on the pH of MRS broth inoculated with the tested *Lactobacillus* spp. and anaerobically incubated for 24 and 48 h at 37 ºC (n=3)

6.3.3 Screening *Lactobacillus* spp. for CLA production

Screening six strains of isolated *Lactobacillus*, two commercial cultures (La5 and C431) and three type strains (*Lb*. *acidophilus*, *Lb*. *casei* subsp. *casei* and *Lb*. *paracasei* subsp*. paracasei*) for CLA production in treated MRS with 0.2 and 0.5 mg/ml free LA was undertaken after anaerobic incubation at 37 ^{o}C for 24 and 48 h (Figure 6.7 and 6.8). It was found that all 11 tested isolates were capable of synthesising CLA in the supernatant with varying conversion percentage.

Figure 6.7 shows the percentage of CLA in MRS+ 0.2 mg/ml LA after 24 and 48 h. After 24 h of incubation isolate Lb11 showed the highest CLA production (33.43%), followed by Lb10 (28.88%) and Lb6 (25.35%); Lb1 presented the lowest conversion (12.06%). After 48 h, the highest conversion was obtained with Lb8 (40.89%), Lb2 (36.22%) and Lb10 (33.69%) and the lowest conversion rate was still observed with Lb1 (12.7%).

According to Figure 6.8, the highest and the lowest converted LA in MRS+0.5 mg LA/ml after 24 h incubation, was recorded for isolates Lb11 (24.95%) and Lb5 (6.41%), respectively. In MRS+0.5 mg LA/ml; however, after 48 h incubation, Lb10 (%35.64) showed the highest rate of conversion and Lb5 exhibited as the lowest conversion (%10.09).

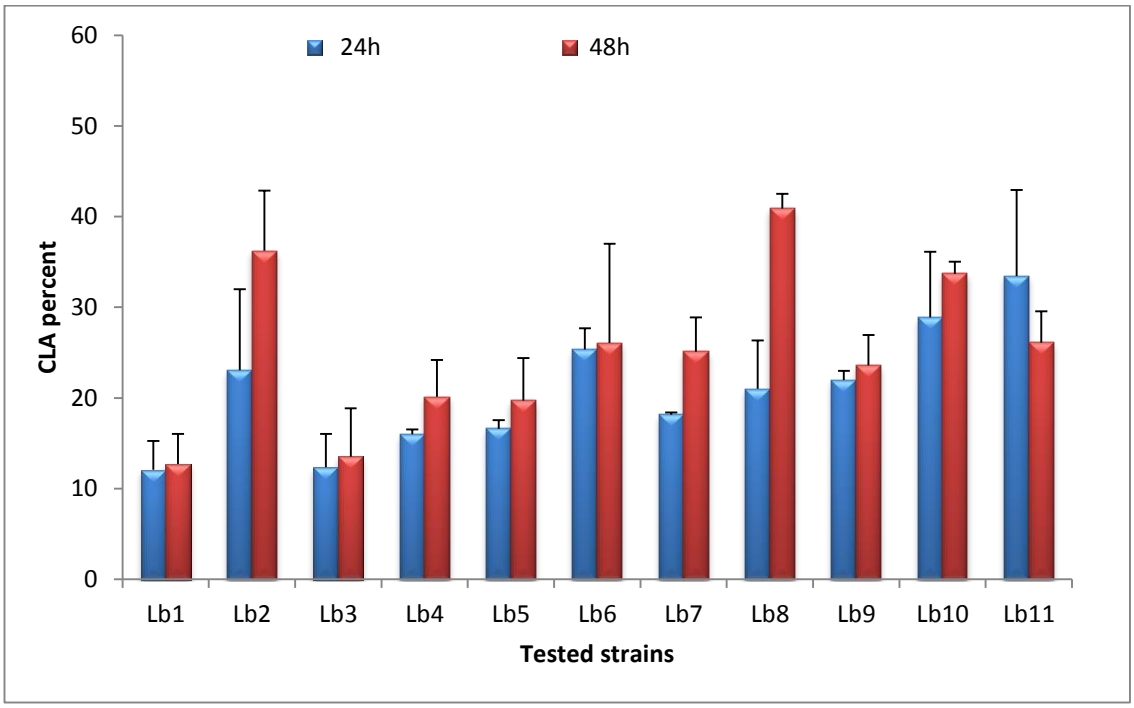


Figure 6.7 Conversion rate (%) of LA to CLA by tested *Lactobacillus* spp. in MRS broth with added 0.2 mg LA/ml, after 24 and 48 h incubation

Data are means ± SD of three replications

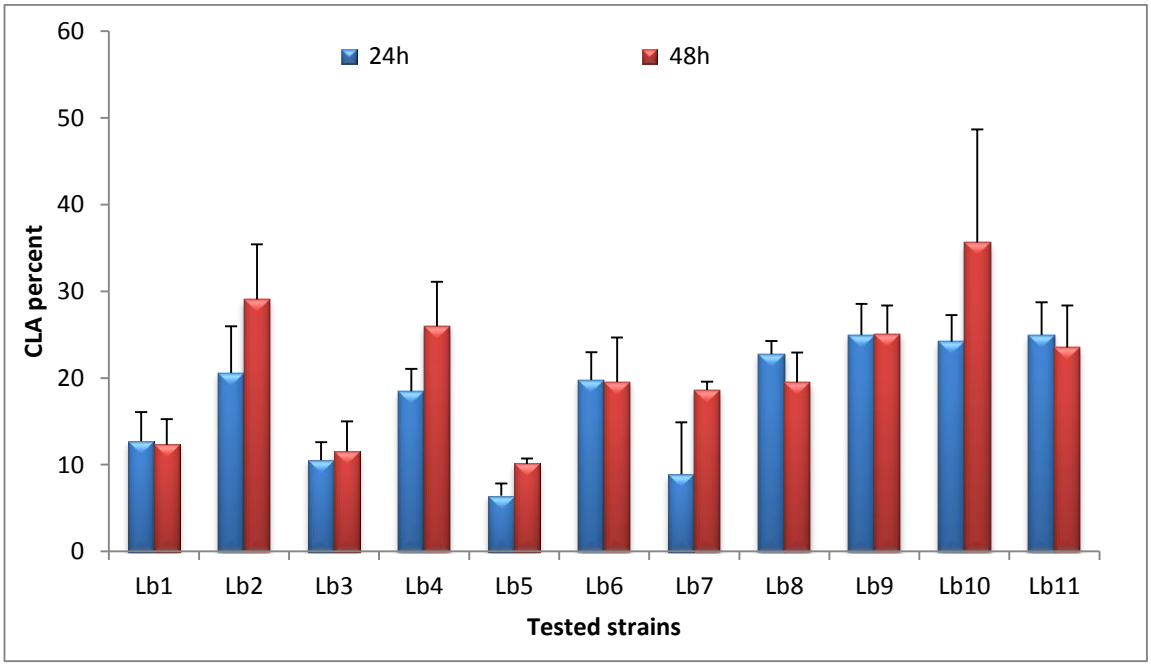


Figure 6.8 Conversion rate (%) of LA to CLA by tested *Lactobacillus* spp. in broth with added 0.5 mg LA/ml, after 24 and 48 h incubation

Data are means ± SD of three replications

The highest level of CLA production was observed after 24 h of incubation in the broth media supplemented with LA (0.2 and 0.5 mg LA/ml) and approximately similar pattern of formation of CLA were obtained with all cultures included in this study.

For all tested isolates, the total amount of CLA in the broth containing LA did not considerably rise with further 24 h increases of the incubation time ($p < 0.05$).

6.3.4 GC analysis of CLA isomers produced by *Lactobacillus* spp.

The percentage of two major isomers of CLA, *cis9- trans11* and *trans10- cis12*, was determined for all tested isolates. By comparing the retention times of the products produced in this study with standards, it was concluded that *cis9-trans11* CLA was the major CLA isomer, which was formed by the fermentation process (Figure 6.9).

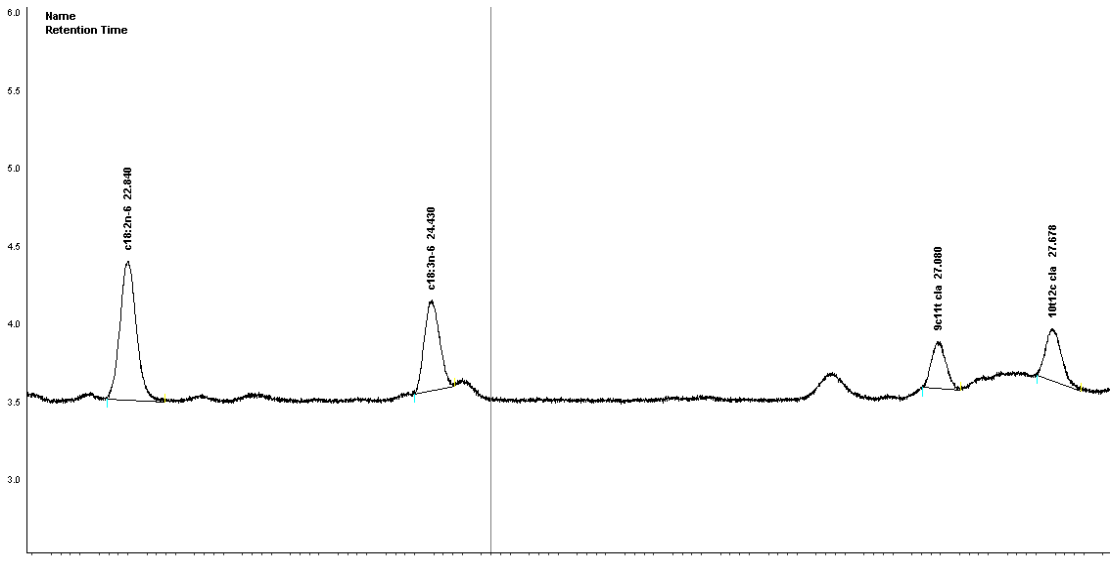


Figure 6.9 Example of a GC chromatogram of the fatty acid composition of the fermented MRS supplemented with LA.

Table 6.2 shows the production of the individual isomers *cis9-trans11* and *trans10-cis12* by all *Lactobacillus* studied, in MRS broth supplemented with 0.2 and 0.5 mg LA/ml LA after 24 and 48 h incubation. The amount of *cis9-trans11* varied from 64.56 to 92.53 % of the total CLA isomer products in MRS+0.2 mg LA/ml, and from 68.11-95.71% in MRS+0.5 mg LA/ml (calculated based on the results presented in Table 6.2).

Table 6.2 Mean percentages of CLA isomers produced by tested *Lactobacillus* spp. in MRS broth + 0.2 and 0.5 mg LA/ml after 24 and 48 h incubation anaerobically at 37 °C

	MRS +0.2 mg LA/ml				**MRS + 0.5 mg LA/ml**			
	***Cis9-trans11* (24h)**	***trans10-cis12* (24h)**	***Cis9-trans11* (48h)**	***trans10-cis12* (48h)**	***Cis9-trans11* (24h)**	***trans10-cis12* (24h)**	***Cis9-trans11* (48h)**	***trans10-cis12* (48h)**
Lb1	9.9±3.67	2.15±0.49	11.61±2.87	1.09±0.45	11.75±3.07	0.96±0.27	11.69±3.05	0.63±0.31
Lb2	20.27±9.0	2.77±0.89	25.71±4.99	10.5±3.45	14.56±3.96	6.01±2.55	20.00±3.52	9.06±3.73
Lb3	**9.13±1.89**	3.22±2.23	**8.74±4.36**	4.79±0.94	7.17±1.59	3.35±1.24	**8.763±2.67**	2.75±0.78
Lb4	13.99±0.82	1.99±0.79	16.04±0.59	1.31±1.09	15.89±0.42	4.52±2.00	14.44±0.67	2.46±1.48
Lb5	11.21±1.49	2.36±0.82	16.17±2.92	3.51±1.80	**5.54±1.41**	0.86±0.28	9.02±0.72	1.60±0.94
Lb6	22.63±2.88	2.72±0.57	21.35±8.33	4.70±3.90	17.39±2.36	2.37±0.95	16.92±4.45	2.61±2.37
Lb7	15.45±0.69	2.69±0.92	17.24±4.79	7.86±2.38	17.69±5.48	1.22±0.77	16.10±1.00	2.51±1.61
Lb8	17.17±4.13	3.82±1.21	**36.07±2.45**	4.81±1.98	21.22±1.01	1.53±0.88	18.14±3.38	1.34±0.66
Lb9	17.81±1.95	4.15±0.93	20.58±3.19	2.99±1.51	**23.60±4.34**	1.361±0.78	23.99±3.72	1.11±0.50
Lb10	24.80±2.48	4.07±1.76	31.17±2.76	2.51±2.99	22.91±1.80	1.28±1.25	**33.00±11.49**	2.64±1.92
Lb11	**30.03±7.53**	3.40±2.09	23.55±4.88	2.54±1.71	23.51±4.32	1.44±1.16	22.53±4.37	1.00±0.71

Data are means ± SD of three replications

6.4 DISCUSSION

Many investigations have been undertaken on the formation of CLA by LAB (Jiang *et al*. 1998; Ogawa *et al.* 2001; Kim *et al*. 2002; Alonso *et al.* 2003; Coakley *et al.* 2003; Lee *et al.* 2009). It has been reported that several parameters, such as pH, media and phase of growth might influence the CLA production (Sieber *et al*. 2004); therefore, very dissimilar results might be obtained in various studies.

Lactobacillus acidophilus La5 is a widely used commercial culture with well-established probiotic properties. It has been reported that *Lb. acidophilus* La5 is able to form CLA when used for the fermentation of dairy products (Kim and Liu 2002; Akalin *et al*. 2007; Ekinci *et al*. 2008), but Bzducha and Obiedzinski (2007) did not find any CLA accumulation in the ripening of cheeses.

Hernandez-Mendoza *et al.* (2008) obtained a higher production of CLA (0.08 mg/ml) by *Lb. reuteri* in MRS broth containing high substrate concentrations (20 mg/ml) free LA, which was incubated aerobically at 10 ˚C for 30 h. However, it should be noted that the initial level of LA added in their study was much higher as compared to this present study, which were 0.2 and 0.5 mg LA/ml. In addition, the purity of LA in their study is not known and also their experiments were carried out at low temperature (10 °C), which might be an advantage. Alonso *et al.* (2003) reported those two strains of *Lb. acidophilus* and also two of *Lb. casei* could produce CLA in MRS broth supplemented with LA (0, 0.05, 0.1, 0.2 and 0.5 mg/ml). They reported that all tested strains were able to form free CLA in all concentrations. Maximum production of CLA (80.14 to 131.63 µg/ml) was recorded at 24 h of incubation in medium containing 0.02% LA.

In the current study, all tested strains were able to produce CLA from free LA, which might be due to the presence of LA hydrogenase enzyme. These bacteria efficiently converted LA to CLA in MRS broth, but the percentage of CLA conversion was variable among them.

The CLA formed by the tested strains was extracted from extracellular phase and of the different CLA isomers, *cis9-trans11* 18:2 was found to represent 64–95% of total CLA formed and this is similar to the proportion indicated by other research (Jiang *et al*. 1998; Kelley *et al*. 2007).

According to Ogawa *et al*. (2001), produced CLA is accumulated into *Lb. acidophilus* cells. They claimed it is considered as an advantageous to the consumer. However, it should be mentioned that cell associated CLA might not be available, as probiotic bacteria are not digested in the human gut. The use of *Lactobacillus* spp. which able to produce free CLA in the fermentation process may offer health benefits, because free CLA in fermented milks is more absorbable than CLA incorporated into the cells of the starter culture (Alonso *et al*. 2003). Therefore, it would be more valuable that cells export CLA to the environment, i.e. the GIT. In this research, bacterial cells were separated from broth by centrifugation and cell free supernatant was assayed only. Therefore, this may not include any cell associated CLA. It is more important for the products that their CLA be available when the products are digested.

The antibacterial effect of free LA has been reported by different research. In this research, also growth of many of the screened stains was rather lower upon inclusion of 0.5 mg/ml linoleic acid into the growth media. It should be stated that toxic effects of free fatty acids for bacteria depends on its concentration and,

in this study, we reported that the tested isolates could grow better in the presence of 0.2 mg LA/ml than 0.5 mg LA/ml. It appeared that addition of greater than 0.2 mg/ml LA exerted an inhibitory effect on CLA production because less was produced in the presence of 0.5 mg/ml LA. This indicates that conversion of free LA to CLA might be part of a detoxification process. Alonso *et al*. (2003) also reported that free LA has a negative effect on the growth and metabolism of tested isolates.

As mentioned earlier, the formation of CLA is usually affected by the media composition. Macouzet *et al.* (2009) reported that MRS broth is not a suitable medium for conversion of LA to CLA. According to their research *Lb. acidophilus* was not able to produce CLA in MRS broth. They stated that *Lb. acidophilus* strains produce higher CLA in a milk-based medium. Coakley *et al.* (2003) reported no CLA formation in a MRS medium with 0.55 mg LA/mL by different strains of lactobacilli, lactococci and pediococci. In addition, several researches demonstrated that milk compounds, such as proteins, are able to neutralize the harmful effects of free fatty acids on the bacterial growth (Boyaval *et al.* 1995; Kim and Liu 2002). 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. 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. However, the results observed in this study indicated that the tested isolates actively convert free LA to CLA in MRS broth, which might be due to the presence of Tween-80 in the medium, that plays an important role in helping

Lactobacillus spp. to recover from the inhibitory effects of LA and became able to produce CLA (Jiang *et al*. 1998).

The mechanism of CLA production by these microorganisms might be due to the presence of LA hydrogenase enzyme, which needs to be studied more. The results obtained in the current research present remarkable perspectives for producing fermented dairy products enriched with CLA.

6.5 CONCLUSION

In conclusion, tested strains were able to produce variable amounts of CLA in the presence of free LA. The largest percentage of CLA was produced during the first 24 h of incubation. *Cis9-trans11* CLA was the main isomer detected, which constituted 64–95% of total CLA produced.

CHAPTER SEVEN:

RESISTANCE/SUSCEPTIBILITY OF *LACTOBACILLUS* SPP. TO THE ANTIBIOTICS AND DETERMINATION OF THE RESISTANCE GENES

7.1 INTRODUCTION

Lactobacillus strains have long history of use in food and extensively been used as probiotics (Shah 2007). Bringing probiotic products containing lactobacilli to the market involves a step-wise process that requires to be carefully controlled in order to obtain a safe product (Saarela *et al*. 2000). Amongst other criteria, it is recommended to screen the potential probiotics for their antibiotic resistance spectrum. Although antibiotics have been developed for the treatment of several infectious diseases, these may have various adverse results, such as antibiotic-associated diarrhoea (Riley 1998; Luchansky 1999). Widespread prescription of antibiotics is often associated with the disruption of the protective flora in the GIT (Forestier *et al*. 2001). Alterations in the microflora by antibiotic therapy also encourage the emergence of resistant strains (Lee *et al*. 2003).

It has been reported that probiotic lactobacilli may have the potential to serve as a reservoir/host for antibiotic resistance genes with the risk of transferring the genes to pathogenic and non-pathogenic bacteria (Teuber *et al*. 1999; Danielsen and Wind 2003; Kastner 2006; Ammor *et al*. 2007).

Before launching a probiotic product into the market, it is important to consider that any single probiotic strain do not contain transferable antibiotic resistance genes.

7.1.1 Antibiotic categories

Antibiotics can be categorised based on their functions and also their specific targets. A summary of groups of antibiotics based on their mechanism of action is shown in Table 7.1.

Table 7.1 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

Antibiotics, such as β-lactam antibiotics and vancomycin, act as inhibitors of cell wall synthesis. Production of a β-lactamase, such as penicillinase or cephalosporinase, is the principal resistance mechanismamong clinically important anaerobes including lactobacilli (Cooksey 1991).

Cell wall impermeability is also one of the main mechanisms of resistance to antibiotic (Condon 1983). Vancomycin may be able to inhibit cell wall synthesis. Vancomycin comes into contact with the peptidoglycan precursors on the cell wall side of the cytoplasmic membrane, and it binds to the D-alanine/D-alanine terminus of the pentapeptide and inhibits the polymerization of peptidoglycan precursors (Gueimonde *et al*. 2013).

Homofermentative lactobacilli group are susceptible to vancomycin (EFSA, 2008), but there are some *Lactobacillus* species such as *Lb. plantarum*, *Lb. casei* subsp. *Casei*, *Lb. salivarius*, *Lactobacillus leishmannii* and *Lb. acidophilus* (excluding obligate heterofermentative species) that carry intrinsic resistance towards vancomycin, which can be associated to the presence of D-alanine/D-alanine ligase-related enzymes (Elisha and Courvalin 1995). It is thought that the reduced susceptibility of lactobacilli to vancomycin occurs because of membrane impermeability (Elkins and Mullis 2004).

Aminoglycosides (streptomycin, kanamycin, gentamycin and amikacin) and tetracycline are able to bind the 30S ribosomal subunit and act as inhibitors of protein synthesis. The aminoglycosides irreversibly bind to the 30S ribosome and freeze the 30S initiation complex and no further initiation can occur. Chloramphenicol, lincomycin and macrolides, such as erythromycin bind to the 50S ribosome and inhibit peptidyl transferase activity.

Antibiotics, which inhibit the nucleic acid synthesis are enoxacin, pefloxacin, norfloxacin, nalidixic acid, sulphamethoxazole, trimethoprim, co-trimoxazole and metronidazole (Coppola *et al*. 2005). Some resistance mechanisms are associated with enzymatic actions. Resistance of lactobacilli to metronidazole might be related to the absence of hydrogenase activity (Church *et al*. 1996).

Antimetabolites antibiotics, such as sulfonamides, mainly inhibit folic acid synthesis. They bind to dihydrofolate reductase and inhibit formation of tetrahydrofolic acid.

7.1.2 The mechanisms of antibiotic resistance in lactobacilli

Resistance mechanisms of the lactobacilli to antimicrobial agents are different. Physiological resistance is expressed when bacteria are in a particular physiological condition like when they produce biofilm and the bacteria adopt a 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. Some of other reasons for defensive functions and phenotypic resistance to antimicrobial agents are:

- decreased uptake of the antibiotic by microorganisms
- increased export of the antibiotic,
- introduction of a new antibiotic resistant target,
- inactivation of the antibiotic target,
- hydrolysis and/or modification of the antibiotic (Normark and Normark 2002).

7.1.3 Resistance genes in lactobacilli

The intrinsic resistance of lactobacilli to several antibiotics can be partially due to genes that encode multidrug resistance efflux pumps, which expel different kinds of antibiotics and chemicals such as biocides, organic solvents, dyes, detergents and metabolic products (Munoz *et al*. 2014). In a study on *Lb. sakei* Rits 9, strain isolated from Italian Sola cheese, transposon-associated *tet*(M)

gene and plasmid-carried *tet*(L) gene, which mediate two resistance mechanisms to tetracycline have been described (Ammor *et al*. 2008). Toomey *et al*. (2010) have shown that *Lb. paracasei*, *Lb. reuteri* and *Lb. curvatus* were resistant to erythromycin containing *erm*(B) and *msr*A/B genes. Also in their study, *Lb. plantarum* was resistant to tetracycline because it contains a resistance gene such as *tet*(M).

Huys *et al*. (2008) indicated that *Lb. paracasei* and *Lb. casei* were uniformly susceptible to ampicillin and clindamycin but exhibited natural resistance to gentamicin and streptomycin. Kastner *et al*. (2006) showed that *Lb. reuteri* SD 2112 harbour tetracycline resistance gene *tet*(W) and the lincosamide resistance gene *lnu*(A). Rosander *et al*. (2008) identified two plasmids carrying *tet*(W) tetracycline and *lnu*(A) lincosamide resistance genes in *Lb. reuteri* ATCC55730 .

In a study by Mayrhofer *et al*. (2010) *Lb. johnsonii,* which was phenotypically susceptible to clindamycin and erythromycin seemed to harbour the *erm* (B) gene. The *tet(W)* gene has been reported in strains of *Lb. crispatus*, *Lb. johnsonii*, *Lb. paracasei* and *Lb. reuteri* (Egervarn *et al*. 2009). Chloramphenicol resistance *cat* gene has been found in *Lb. plantarum* (Ahn *et al.* 1992) and *Lb. reuteri* (Lin *et al.* 1996).

7.1.4 Transferability of resistance genes

Antibiotic resistance among *Lactobacillus* strains may be desirable (Charteris *et al*. 1998), but continuous attention should be paid to the selection of *Lactobacillus* strains free of transferable antibiotic resistance (Temmerman *et al*. 2003).

The dissemination of antibiotic resistance determinants in microbial communities are vertical or horizontal. Vertical dissemination is through the clonal spread of a special resistant strain, while horizontal gene transfer is through conjugation, transformation and transduction (Davison, 1999). The three types of resistance to a given antibiotic can be intrinsic, acquired and mutational (Ashraf and Shah 2011).

a) Intrinsic (inherent/innate/natural) resistance:

Intrinsic resistance to a bacterial species or genus is not horizontally transferable, and poses no risk in non-pathogenic bacteria. Inherent resistance to vancomycin for *Lb. paracasei*, *Lb. salivarius* and *Lb. plantarum* has been confirmed in Italian probiotic products (Blandino *et al*. 2008). In the study of Toomey *et al*. (2010) intrinsic streptomycin resistance has been observed in *Lactobacillus* strains isolated from Irish pork and beef abattoirs. It has been noted that in different lactobacilli natural resistance to vancomycin is not transmissible (Klein *et al.* 1998; Bernardeau *et al.* 2008).

b) Acquired resistance:

Some bacteria strains or species, which were usually susceptible to some antibiotics may acquire antibiotic resistance elements that might further be horizontally spread among other bacteria (Mathur and Singh 2005).

It has been shown that antibiotic resistance of *Lb. acidophilus*, *Lb. crispatus*, *Lb. gasseri* and *Lb. plantarum* may be acquired in the intestinal tract during transit (Cataloluk and Gogebaken 2004). Acquired resistance to tetracycline is well known in *Lb. acidophilus* strains (Delgado *et al*. 2005; Cauwerts *et al*. 2006;

Klare *et al*. 2007). In acquired resistance, horizontal transfer increases the evolution of antibiotic resistance in microbial communities. It is happened by moving resistance genes across species borders via conjugative plasmids, integrons, transposons, insertional elements, lytic and temperate bacteriophages (Davies 1994). Among different mechanisms, conjugation is the main way of antibiotic resistance gene transfer (Salyers 1995), because conjugation allows DNA to move across genus and species lines, whilst transformation and transduction are restricted to within the same species (Mathur and Singh, 2005).

There are plasmids, which encode resistance to tetracycline, erythromycin, chloramphenicol in *Lb. fermentum* (Fons *et al.* 1997), *Lb. plantarum* (Danielsen 2002) and *Lb*. *reuteri* (Lin *et al*. 1996).

In vitro and *in vivo* studies in mice have revealed the transfer of vancomycin resistance (gene *vanA*) from enterococci to a commercial *Lb. acidophilus* strain (Mater *et al*. 2008). Such evidence may suggest that intestinal bacteria, such as lactobacilli, can acquire resistance especially by horizontal transfer of resistance genes from other intestinal species (Liu *et al*. 2009). Thus, one of the safety traits of lactobacilli, which should be evaluated, is their capability of acquiring and disseminating resistance determinants.

c) Mutation:

Mutations may cause genetic changes in multiple regions of the genome; they play a minor role in the development of resistance (Howden *et al*. 2006). It needs to be stated that intrinsic resistance and the resistance caused by mutation of

chromosomal genes have a low risk of horizontal transfer, and such strains should be acceptable to use in food (Ashraf and Shah 2011).

The aim of this part of the research was to study the antibiotic resistance profiles of isolated lactobacilli. The objectives included:

a) To determine the antibiotic resistance profile of the isolates, type strains and commercial lactobacilli.

b) To detect possible antibiotic resistance genes in these isolates, type strains and commercial lactobacilli.

c) To screen of the isolates, type strains and commercial cultures ability to transfer resistance genes to other bacteria.

7.2 MATERIALS AND METHODS

7.2.1 Microorganisms

A) Strains tested for antibiotic resistance

The bacteria tested in this experiment were shown in Table 3.1.

B) Positive control organisms for the gene transfer study (Table 7.2) which werekindly provided by the Technical University of Denmark, (National Food Institute, Antimicrobial Resistance Centre, Denmark).

Table 7.2 The organisms used as positive controls for the detection of antibiotic resistance genes

Bacteria	**Gene related**
Staphylococus rissem 7522486-1	*aph*(3'')-I
Entrococus faecalis	*aadE*
Salmonella Typhimurium	*aadA*
Staphylococus aureus RN422	*erm*(C)
Enterococus Faecalis JH2-2	*erm*(B)
Staphylococus aureus Tn554	*erm*(A)
Staphylococus aureus PSTS 9-like	*tet*(L)
Staphylococus aureus PT181-like	*tet*(K)
Staphylococus intermedius 2567	*tet*(M)
Escherichia coli	*tet*(Q)
Listeria monocytogenes BM4210/PIP811	*tet*(S)
Escherichia coli K2	*ant*(2'')-I
Enterococus faecalis JH2-1-5	*aph(*3'')-III
Escherichia coli	*tet*(W)
Entrococus faecium BM4147	*Van A*
Entrococus faecalis V583	*Van B*
Entrococus faecalis SF 350 la	*aac(6')aph(2")*
Salmonella Typhimurium DS611	*cmlA*
Salmonella Weltevreden TA 428/97	*Cat1*
No positive control	*Van X, tet O, Cat, Str A, Str B, aac(3")IV*

7.2.2 Determination of minimum inhibitory concentration (MIC)

Minimum 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. MIC for 25 antibiotics was determined using a sensititre plate of 96 wells containing variable amounts of antibiotics (Trek, Diagnostic Systems Ltd., East Grinstead, UK). After two consecutive subcultures of each organism (table 3.1), a single colony on MRS (CM0361, Oxoid, UK) plate 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 the stock inoculum in 5 ml MRD in a glass boiling tube 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 MacFarland standard (10^7-10^8 CFU/ml). The liquid medium used was MRS broth (CM0359, Oxoid, UK). The working inoculum suspension was then diluted 100-fold in fresh MRS broth and 50µl of inoculated MRS broth 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 96 well 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/susceptibility a breakpoint for each antibiotic was established based on published literature (Table 7.3).

Table 7.3 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.* (2008b)
Ampicillin	1-32	>1	EFSA
Cefoxitin	0.5-32	>32	Moubareck *et al*. 2005
Ceftiofur	0.12-8	8	Ouoba *et al.* (2008b)
Ceftriaxone	0.25-64	>4	Karlowsky and Jones (2003)
Ciprofloxacin	0.015-4	4	Ouoba *et al.* (2008b)
Chloramphenicol	2-32	>4	EFSA
Daptomycin	0.25-16	≥8	King and Phillips (2001)
Erythromycin	0.25-8	>1	EFSA
Gentamycin	0.25-1024	>16	EFSA
Kanamycin	8-1024	>256	Ouoba *et al.* (2008b)
Lincomycin	1-8	≥1	UKPAR
Linezolid	0.5-8	≥8	Ouoba *et al.* (2008b)
Nalidixic acid	0.5-32	32	Ouoba *et al.* (2008b)
Nitrofurantoin	2-64	≥128	Otero *et al*. 2007
Penicillin	0.25-16	4	Ouoba *et al.* (2008b)
Quinupristin/dalfopristin	0.5-32	>4	EFSA
Streptomycin	512-2048	>64	EFSA
Sulfisoxazole	16-256	256	CLSI
Tetracycline	4-32	>4	EFSA
Tigecycline	0.015-0.5	≥8	Nord *et al.* (2006)
Trimethoprim/sulfamethoxazole	0.12/2.38-4/76	>32/512	Ouoba *et al.*(2008b)
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
CLSI: Clinical and Laboratory Standards Institute
UKPAR: United Kingdom Public Assessment Report

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. If any tested organisms show resistance to any antibiotic, it might indicate intrinsic resistance. However, if for any individual antibiotic the tested strains react differently, then there is a possibility of acquired resistance. For such cases, further investigation was carried out to examine the presence of selected genes.

7.2.3 Detection of the resistance genes

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. The genetic background of antibiotic resistance genes was studied by PCR using specific primers for antibiotic resistance genes. For an antibiotic to which a tested isolate showed resistance, PCR was carried out for most frequent genes. The following antibiotic genes were screened.

- 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)*
- Gentamycin: *aac(6'), aph(2"), ant(2")-I, aac(3")IV*,
- Vancomycin: *van(*A*), van(*B*), van(*X*)*
- Chloramphenicol: *Cat, Cat1, CmlA*

DNA extraction in all isolates was carried out as described in section 2.2.7.1. The PCR reaction mix (49µl) used for tetracycline, kanamycin, vancomycin, gentamycin and erythromycin contained the following reagents and for each investigated gene the corresponding set of its primers were applied (Table 7.4).

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 extract 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 and also chloramphenicol, 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 extract 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 extract 2 µl and volume in total was 50 µl.

The PCR reagents for investigation of resistance gene *aadE* 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 extract 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 7.4) and 72 °C for 1 min and a final extension step at 72 °C for 10 min. A volume of 10 µl of PCR products were separated by electrophoresis on 1.5% w/v agarose gel and visualised by ethidium bromide staining (see section 2.2.7.3 for more details).

Table 7.4 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

Resistance genes	Primers	Annealing temperature(°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
aac(6′)aph(2″)	5′-CCA AGA GCA ATA AGG GCA TA-3′ 5′-CAC TAT CAT AAC CAC TAC CG-3′	48°C
aac(3″)II	5′-TGA AAC GCT GAC GGA GCC TC-3′ 5′-GTC GAA CAG GTA GCA CTG AG-3′	55°C
aac(3″)IV	5′-GTG TGC TGC TGG TCC ACA GC-3′ 5′-AGT TGA CCC AGG GCT GTC GC-3′	63°C
Van(A)	5′-AAC AAC TTA CGC GGC ACT-3′ 5′-AAA GTG CGA AAA ACC TTG -3′	55°C
Van(B)	5′-GAT ATT CAA AGC TCC GCA GC-3′ 5′-TGA TGG ATG CGG AAG ATA CC-3′	55°C
Van(X)	5′-TGCGATTTTGCGCTTCATTG -3′ 5′-ACTTGGGATAATTTCACCGG -3′	55°C
cmlA	5′-TACTCGGATCCATGCTGGCC -3′ 5′-TCCTCGAAGAGCGCCATTGG -3′	65°C
Cat	5′-GGATATGAAATTTATCCCTC -3′ 5′- CAATCATACCCTATGAAT-3′	47°C
Cat1	5′-CGCCTGATGAATGCTCATCCG -3′ 5′- CCTGCCACTCATCGCAGTAC -3′	60°C

7.3 RESULTS

Eleven strains of *Lactobacillus* 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 also studied by PCR reactions.

7.3.1 Susceptibiliy to antibiotics

Table 7.5 shows the results for MIC for the tested isolates.

Table 7.5 Minimum Inhibitory Concentrations (µg/ml) of tested lactobacilli with their susceptibility status (resistance/sensitive) according to recommended breakpoints (Table 7.3)

Anibiotic \ Strains	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6	Lb7	Lb8	Lb9	Lb10	Lb11
Amikacin	>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	2/1 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
Cefoxitin	32 S	32 S	32 S	>32 R	>32 R	8 S	>32 R	16 S	16 S	16 S	>32 R
Ceftiofur	1 S	0.25 S	0.25 S	1 S	1 S	0.25 S	0.25 S	1 S	0.25 S	0.25 S	1 S
Ceftriaxone	8 R	2 S	2 S	16 R	16 R	2 S	1 S	2 S	2 S	4 R	8 R
Ciprofloxacin	4 R	>4 R	>4 R	>4 R	>4 R	>4 R	>4 R	>4 R	>4 R	>4 R	>4 R
Chloramphenicol	8 R	8 R	8 R	8 R	8 R	<2 S	8 R	8 R	8 R	8 R	8 R
Daptomycin	>16 R	>16 R	>16 R	>16 R	>16 R	>16 R	>16 R	>16 R	>16 R	>16 R	0.5 S
Erythromycin	2 R	0.5 S	0.5 S	2 R	<0.25 S	<0.25 S	1 S	1 S	1 S	1 S	<0.25 S

Table 7.5 continued

Strains / Antibiotic	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6	Lb7	Lb8	Lb9	Lb10	Lb11
Gentamycin	>16 R	>16 R	>16 R	>16 R	8 S	8 S	>16 R	>16 R	>16 R	>16 R	>16 R
Kanamycin	512 R	64 S	1024 R	512 R	64 S	<128 S	256 S	1024 R	512 R	256 S	512 R
Lincomycin	8 R	>8 R	>8 R	<1 S	<1 S	8 R	<1 S	>8 R	>8 R	>8 R	<1 S
Linezolid	4 S	<0.5 S	2 S	4 S	<0.5 S	1 S	4 S	2 S	4 S	2 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
Nitrofurantoin	16 S	>64 S	16 S	16 S	64 S	<2 S	16 S	4 S	16 S	8 S	<2 S
Penicillin	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S	<.025 S
Quinupristin/ Dalfopristin	1 S	1 S	2 S	<0.5 S	<0.5 S	4 S	<0.5 S	2 S	2 S	<0.5 S	<0.5 S
Streptomycin	>64 R	<32 S	<32 S	>64 R	>64 R	<32 S	>64 R	>64 R	<32 S	<32 S	>64 R
Sulfisoxazole	>256 R	>256 R	>256 R	>256 R	>256 R	>256 R	>256 R	>256 R	>256 R	>256 R	>256 R

Table 7.5 continued

Strains / Antibiotic	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6	Lb7	Lb8	Lb9	Lb10	Lb11
Tigecycline	0.12	0.12	0.12	0.12	<0.015	0.12	0.12	0.25	0.12	0.12	<0.015
	S	S	S	S	S	S	S	S	S	S	S
Trimethoprim/ Sulfamethoxazole	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76
	S	S	S	S	S	S	S	S	S	S	S
Tylosin tartrate	8	>32	0.5	4	1	0.5	1	1	0.5	0.5	<0.25
	S	R	S	S	S	S	S	S	S	S	S
Tetracycline	4	2	<1	>32	<1	<1	4	2	<1	2	<1
	S	S	S	R	S	S	S	S	S	S	S
Vancomycin	>32	1	1	>32	>32	1	>32	4	1	2	>32
	R	S	S	R	R	S	R	R	S	S	R

(R) Resistant (S) Sensitive

The β-lactam antibiotics appeared to be the most effective antibiotics and all tested bacteria showed susceptibility to them, which inhibit the cell wall synthesis. Penicillin was the most active and had a MIC value below 0.25 µg/ml for the all tested bacteria. According to the breakpoints, the isolates were considered susceptible to penicillin. The MIC value determined for ampicillin also was below 1 µg/ml. Also some of the tested bacteria (Lb2, Lb3, Lb6, Lb9 and Lb10) showed susceptibility to vancomycin. Their MIC for vancomycin was 1µg/ml. Vancomycin acts on the cell wall synthesis. However, the rest of the tested isolates showed resistance to vancomycin and the MIC was detected above 32 µg/ml.

With the exception of Lb2, Lb5, Lb6, Lb7 and Lb10, all others were resistant to kanamycin where the MIC value ranged from 512 to <1024 µg/ml. Also for streptomycin, Lb2, Lb3, Lb6, Lb9 and Lb10 were susceptible to streptomycin (MIC <32 µg/ml), but other isolates exhibited resistance toward the antibiotic (MIC value was >64 µg/ml).

All isolates except Lb6, showed resistance toward chloramphenicol and only Lb4 showed resistance to tetracycline. However, the reaction of the isolates toward other 50S ribosomal subunit directed antibiotics, such as erythromycin was variable. Nine out of 11 isolates were susceptible to erythromycin, but Lb1 and Lb4 showed reduced susceptibility (MIC 2µg/ml).

All strains tested in this study showed low susceptibility to nalidixic acid and ciprofloxacin. The MIC for nalidixic acid and ciprofloxacin were >32 and >4 µg/ml, respectively.

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

7.3.2 Detection and characterization of possible resistance genes

Based on the results obtained for bacterial resistance to antibiotics (Table 7.5), seven out of 25 tested antibiotics were selected to be assessed for resistance determinants. As seen in Table 7.5, 12 antibiotic affected the tested strains differently (some inhibitory and some not). It was, therefore, decided to screen the resistant isolates for the presence of common genes related to seven of these antibiotics based on our access and availability of these genes.

The DNA of selected isolates (Table 7.5) was subjected to genetic screening for a selection of different resistance genes using PCR (section 7.2.3) with primers specific for the respective antibiotic resistance genes.

For all resistance genes screened, no positive amplicon was observed for all tested strains. It should be pointed out that positive control was used for accuracy of the experiment, and in all related experiments, they produced a clear positive amplicon.

As a representative, Figure 7.1 shows the result of five isolates (Lb2, Lb4, Lb5, Lb8 and Lb9) for six popular genes including tetracycline, *tet(L)*, (M), (W), (K), (Q) and *tet(S)* where there is no clear amplicon for any of the tested isolates except for the positive control.

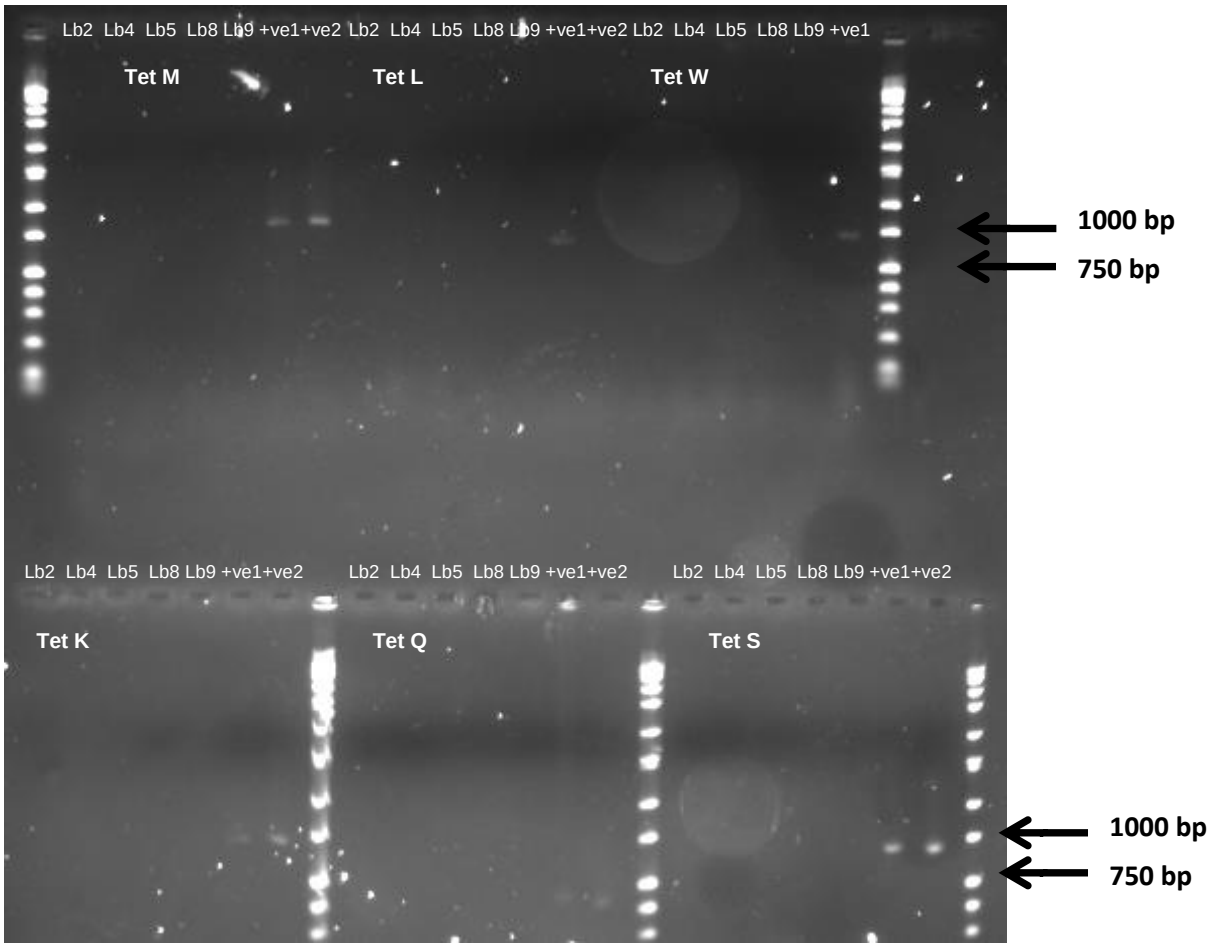


Figure 7.1 Representative gel image of tested isolates for presence of selected tetracyclin genes; *tet*(L), *tet*(M), *tet*(W), *tet*(K), *tet*(Q) and *tet*(S) by PCR

7.4 Discussion

Special attention has been paid to prevalence of antibiotic resistance in lactobacilli, and due to growing interest in probiotic bacteria, the number of studies on this issue has recently increased (Ashraf and Shah 2011). The Scientific Committee for Animal Nutrition (SCAN) has concluded that some probiotic products pose a risk to human/animal health, because they include bacterial strains having transferable resistance genes (European Commission, 2001). The presence of the genes, which are not considered intrinsic, is slightly

alarming because they may be found in commercial starter/probiotic lactobacilli, which are consumed at higher dosages (Teuber *et al*. 1999).

In this research, the reaction of 11 tested strains was studied against 25 different antibiotics. Results obtained demonstrated that all tested isolates might be intrinsically resistant to amikacin, ciprofloxacin, daptomycin, nalidixic acid, sulfisoxazole. Resistance of *Lb. acidophilus* strains to nalidixic acid was reported by Gupta and Mittal (1995).

This study showed that they are sensitive to ampicillin, penicillin, ceftiofur, linezolid, nitrofurantoin, tigecycline, trimethoprim/sulfamethoxazole, quinupristinm/dalfopristin and amoxicillin/clavulanic acid. Huys *et al*. (2008) indicated that *Lb. paracasei* and *Lb. casei* were uniformly susceptible to ampicillin, but exhibited natural resistance to gentamicin and streptomycin.

Lactobacilli has been reported to be intrinsically resistant to streptomycin and kanamycin, gentamicin, fluoroquinolones and vancomycin (Elisha and Courvalin 1995; Danielsen and Wind, 2003; Liu *et al*. 2009). Intrinsic resistance to aminoglycosides, such as streptomycin and kanamycin, has been indicated as a general feature of lactobacilli (Danielsen and Wind 2003).However, this piece of research reported that resistance to cefoxitin, ceftriaxone, chloramphenicol, erythromycin, gentamycin, kanamycin, lincomycin, streptomycin, tylosintartarate, tetracycline and vancomycin were rather variable among tested strains and they react differently, therefore it may be considered as acquired resistance. Based on the method used, none of the tested bacteria in this research presented positive PCR for the resistance genes investigated.

Therefore, they may be considered safe regarding their potential to transfer resistance genes.

Vancomycin resistance is considered a major concern because it is widely effective against clinical infection (Zhou *et al*. 2005). The literature shows contradictory reports concerning the susceptibility of lactobacilli to vancomycin. It should be stated that these studies were carried out using different methods; accordingly, the contradictions might be because of variations in methodologies (Zhou *et al*. 2005).Therefore, it is necessary to standardize the applied methods.

As mentioned earlier, vancomycin inhibits the synthesis of cell walls in susceptible microrganisms. They bind to D-alanine/D-alanine in peptidoglycan. Liu *et al*. (2009) ascribed the intrinsic resistance of *Lb. plantarum* LP1 and LP2 to vancomycin to this fact that peptidoglycan precursors of *Lb. plantarum* composed of D-lactate rather than D-alanine. Another study has also stated that the resistance could be due to the presence of D-alanine/D-lactate as the normal peptide in the peptidoglycan (Florez *et al*. 2005). In our research, resistance to vancomycin was observed, but the screened resistance gene (*van*(A), *van*(B), *van*(X) was not found for this antibiotic.

Klein *et al*. (2000) indicated that *Lb. rhamnosus* GG (ATCC 53103) is resistant to vancomycin, but do not possess the *van(A)*, *van(B)*or *van(C)* genes. According to their results the safety of this strain was established for use as probiotic regarding its vancomycin resistance.

The *tet* genes are the most abundant antibiotic resistance determinants, which were described for resistance to tetracycline resistance and coding for ribosomal protective proteins (Gueimonde *et al*. 2010).The *tet(W)* gene has

been reported in strains of *Lb. johnsonii*, *Lb. paracasei* and *Lb. reuteri* (Egervarn *et al*. 2009). Huys *et al*. (2008) found that *Lb. paracasei* strains from cheese showed acquired resistance to tetracycline, which were related to *tet*(M) or *tet*(W) genes. They also reported resistance to erythromycin, which was associated with *erm*(B) gene. However, no resistance gene neither for tetracycline nor for erythromycin was seen in this study.

Ouoba *et al*. (2008b) reported the *aph* (3´)-III gene conferring kanamycin resistance in *Lb. casei* and *Lb. paracasei* strains of human origin, but in the current research no gene associated with kanamycin resistance was found.

Resistance to certain antibiotics is not an unusual feature of starter and probiotic cultures. Many of these resistances are due to complex intrinsic features such as cell wall structure or metabolic properties. This is perhaps the reason why the resistances detected in this study could not be traced back to specific genes by PCR. The absence of the investigated genes in this study is not surprising since the study was on isolates from commercial probiotic cultures, which are consumed at high dosages and may have undergone tests to assure the absence of transferable resistance genes before their use.

7.5 CONCLUSION

In conclusion, tested isolates presented intrinsically resistant to amikacin, ciprofloxacin, daptomycin, nalidixic acid, sulfisoxazole. They showed susceptibiliuty to ampicillin, penicillin, ceftiofur, linezolid, nitrofurantoin, tigecycline, trimethoprim/sulfamethoxazole, quinupristinm/dalfopristin and amoxicillin/clavulanic acid. However, their resistance to cefoxitin, ceftriaxone,

chloramphenicol, erythromycin, gentamycin, kanamycin, lincomycin, streptomycin, tylosin tartarate, tetracycline and vancomycin were rather variable and it might be considered as acquired resistance.

The presence of associated resistance genes was investigated in tested isolates which based on the method used in this research, no resistance gene was discovered. Therefore, they can be considered safe regarding their potential to transfer resistance genes.

CHAPTER EIGHT: GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

8.1 General framework of the study

This research primarily studied the variety and viability of probiotic lactobacilli in fermented milk marketed in the UK and Europe throughout their shelf-life and also identified the isolated probiotic *Lactobacillus* spp. with molecular techniques. In addition, other major objectives of this research were *in vitro* studies of the possible physiological and functional properties of the isolates, such as resistance to simulated gastric secretions in stomach and bile salts, antibiotic resistance and detection of any gene associated with antibiotic resistance, biofilm formation, antimicrobial activity and production of CLA.

8.2 Scope of research

In order to achieve the overall aim, this study was carried out in two steps:

In the first step, the presence of probiotic lactobacilli in 36 probiotic fermented dairy products from the UK/Europe market at the time of purchase and at the end of shelf-life was assessed. The isolated lactobacilli were further studied to identify them by means of genotypic methods at subspecies/strain level by sequencing of 16S rRNA and *rpoA* genes, and also discriminatory examination using rep-PCR.

To achieve the first aim of this study, isolation and identification of lactobacilli from fermented milks, it was considered essential to use a suitable medium for isolation of lactobacilli (chapter 2). Evaluation of three selective media (MRS-Clindamycin, MRS-Sorbitol and MRS-IM Maltose) for the selective enumeration of *Lactobacillus* spp. demonstrated that MRS-Clindamycin and MRS-Sorbitol were effective for enumeration of *Lactobacillus* spp. in fermented dairy products.

However, MRS-Clindamycin was chosen for its selectivity as well as good recovery of lactobacilli and simplicity of preparation.

Lankaputhra and Shah (1995) reported that MRS-clindamycin has the potential to differentiate *Lb. acidophilus* in mixed probiotic cultures in yogurt. In their study, 0.5 ppm clindamycin was added to MRS medium and incubated anaerobically at 37 °C for 2–3 days. Van de Casteele *et al*. (2006) used MRS-clindamycin for enumeration of commercial probiotic culture. According to their suggestion, MRS-clindamycin was considered as the preferred medium for the selective enumeration of commercial *Lb. acidophilus* strains La-145 and Lafti L10 in the presence of yogurt and starter. MRS-clindamycin-ciprofloxacin agar has been used by ISO (2006) for enumeration of *Lb. acidophilus* in milk products in the presence of other LAB and bifidobacteria.

The clindamycin and ciprofloxacin both can inhibit the growth of the *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, *S. thermophilus*, *Lb. casei* subsp. *casei, Lb*. *paracasei* subsp. *paracasei*, *Lb. rhamnosus*, *Lb. reuteri*, bifidobacteria, lactococci and *Leuconostoc* species. Moreover, it has been stated that this method is not applicable when the number of *Lb. acidophilus* is less than 10^4 CFU/g and the numbers of *Lb. rhamnosus, Lb. reuteri* and *Lb. paracasei* subsp. *paracasei* are greater than10^6 CFU/g. It is necessary to note that *Lb. acidophilus* is closely related to *Lb. johnsonii*, *Lb. gasseri* and *Lb. crispatus*. This method cannot distinguish between them and, therefore, only presumptive *Lb. acidophilus* is mentioned (ISO, 2006).

The results of *lactobacillus* counting in 36 samples of probiotic dairy products revealed that 31 out of 36 fermented milks contained more than 10^6 CFU/g at the time of purchase. By the end of shelf-life, and depending on the tested

product, the number of *Lactobacillus* spp. reduced between 0.00 and 2.62 $\log_{10}$ unit. There are various studies regarding the decrease/increase population of the probiotic lactobacilli in dairy products (Cruz *et al*. 2009b). In some studies, the viability of probiotics dramatically decreased about three $\log_{10}$ cycles or more during the storage (Phillips *et al.* 2006). In some other studies, the viable counts of probiotic bacteria increased one log cycles or more throughout the storage period of dairy products (Kasımoglu *et al*. 2004; Bergamini *et al*. 2005; Buriti *et al.* 2005). The most of isolated lactobacilli, in this research, were demonstrated to be technologically viable, and 61.1% of tested samples contained greater than 10^6 CFU/g viable *Lactobacillus* spp. at the end of shelf life, which is the suggested minimum level required for functional and therapeutic benefits.

A correct identification of the employed probiotic strains is certainly of fundamental importance. In this research, genotypic techniques made it possible to achieve a reliable identification. Rep-PCR was used as a valuable technique for rapid and accurate differentiation of probiotic strains in combination with other genotypic analysis to identify and discriminate bacteria contained in commercial dairy products. Using rep-PCR, this study revealed that *Lactobacillus* spp. in fermented milks in the UK covers a diverse range of species. Eighty five *Lactobacillus* spp. were isolated from a total of 36 fermented milk samples and were identified mainly as *Lb. acidophilus, Lb. casei* and *Lb. paracasei* using genotypic analysis. The presence of these organisms in fermented milks, as an adjunct culture, is in agreement with results of other studies. Pyar and Peh (2014) isolated probiotic *Lactobacillus* species from commercial yogurt and they were identified as *Lactobacillus acidophilus*.

Coeuret *et al.* (2004) studied the different probiotic food in Europe market for claimed probiotic lactobacilli stated on their labels. They could identify isolated bacteria as *Lb. casei* defensis, *Lb. acidophilus*, *Lb. casei* Shirota and *Lb. casei* subsp. *rhamnosus*.

It has been believed that reduction in the number of probiotic lactobacilli in fermented products might be avoided or limited by choosing of appropriate carrier, technological care during processing and the use of higher initial inoculum levels.

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. Further research stated on mislabelling of the probiotic products. Microbial investigation of probiotic products have indicated that the number and identity of recovered species do not always correspond to those declared on the labels of products (Hamilton-Miller and Shah 2002; Temmerman *et al.* 2003). However, this study also signifies that the current situation still needs to be improved.

8.3 Assessment of probiotic characteristics of *Lactobacillus* spp. isolates

Out of 20 identified isolates of probiotic *Lactobacillus*, six were selected to be screened for physiological and functional properties. These isolates along with two commercial cultures of *Lactobacillus* spp. from Chr. Hansen (La5 and C431), and also three different type strains from National Collection of Industrial Food and Marine Bacteria (NCIMB) (*Lb. casei* subsp. *casei, Lb*. *paracasei* subsp. *paracasei* and *Lb. acidophilus*) were used throughout this part of the study.

8.3.1 Resistance to gastrointestinal tract conditions

In chapter three, further characterisation of *Lactobacillus* spp. isolates based on their ability to tolerate conditions in the GIT was carried out. 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 and/or intestinal tract
- 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.

The strains studied showed very poor survival at pH 2, but some degree of protective effects were seen in presence of pepsin at pH 2. No significant reduction in the viable counts of tested *Lactobacillus* spp. was observed at pH values of 3 and 4 in presence of pepsin.

This study confirmed that capability to survive in acidic condition is a characteristic feature of *Lactobacillus* spp., which is entirely depend on pH values and time. It has been reported that *Lb. acidophilus* La5 reduced only 1 $\log_{10}$ cycle after 2 h of exposure to pH 2, but were entirely destroyed after 1 h at pH 1 (Favaro and Grosso 2002). Pan *et al*. (2009) evaluated the resistance of *Lb. acidophilus* NIT to pH 2–4 and, at pH 2, the counts of lactobacilli had decreased to an undetectable level after 2 h; however, within 1 h treatment, the survival rate was >20%. The lactobacilli survival at pH 3 was higher than at pH 2. After 3 h interaction, the viable rate of *Lb*. *acidophilus* NIT was about 10%. When the test pH was enhanced to pH 4, a high survival was notable. They observed that with increasing the incubation time, the count of viable lactobacilli was decreased.

Tested isolates in this research showed extreme sensitivity to acidic condition at pH 2, and the protective effect of pepsin in pH 2 was not as noticeable as pH 3 and 4.

Supporting evidence for protective effects of pepsin on *Lactobacillus* spp. in low pH has been accumulating in recent years (Schillinger *et al.* 2005; Madureira *et al*. 2005). According to the results of Schillinger *et al*. (2005), the reduction of *Lb. acidophilus* strains isolated from probiotic yogurts varied from 0.1 to 2.5 $\log_{10}$ cycles during 90 min exposing to a simulated gastric buffer containing pepsin at pH 2.0, while *Lb. paracasei* and *Lb. rhamnosus* indicated a complete loss of viability within 30 min of experiment. Such protective effect might be due to decreased hyper-polarisation of the cells during exposure to low pH, and it might be linked to the H+-ATPase activity of the cells (Matsumotoa *et al*. 2004; Matto *et al*. 2006). This was not studied and is considered worthy of examination in future.

In contrast to acid resistance, all tested strains in this study presented good resistance to different percentage of bile salts, and were able to retain their viability. 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*. 1994; 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 the 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.* (2000a) 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 more investigated in future.

In this study, in spite of good tolerance of tested strains to bile salts, most of them died in a sequential model after exposing to gastric condition followed by exposure to bile salts (without pancreatin). But, viable numbers was retained when duodenal juice was complemented by pancreatin. It seems that all tested strains not only are intrinsically resistant to pancreatin, but also pancreatin facilitated to recover the strains from harsh conditions in duodenum, and it is a remarkable result in this research which need to be studied further.

8.3.2 Biofilm formation

Another trial undertaken in this study enabled measurement of biofilm formation by *Lactobacillus* spp. The importance of this study originates from the fact that few such studies have been carried out for *Lactobacillus* spp. Such characteristic might be considered valuable to *Lactobacillus* spp. since it helps them to support the colonisation in different ecosystems. After covering of epithelial receptors by *Lactobacillus* spp. biofilm, undesirable microorganisms will not be able to colonise.

It is reported that many parameters influence biofilm formation and composition of growth media might be one of them (Djordjevic *et al*. 2002). Various growth media with normal and altered compositions were applied and, the capability of tested isolates for growing in these media either in planktonic or biofilm form, were assessed (Chapter 4). It was revealed that potential of tested strains for

biofilm formation in different growth media is completely strain dependent, as all tested isolates were not able to produce considerable biofilm in the tested media. It is not easy to interpret which isolate is the most biofilm producer, as it varies among tested isolates in different tested media and at different incubation times.

The evaluation of biofilm formation by tested strains concluded that they produced more biofilm in nutrient rich medium. In general, there was no direct relationship between the extent of biofilm formation and planktonic growth in tested media. Nutrient limitation in diluted media and also replacing of glucose by inulin, caused inadequate or no growth. But, growth limitation was not enough to induce biofilm formation.

Lebeer *et al*. (2007) have reported that *Lb*. *rhamnosus* GG can form biofilms on abiotic surfaces in contrast to the strains of the *Lb. casei*. Biofilm formation by *Lb. rhamnosus* GG was prevented at an initial pH of 4.0 in contrast to neutral pH. Also, the addition of 0.2% bile to the medium increased biofilm formation, but in presence of 1.5% bile, this effect was much less. Contradictory to the observation of Lebeer *et al.* (2007), this study showed that the effect of bile salts does not appear to be dependent to its concentration, since the effect was similar when two different concentration of bile (0.3% and 1.5%) was added to the medium.

Hood and Zottola (1997) studied biofilm production by five different bacterial species. They reported that the growth medium, which induced the formation of the maximum amounts of biofilm, varied for each tested bacteria. They also reported that *Salmonella enterica* serovar Typhimurium produced more biofilm in nutrient limited medium than *Listeria monocytogenes* which produced more

biofilm in nutrient rich media. Nevertheless, the composition of the used medium did not show the same extent of influence on all tested isolats. Based on these results, it is concluded that the tested strains have the ability to form a well-structured biofilm.

8.3.3 Antimicrobial properties

This research reported that culture supernatants (un-concentrated) of all tested isolates did not show suppressive activity against indicator bacteria. Failure of well diffusion method to detect any inhibition activity does not necessarily imply the absence of suppression activity, but might be due to a low concentration of antimicrobial compounds. Confirming this hypothesis the antibacterial activity of tested strains was recovered by concentrating their filtered supernatant.

Concentration of supernatants by vacuum evaporation increased the amount of antibacterial compounds in the supernatant which then showed an inhibition zone of more than 10 mm diameter. However, when concentrated supernatants were neutralized, then antimicrobial activities disappeared or reduced. In this research, production of organic acid besides other unknown substances was detected to suppress the growth of indicator bacteria. There is, however, a possibility of antimicrobial compound production other than organic acids by these organisms. Regardless of organic acids and bacteriocins, there are some investigations about bacteriocin-like inhibitory substance (BLIS) produced by some lactobacilli with inhibitory activities against a wide range of Gram-positive and Gram-negative bacteria (Cheikhyoussef *et al*. 2009; Batdorj *et al*. 2006).

Zhang *et al*. (2011) assessed the antimicrobial substances of 4 lactobacilli strains (*Lb*. *paracasei* subp. *paracasei* M5-L, *Lb. rhamnosus* J10-L, *Lb. casei*

subsp. *casei* Q8-L and *Lb*. *rhamnosus* GG, against *Shigella sonnei*, *E. coli* and *S.* Typhimurium. Organic acids, in particular lactic acid, were identified as the main antimicrobial substances because such activity disappeared when the pH was buffered, and also proteinase K did not affect the extent of the inhibition zones, which indicated that bacteriocins were not involved in the antimicrobial activity.

8.3.4 Production of secondary metabolite (CLA)

Using bacterial strains for bioconversion of LA to produce dietary CLA, is attracting more attention (Nieuwenhove *et al*. 2007; Sieber *et al.* 2004). The use of *Lactobacillus* spp., which are able to produce free CLA in fermentation process, may offer health benefits. The potential of *Lactobacillus* spp. strains in production of CLA in presence of 500 µg LA/ml in MRS broth was studied (Chapter 6). The results obtained in this research showed that tested isolates efficiently converted free LA to CLA in MRS broth, which might be due to presence of Tween-80 in MRS, and plays an important role in the recovery of inhibitory effects of LA on the growth of bacteria and hence, the production of CLA (Jiang *et al*. 1998).

Alonso *et al*. (2003) tested 4 strains of *Lb. acidophilus* and *Lb. casei* subsp. *casei* for their ability to produce CLA in MRS broth containing different concentrations of LA. All strains were able to produce CLA. Maximum production of CLA was observed after 24 h of incubation in MRS broth containing 0.02% of LA.

The percentage of CLA conversion was variable among the isolates in our research. The CLA produced by the tested isolates was recovered from

extracellular phase. It was reported that *cis9-trans11* 18:2 represent 64–95% of total CLA formed and this is similar to the proportion indicated by other researches (Jiang *et al.* 1998; Kelley *et al*. 2007).

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. The mechanism of CLA production by these bacteria might be due to LA hydrogenase enzyme, which needs to be studied further.

8.3.5 Antibiotic resistance

Qualified Presumption of Safety (QPS) is an approach for assessment of selected microorganisms referred to European Food Safety Authority (EFSA 2007). Experience has shown that a tool is required to set priorities in the risk assessment of food/feed associated microorganisms referred to EFSA and formal assessment of safety. Some of the microbial species used in food and feed production have a long history of safe use, while others may represent risks for consumers.

This study reported that all tested strains were intrinsically resistant to amikacin, ciprofloxacin, daptomycin, nalidixic acid, sulfisoxazole. Also it was shown that they are susceptible to amoxicillin/clavulanic acid, ampicillin, ceftiofur, linezolid, nitrofurantoin, penicillin, quinupristinm/dalfopristin, tigecycline and trimethoprim/sulfamethoxazole. However, resistance to cefoxitin, ceftriaxone, chloramphenicol, erythromycin, gentamycin, kanamycin, lincomycin, streptomycin, tylosintartarate, tetracycline and vancomycin were relatively variable among tested isolates.

Susceptibility of *Lactobacillus* species to chloramphenicol has been reported in many studies (Baumgartner *et al.* 1998; Charteris *et al.* 1998; Katla *et al*. 2001; Arici *et al*. 2004) but, in some studies, a resistance to this antibiotic has been seen (Lin *et al.* 1996; Chang *et al.* 2001). However, resistance to chloramphenicol was varying among tested strains in this study.

Temmerman *et al.* (2003) observed the relatively high percentage of kanamycin and vancomycin resistance among a large collection of lactobacilli. In their study, the studied lactobacilli (187 isolates) comprised strains resistant to kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillinG (23%), erythromycin (16%) and chloramphenicol (11%). They reported that the majority of the *Lactobacillus* spp. are intrinsically resistant to glycopeptides. This study screened the presence of the transferable glycopeptide resistant genes *vanA*, *vanB* and *vanX*, but no amplicons were obtained, and it confirmed that resistance of lactobacilli to glycopeptides is intrinsic. These findings are also in line with the results of Ouoba *et al*. (2008b).

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 in Chapter 7.

According to the results, none of the tested strains in current study presented positive PCR for the resistance genes investigated. Consequently, they can be considered safe regarding their potential to transfer resistance genes. The absence of the transferable resistance genes in this research is not unexpected, because they were isolated from commercially probiotic products where the commercial cultures are used at high dosages and which may have undergone

several assessments to guarantee the absence of transferable resistance genes before their use. Also, resistance to certain antibiotics is not considered as an abnormal characteristic of probiotic cultures. The complex intrinsic characteristics such as cell wall structure or metabolic properties are considered the reasons for many of these resistances. It could be argued, however, that the resistances detected in this study could not be traced back to specific genes by PCR.

Outcomes of this research indicated that as part of the commercialisation procedure for probiotic strains, such important safety measures need to be carried out. However, such data by no means are inclusive and, as a controversial topic, needs further investigations.

8.4 Strengths and limitations of this study

Characterisation of probiotic microflora is needed for the legal requirements to sustain associated claims in functional dairy foods (Sanders 2008). The manufacturers of probiotic products always need to assure consumers for viability of probiotic microorganisms in functional products and also whether they provide any benefits for them. Recently, European Food Safety Authority (EFSA) has rejected numerous health claims for probiotics, and banned on the descriptive usage of the term ‘probiotic’ as it implies health benefit. The reason for this has been the insufficient reports of evidence available to trace back such health claims (Katan 2012; Salminen and van Loveren 2012; Binnendijk and Rijkers 2013).

This research was considered necessary because of the gaps in knowledge about the exact mechanisms by which probiotics can exert their health benefits. However, there are very few studies on diversity of lactobacilli to demonstrate their probiotic beneficial properties, specifically after exposure with low pH condition in commercial fermented products.

Even though probiotic products, such as fermented milks are generally regarded as safe and stable, this research emphasises the necessity for continuing studying and publication by researchers which may persuade manufacturers to improve the quality of their products. This research has attempted to understand what could happen to probiotic bacteria which are not protected. 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. This study revealed a considerable diversity in probiotic properties among the different isolates of lactobacilli. From the results of this study, it could be suggested that tested isolates are justified in their use as a probiotic in most of occasions, while these strains would need to be protected from very harsh condition (i.e. pH 2) in order to increase the likelihood of obtaining benefit from their probiotic properties.

Our findings are good indication of the improvement that has been made over the years in selecting and applying more resilient strains of lactobacilli in fermented dairy products. Also, 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.

Although *Lactobacillus* spp. has many technological advantages, such as resistance to harsh conditions in the products and even in the GIT, 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.

The difficulties involved in the analysis of probiotic *in vivo* have led to the development of *in vitro* models for the selection of potentially adherent strains. But *in vitro* studies and animal experimental analyses only give indications to possible health relevant effects. It has been found that due to the complexity of the immune system and the numerous interactions with the indigenous gut microbiota and administered probiotic bacteria, interpretation of obtained data is often difficult.

Indeed, *in vivo* studies are necessary to confirm the potential influences prior to introducing the probiotic species to clinical intervention studies (Collado *et al*. 2007). Besides, individual strains should be tested for each attributes because characteristics ascribed to a probiotic are strain-specific (Verdenelli *et* al. 2009).

8.5 Suggestions for future works

- Continued search for finding more diverse range of *Lactobacillus* species/strains with other beneficial properties for use in fermented dairy products and also an expansion to other probiotic groups, such as *Bifidobacterium* spp., *Lactococcus* spp., etc.

- Further research on labelling of the probiotic products and suggestions for improvement of current situation.

- Improving 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 in order to give an even more realistic representation of how gastrointestinal stress could affect functional properties.

- Research on possible protective mechanisms of pepsin and pancreatin on probiotic bacteria during exposure to low pH.

- Further investigations and more evidences as potential warning that risk of transfer of antibiotic resistance genes, might be associated with commercial probiotic foods.

- Research on gene associated with biofilm formation and its expression after being exposed to conditions similar to GIT, such as low pH, presence of bile salts and mucus.

- Further investigations on interactions of lactobacilli 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.

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APPENDIX

Media and composition

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

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

Maximum Recovery Diluent (MRD, CM0733, Oxoid Ltd)	g/l
Pepton	1
Sodium chloride	8.5

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

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 using sterile membrane filter (0.2 µm, 7187, Whatman Filters, Maidstone, UK) or with sterile syringe-driven membrane filters (0.2, 190-2545, Nalgene, Fisher Scientific, Loghborough, UK).

QIAquick® PCR Purification Kit

Notes before starting

- This protocol is for the purification of up to 10 µg PCR products (100bp to 10 kb in size).

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. The adsorption of DNA to the membrane is only efficient at pH ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.
- Symbols: ● centrifuge processing; ▲vacuum processing.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ● a provided 2 ml collection tube or into ▲ a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ● centrifuge for 30–60 s or ▲ apply vacuum to the manifold until all the samples have passed through the column. ● Discard flow-through and place the QIAquick column back in the same tube.

4. To wash, add 750 µl Buffer PE to the QIAquick column ● centrifuge for 30–60 s or ▲ apply vacuum. ● Discard flow-through and place the QIAquick column back in the same tube.

5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0– 8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.