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CHARACTERIZATION OF THE NON-STARTER BACTERIAL FLORA OF STILTON CHEESE

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Abstract

This study characterised the bacterial flora of a commercially produced Stilton cheese in an effort to determine the contribution of non-starter lactic acid bacteria (NSLAB) to its aroma profile. A total of 123 microbial strains previously isolated from different sites (outer crust, blue veins and white core) of the cheese sample obtained at the end of ripening (~8 weeks) were recovered in MRS and BHI broths and preliminarily identified using conventional microbiological methods in order to establish population diversity and to screen out yeasts and moulds. Organisms identified with partial 16S rDNA sequence analysis were *Lactobacillus plantarum*, *Lactobacillus brevis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Acinetobacter baumanii* and *Psychrobacter* spp., with the genus *Lactobacillus* being the dominant (75%) group detected in all the sampled sites. Cluster analysis of pulse-field gel electrophoresis patterns associated the *Lactobacillus* isolates according to their site of isolation.

Lb. plantarum isolates, two from each of the cheese sites, were evaluated for tolerance to heat stress and to different levels of salt, acid and relative humidity (RH) in order to ascertain whether the stress conditions associated with the isolation site could select the phenotype of microbial species recovered. The $D_{72^{\circ}C}$ values revealed that isolates obtained from the outer crust were more heat sensitive suggesting they may have colonised the cheese post-pasteurisation. All the isolates were sensitive at pH range 3-4 but could grow at pH range 4.5-5. Similarly, isolates could grow at 3.5-5% (w/v) sodium chloride but were suppressed at 10%. Lactobacilli from the outer crust were the most halo-tolerant growing at 8% sodium chloride. For all strains, survival was low at 33-54% RH when cells were suspended in sterile de-ionised water but survived better at 33% RH in maximum recovery diluent (MRD) suggesting cellular protection by MRD.

Lb. plantarum isolates from each site (outer crust=7; blue veins=19; white core=24) were tested for antimicrobial activity against *Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Staph. aureus, Salmonella* Typhimurium, *Clostridium sporogenes, Lb. pentosus* and *Lactococcus lactis* using the plate agar overlay and paper disc diffusion assays. All the 59 *Lactobacillus* isolates were tested for plantaricin EF genes using PCR. The nature of antimicrobial activity was examined using cell-free supernatants treated to neutralise acids and/or hydrogen peroxide. Treatment with proteinase K was used to ascertain whether activity was due to bacteriocin (putative plantaricin) production. On solid medium, the isolates had antimicrobial activity against Gram-negative and Gram-positive bacteria, each isolate showing activity against more than one species. *Lb. pentosus, Ps. aeruginosa, E. coli* and *L. monocytogenes* were the most sensitive whereas *Cl. sporogenes* was the most resistant spp. Activity against these organisms was mainly attributed to acid, and to a less extent, hydrogen peroxide and plantaricin production. Whereas *Lb. plantarum* isolates had a high prevalence of plantaricin EF genes, there was weak evidence for plantaricin production in liquid medium assays. Plantaricin production was only demonstrable among *Lb. plantarum* isolates from the veins and core against *Lb. pentosus*, implying the phenomenon was largely dependent on the genotype/strain of *Lb. plantarum* and was only active against closely related lactic acid bacteria.

Subsequently, the effect of growth and survival dynamics of the different genotypes of the organism on the volatile aroma profiles of milk was examined. Individual isolates, one from each of the cheese sites, were co-cultured with acid-producing *Lc. lactis* (APL) and non acid-producing *Lc. lactis* (NAPL) in UHT milk under simulated cheese ripening conditions. During early fermentation (0-48 h, 30^{o}C), the isolate obtained from the blue

veins stimulated more growth of *Lactococcus* strains in mixed culture when compared to single cultures and to *Lactobacillus* isolates obtained from other sites in mixed culture. The volatile profiles of all *Lb. plantarum* strains grown alone were not significantly different (p>0.05). The type and levels of volatiles detected in mixed culture depended on the genotype/strain of *Lb. plantarum* inoculated as well as the acidification capability of *Lc. lactis* with which it was co-cultured. Co-culture of *Lactobacillus* isolates with APL resulted in increased aldehyde and alcohol production, whereas with NAPL only acetoin synthesis was enhanced. Salt addition had minimal effect on the volatile profiles. During further incubation (12 weeks, 18^{o}C), growth of *Lb. plantarum* strains was better in salted samples inoculated with NAPL. The NAPL strain remained stable at 7 $\log_{10}$ CFU/ml throughout, while the APL rapidly declined from 9 to less than 5 $\log_{10}$ CFU/ml. The highest level of alcohols, organic acids and acetoin was detected from samples inoculated with the pure culture of the *Lactobacillus* isolate obtained from the blue veins. Co-culture of the isolate with APL enhanced acid and alcohol production, whereas its co-inoculation with NAPL increased acetoin synthesis. As *Lb. plantarum* is an incidental organism in cheese, its presence is unpredictable; it was therefore concluded that occurrence of different genotypes of the organism could be a major contributory factor to the variations in the cheese quality characteristics from batch to batch.

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List of Abbreviations

AHDB	Agriculture and Horticulture Development Board (UK)
AMOVA	Analysis of molecular variance
APL	Acid-producing *Lactococcus lactis*
AU	Area units
FFA	Free fatty acids
FSAI	Food Safety Authority of Ireland
IDF	International Dairy Federation
IDFA	International Dairy Foods Association
NAPL	Non acid-producing *Lactococcus lactis*
NSLAB	Non-starter lactic acid bacteria
PCA	Principal component analysis
PDO	Protected Designation of Origin
SCMA	Stilton Cheese Makers Association
SPME GC-MS	Solid phase micro-extraction gas chromatography- mass spectrometry
TAPPI	Technical Association of Pulp and Paper Industry
UHT	Ultra high temperature
UNFCC	University of Nottingham Food Sciences laboratory culture collection
USDA-FAO	United States Department of Agriculture-Food and Agricultural Organisation

CHAPTER 1

GENERAL INTRODUCTION

1.0 Background

Microorganisms are important in the production of all fermented food products and are responsible for their organoleptic characteristics (Bylund, 1995). In many fermented foods, microorganisms are added as starter cultures but often the final flora of the product varies widely, as other microorganisms enter the food as contaminants and survive the processing stages (Yim and Glover, 2003). These non-starter organisms can dominate the microflora of the final product and they may contribute substantially to aroma and other properties of the matured food. This makes quality control of most fermented foods difficult as batch-to-batch variation occurs depending on which organisms are present.

Milk fermentation is one of Man's most ancient traditions and has resulted in worldwide development of a variety of cultured milk products (Wouters *et al*., 2002). The evolution of these products has mainly been dependent on the type of milk, microorganisms involved and the technologies used for fermentation and microbial metabolites that accumulate in the final product (Platt, 1987; Robinson, 1990). The microbial ecology of different regions of the world, as well as the differences in dietary habits and social structures, have had profound influence on the various types of fermented milk products available on the market to such an extent that makes it difficult to classify them effectively due to their wide variation (Robinson and Tamime, 2006).

Milk for manufacture of fermented products must be of high quality and free from antibiotics and sanitising agents that may inhibit the growth of acid-forming bacteria. The milk is often pasteurised to eliminate spoilage and pathogenic microorganisms (IDF, 1988). The process results in denaturation of whey proteins and improves texture of the fermented product (Rynne *et al*., 2007). Cheeses made from pasteurised milk often ripen less rapidly and less extensively giving less intense flavours than their counterparts made from raw or lightly heat-treated milk (IDF, 1988; Buchin *et al*., 1998). For this reason, pasteurised milk for cheese manufacture is normally inoculated with both the fermenting

microflora (starter cultures) and rennet which promotes rapid curdling and ripening (Tamime and Robinson, 1999). Cheese ripening brings about the chemical changes necessary for transforming the fresh curd into a distinctive aged cheese. These changes are catalysed by rennet and microbial enzymes and largely depend on temperature and humidity in the ripening room, as well as on the type and levels of substrates (citrate, lactose, proteins and lipids, etc) and moisture content of the cheese. The ripening time can be as short as one month, such as for Brie, to more than a year, as in the case of some Cheddar varieties.

The thermophilic bacteria *Lactobacillus helveticus* and *Lactococcus thermophilus* are usually used as starter cultures for acid and flavour development in Emmenthal type cheeses (not typical of other cheeses) where the curd is normally heated at temperatures greater than 40^{o}C prior to moulding and ripening (Beresford *et al*., 2001). Citrate-negative mesophilic strains of *Lactococcus lactis* subsp. *lactis* or *cremoris* (O-cultures) are often used for acid production in Cheddar, Feta, Camembert and Stilton cheeses, whereas citrate-positive species such as *Leuconostoc cremoris* (also called L-culture), *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*, and *Lactobacillus casei* (D-cultures) are mostly applied for aroma production in hard and semi-hard yellow cheeses (Morgana *et al*., 2002). When both acid and aroma-producing strains are present, the mixed culture is referred to as DL-type. In cheese manufacture, DL cultures are selected on the basis of their ability to produce carbon dioxide that contributes to an open texture, and diacetyl which give the product its essential buttery flavour (Vallejo *et al*., 2008). Citrate metabolism, via the reductive tricarboxylic acid pathway, results in formation of succinate or diacetyl, and carbon dioxide (McSweeney, 2004). Succinate contributes a savoury flavour to Swiss-type cheeses. Diacetyl, formed by oxidative decomposition of α-acetolactate, imparts a buttery note in butter, buttermilk and some cheese types, whereas carbon dioxide aids the formation of an open texture in some soft/semi soft cheeses. Knowledge of citrate metabolism and diacetyl production has led to effective strategies for engineering *Lc. lactis* strains to enhance diacetyl production.

In the United Kingdom (UK), milk is mainly obtained from cows but other sources include sheep and goats, as well as a number of plant-based substitutes such as soya, rice, oat and almond milk for people with lactose intolerance (UK milk statistics, 2009). The milk is either consumed fresh or processed into cheese, butter and yoghurt using

traditional and modern technologies. Cheese is manufactured through a series of carefully controlled processing steps involving fermentation with a composite of more than one microbial species to allow the development of desired organoleptic properties. Traditional cheese processing technologies often result in products of variable quality and microbiological characteristics due to complex sequential microbial succession dynamics involving non-starter lactic acid bacteria (NSLAB), yeasts and moulds (Martley and Crow, 1993). The ripening temperature, time, type and level of starter cultures added vary with different producers, countries and regions giving rise to thousands of cheese varieties in the world. Upgrading traditional cheese production, through identification and development of the natural microflora contributing to desirable aroma and other organoleptic characteristics (starter culture adjuncts) would enhance products with good and consistent quality characteristics (Ibourahema *et al*., 2008).

Ripened cheese is manufactured using either individually or a combination of rennet, acid or heat treatment of the milk, whereas for cottage cheese, milk coagulation is mainly induced solely by acidification (Fernández *et al*., 2009). The majority of semi-soft blue cheeses are manufactured by acid-heat coagulation (Fernández *et al*., 2009), but in the case of Stilton, milk coagulation is induced by acid/rennet treatment.

Production of blue cheese involves inoculation of the milk with spores of *Penicillium roqueforti* which imparts the typical blue venation and flavour (Lawlor *et al*., 2003). The ripening process is associated with changes in the cheese microenvironments, microorganisms that contribute to the process and various biochemical changes such as lipolysis and proteolysis which are important for aroma formation. In general, the microenvironments in blue cheese are heterogeneous with pronounced gradients of pH, salt, water activity (a_w) and redox potential (Cantor *et al*., 2004; Fernández *et al*., 2009) depending on the method of salting, solute concentration in the cheese mass, metabolic activities of inherent microorganisms and prevailing conditions in the ripening room including aeration, relative humidity and temperature (Prieto *et al*., 1999). This consequently leads to considerable structural differences within the cheese which influences the level and distribution of oxygen and carbon dioxide (Cantor *et al*., 2004). The above factors directly or indirectly impact on the growth, interaction and biochemical activity of the various microorganisms present in different sites of the cheese and consequently affect the quality characteristics of the final product. Elucidation of such

interactions would greatly add to an understanding of the cheese ripening process and would enable a more targeted approach to starter culture and culture adjunct selection for quality improvement and maintenance of ripened Stilton cheese.

Many countries have developed their own types of blue cheeses, each with different characteristics and involving different production methods developed over a long period of time. Today, the best known blue veined cheeses are considered to be Gorgonzola (Italy), Roquefort (France), Stilton (UK) and Danablu (Denmark), all of which have been granted the status of protected designation of origin (PDO) (Fernández *et al*., 2009), a classification awarded by the European Union to protect Native rights of traditional foods. A PDO covers the term used to describe foodstuffs and agricultural products which are produced, processed and prepared in a given geographical area using recognised know-how (FSAI, 2003). This EU law aims to protect the reputation of the regional foods, promote rural and agricultural activity, help producers obtain a premium price for their authentic products, and eliminate unfair competition and misleading of consumers by non-genuine products, which may be of inferior quality or of different flavour. In general, a PDO is the name of an area, a specific place or, in exceptional cases, the name of a country, used as a designation for an agricultural product or a foodstuff which comes from such an area, place or country whose quality or properties are significantly or exclusively determined by the geographical environment, including natural and human factors. Production, processing and preparation must take place within the determined geographical area and the entire product must be traditionally and entirely manufactured (prepared, processed and produced) within the specific region and thus acquire unique properties.

There are two varieties of Stilton cheese; blue and white Stilton both of which are produced in only six creameries in the counties of Nottinghamshire, Leicestershire and Derbyshire following a standard procedure (SCMA, 2010). Blue veined Stilton cheese is mould-ripened and has a rich and mellow flavour, whereas the white variety is produced without inoculation of *Pen. roqueforti*. Although there are only six dairies in the world (in UK) licensed to make Stilton cheese, the word 'Stilton' is a certification trade mark registered not only in the UK but in another 15 countries around the world. As such, there are legal requirements relating to the labelling of these products and products where the

cheese may form a predominant part, such as white Stilton cheese with apricot (SCMA, 2010).

The presence of non-starter bacteria in inoculated and non-inoculated fermented foods as well as their contribution to biochemical changes and aroma characteristics of these products is well documented (Williams and Banks, 1997; Yim and Glover, 2003). Cheese ripening generally follows a gradual succession of different bacteria, yeasts and moulds. The dominant species in the majority of the cheeses include staphylococci, corynebacteria, micrococci, *Debaryomyces hansenii*, *Geotrichum candidum* and *Penicillium camemberti*, all of which are sometimes deliberately applied as secondary starter culture adjuncts to enhance organoleptic properties of the ripened products (Corsetti *et al*., 2001). In fact, improvements in the microbiological quality of raw milk, sub-pasteurisation of milk for 15-30 s at 60-65^{o}C (thermisation) to inhibit bacterial growth for 1-2 days, relatively higher level of hygiene and modifications in the cheese manufacturing plants have all been reported to reduce sources of indigenous secondary microbiota in ripened cheeses (Chamba and Irlinger, 2004). Consequently, many cheeses have frequently developed bland tastes; this has led to increasing awareness about the role of the non-starter microflora in production of high quality cheese. This has led to the demand for development of starter culture adjuncts for consistent product quality characteristics (Chamba and Irlinger, 2004; Cogan *et al*., 2007).

The NSLAB that grow in ripened cheese sometimes additionally provide protective advantage against spoilage and pathogenic microorganisms through formation of acetic and lactic acid, hydrogen peroxide, acetaldehyde, diacetyl and bacteriocins (Amin *et al*., 2009; Essid *et al*., 2009; Sawitzki *et al*., 2009). In Cheddar cheese for example, *Lactobacillus casei* and *Lactobacillus plantarum* are the most common NSLAB used as culture adjuncts for flavour enhancement and to reduce microbial spoilage of the cheese (Peterson and Marshall, 1990). In Swiss cheese, *Propionibacterium freudenireichii* is used for riddling holes known as "eyes", whereas *Brevibacterium linens* is often used as an outside smear culture adjunct in Gruyere, Brick and Limburger cheeses (Prabhakara *et al*., 2011). There is no information on the application of NSLAB as culture adjuncts during the production of Stilton cheese; which calls for scientific research on this aspect.

As blue cheeses become more popular, there is increasing interest in identification and characterisation of their dominant NSLAB that enhance products with more consistent

quality characteristics (Cantor *et al.*, 2004). The microflora of Stilton cheese has previously been established using conventional culture-dependent microbiological methods and molecular techniques (Fitzpatrick, 1971; Whitley, 2002; Ercolini *et al.*, 2003, Hiscox *et al.*, 2008; Gkatzionis, 2010). Acidification usually begins with the primary starter culture *Lc. lactis*, which is gradually replaced by the more acid-tolerant NSLAB from the environment particularly lactobacilli (Hiscox *et al.*, 2008). Whereas it is believed that the non-starter lactobacilli establish competitive symbiotic interactions with lactococci in the cheese, there is insufficient information on the possible interaction of these organisms with starter culture lactococci and *Pen. roqueforti* (Gkatzionis *et al.*, 2009).

The contribution of *Pen. roqueforti* to the aroma and blueing of Stilton cheese is well recognised (Fernández *et al.*, 2009; Gkatzionis *et al.*, 2009), but there is no evidence to suggest that NSLAB that grow in the cheese interact with starter lactococci and the mould to modulate the organoleptic characteristics of the product. The role of possible interactions between the dominant NSLAB in Stilton cheese and starter culture lactococci as well as *Pen. roqueforti* in development of the cheese aroma properties needs to be established. This study aimed to examine the role of possible interactions between NSLAB that grow in Stilton cheese and *Lc. lactis* in modulating the product aroma profile. The study also aimed to establish whether abiotic stresses associated with the microenvironments in different sites (outer crust, blue veins and white core) within Stilton cheese are important in selecting for the presence of genotypically different strains of the dominant NSLAB within the ripened product. The strains with prospects for development into culture adjuncts for reliable and consistent quality production of Stilton and other blue cheeses would be identified.

CHAPTER 2

LITERATURE REVIEW

2.1 Cheese production and nutrition

World cheese production has increased by 81% in the last 35 years, from under 11 million tonnes in 1977 to over 18 million tonnes in 2012 (AHDB, 2009; USDA-FAO, 2012). France, Germany and United States of America (USA) are the major cheese producers estimated at 1.8, 2 and 4.8 million tonnes respectively in 2007; which is about 50% of the global cheese production (Table 2.1).

Table 2.1: World cheese production, export and consumption patterns for 2004-2012

Country	Production (000 tonnes)	Export (000 US $)	Consumption (kg/capita/year)
Year	2012	2012	2012
Total	18,794		
USA	4,275	-	14.8
Germany	1,927	2,416,973	22.6
France	1,884	2,658,441	26.1
Italy	1,149	1,253,580	
Russia	603 (2007)	-	-
Brazil	495	-	-
Egypt	462	-	-
Argentina	425	-	11.3
Australia	395	643,575	12.0
UK	391 (2007)	374,156	10.9
New Zealand	291 (2007)	631,963 (2004)	-
China	275 (2007)	-	-

Source: AHDB (2009), USDA-FAO (2012), (2004-2007) data for the year 2004-2007

The world's most cheese exporting countries by monetary value are France and Germany. Among the top ten cheese exporters, only Ireland, the Netherlands and Australia have cheese production that is mainly export oriented with 95%, 72% and 65% respectively, of their cheese production exported (USDA-FAO, 2012). In contrast, France (world largest exporter) exports only 30% of cheese production with Emmental (used as a cooking ingredient) and Camembert being its most common cheeses. USA is the largest world cheese producing country but exports marginal levels as most of the cheese is consumed by the domestic market (PM Food and Dairy Consulting, 2009). Mozzarella is America's favourite cheese and accounts for nearly a third of its consumption, because it is one of the main ingredients of pizza (IDFA, 2012). Cheese consumption is increasing in China, with annual sales more than doubling from 1996 to 2003 (AHDB, 2009). Germany, UK and Italy are the world leading importers of cheese. The increase in global cheese production and consumption calls for more studies to improve its quality characteristics.

Cheese is an ancient food with no conclusive evidence of whether it originated from Europe, Central Asia or the Middle East where it is strongly associated with tradition and culture. Although still rarely considered as part of the local ethnic dishes in and outside these regions, cheese has increasingly become known and popular worldwide due to its portability, longer shelf life (especially if it is encased in a protective rind), good flavours and high nutritional value. Cheese has a high calorific value due to its relatively high protein and fat content (Table 2.2). Cheese produced in Europe, where climates are cooler, requires less salt and acidity for preservation making it a suitable environment for microbial growth. This gives aged cheeses their distinctive flavours arising from less hindered starter culture growth and metabolism.

Table 2.2 Average composition of some cheese varieties

Cheese variety	Component (per 100 g)						
	water (g)	energy (kcal)	fat (g)	protein (g)	lactose (g)	sodium (mg)	calcium (mg/100 g)
Cheddar	-	402	33	25	1.4-2.1	621	720
Mozzarella	-	280	17	28	0.3-3.2	-	731
Cottage	-	<289	3.9	49	4.9-28	-	243
Blue Stilton	38.0	410	35.0	23.7	-	788	-
Danablu	45.3	347	29.6	20.1	-	1360	-
Gorgonzola	45.0	357	28.9	21.8	-	1000	-
Roquefort	41.3	375	32.9	19.7	-	1670	-

Source: SCMA (2009)

Some of the main factors hindering consumption of cheese and other dairy products include personal selective tastes and preferences, prevalence of lactose intolerance in some communities such as in East Asia and strict religious and cultural taboos which restrict consumption of cheeses made with rennet from animals that are not slaughtered in a manner that does not adhere to halal or kosher laws (Watanabe *et al*., 1999). Currently, almost all cheese types are made with rennet produced from the fungus *Mucor miehei* (De Lima *et al*., 2008) or by cloning the rennet gene into a host microbe where it is expressed. This has consequently increased cheese consumption, particularly in some parts of Asia.

2.1.1 Classification of cheese

There are several types of cheese, with about 500 different varieties recognised by the International Dairy Federation (IDF). Cheese varieties are classified according to criteria (singly or combination) such as length of ripening, texture, method of manufacture (e.g. cooking step to enhance syneresis), fat content, animal milk used, country or region of origin, etc (Banks, 2006). Although there is no universal method of classification, cheese is commonly categorised on the basis of moisture content, which is then further discriminated by fat content, primary and secondary starter culture used and ripening methods employed (Little *et al*., 2005).

There are three major types of cheese with regard to the fat content (FC), i.e. >60%, 45-60% and 30-45% (Gunasekaran and Mehmet, 2003). Soft cheeses such as Brie are classified as high FC cheeses. Moisture content (MC) of cheese varies from less than 35% to over 60% (Farkye, 2004). Fresh or cottage cheeses such as Ricotta and Feta contain the highest MC while hard-curd cheeses such as Cheddar have the lowest. In general, cheeses are classified on the basis of their MC as: fresh (>60%), soft (45-60%; such as Camembert and Roquefort), semi-soft (35-45%; such as Edam, Stilton and Brick) and hard-type cheeses (<35% MC; such as Gruyere, Parmigiano Reggiano and Gouda) (Farkye, 2004). Soft and semi-soft cheeses are normally consumed within 1-3 months of production when they still retain their MC, have a rubbery texture and buttery flavour.

Cheese may also be classified according to the type of rind (bloomy, artificial or washed) (Banks, 2006). Washed-rind cheeses, such as Langres, are rubbed or washed with water, wine, beer or brine in order to maintain high internal MC and attract the bacterium *Brevibacterium linens* that forms the characteristic red/orange sticky rind and a smooth texture at maturity. In bloom-rinded cheeses such as Brie and Camembert, the cheese surface is seeded with *Penicillium candidum* and *Pen. camemberti* respectively, which grow and cover the curd with a white velvety layer referred to as the "bloom" (Shaw, 2007). Smeared rinds progressively change in colour, aroma and flavour as the ripening process evolves (Lawrence *et al*., 1987). Cheese types with well-formed rinds such as Stilton can be stored for longer time with minimal changes to their aroma and other quality characteristics.

2.1.2 Methods of analysis of microbiology of cheese

In the past, studies on microbial interactions which occur during the manufacture and ripening of cheese and other food products were mainly dependent on the classical culture-based microbiological techniques, which are not only time consuming and biased due to selectivity of media applied, but also unsuitable for handling large numbers of samples and generally not suitable to studies at sub-species level (Beresford, 2001). Today, there is a wide range of molecular protocols, which enable rapid identification of microorganisms to species and strain levels. Techniques such as pulse-field gel electrophoresis (PFGE) and polymerase chain reaction with denaturing gradient gel electrophoresis (PCR-DGGE) can provide a reliable distinction between the strains involved in complex microbial population dynamics and they have been successfully applied to study microbial interactions and succession in many ripened cheeses and other food fermentation systems (Beresford, 2001; Blaiotta *et al*., 2001; Bouton *et al*., 2002; Ercolini *et al*., 2003).

Within the genus *Lactobacillus*, species and subspecies have been also discriminated by using the following methods: ribotyping, amplified rDNA restriction analysis (ARDRA) and amplified fragment length polymorphism (AFLP) (Singh *et al*., 2009). Ribotyping employs probes designed against 16S or 23S rDNA genes. Species-specific oligonucleotide probes based on the gene *groEL* which encodes the Hsp60 have also been used to differentiate more than 40 species of *Lactobacillus* (Blaiotta *et al*., 2008). Probes based on other genes including *rpoB*, *pyrDFE* (Singh *et al*., 2009), *tuf* (Ventura *et al*., 2003) and *recA* (Torriani *et al*., 2001) have also been successfully applied to infer phylogenetic relationships among species of lactobacilli and bifidobacteria. This approach has been used to discriminate *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum* (Torriani *et al*., 2001). Species-specific fragments obtained from randomly amplified polymorphic DNA (RAPD) or DNA fragments obtained from restriction digests of plasmids or genomic DNA, have also been utilised to obtain specific probes for some *Lactobacillus* species (Kunene *et al*., 2000).

Ribotyping involves separation and identification of fragments of the genome containing the 16S or 23S rDNA genes and detecting variation in restriction patterns by hybridisation with a labelled probe, within the restriction endonuclease pattern. The resultant profile (ribopattern) is simpler than the original restriction profile since only DNA fragments

complimentary to the rDNA probe are visualised. Ribotyping is widely used for both species and subspecies discrimination within *Lactobacillus* (Kunene *et al*., 2000). The technique is highly reproducible and has successfully been applied to detect individual species or strains within the *Lb. acidophilus* complex, *Lb. casei*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. helveticus*, *Lb. plantarum*, *Lb. reuteri*, *Lb. rhamnosus* and many other lactobacilli (Singh *et al*., 2009). ARDRA, a technical variation of ribotyping, is equally efficient for species level identification of *Lactobacillus* species and involves the restriction enzyme analysis of 16S rDNA PCR amplicons. AFLP employs a combination of PCR and RFLP and is based on selective PCR amplification of restriction fragments from digested DNA. The amplified fragments are then separated by polyacrylamide gel electrophoresis. The method is highly reproducible, allows a quick scan of the whole genome for polymorphisms and has been found good enough to discriminate between *Lb. pentosus*, *Lb. plantarum* and *Lb. paraplantarum* (Torriani *et al*., 2001). Sequence-based approaches such as multilocus sequence typing (MLST) are also gaining popularity for species and subspecies differentiation of lactobacilli (Singh *et al*., 2009; Tanigawa and Watanabe, 2011).

Other techniques which have been used to characterise and classify LAB include analysis of whole-cell protein, cell wall composition, morphology, physiology and biochemical characteristics (Kunene *et al*., 2000). While the combined use of these methods is invaluable for distinguishing LAB at species level, the methods are not sufficiently discriminatory to differentiate the organisms at subspecies and strain levels (Kunene *et al*., 2000). Determining the electrophoretic patterns of total soluble proteins (Khalid, 2011) and genomic analysis with PFGE (Yeung *et al*., 2004) followed by computer-assisted analysis of the resulting profiles are well established procedures in bacterial taxonomy. The latter technique, also the main focus of this work, has been previously used for taxonomic discrimination of LAB at sub-species and strain levels (Sanchez *et al*., 2004; Yeung *et al*., 2004). The precision of PFGE can be improved by creating a digitised and normalised restriction profile of LAB of known species, subspecies and strains (Benson and Ferrieri, 2001), as well as by employing 2-3 restriction enzymes (Singh *et al*., 2009). Other techniques such as RAPD and determination of rRNA sequence homology have been shown to have their limitations of lack of reproducibility of the results (RAPD) and requirements for long periods of time and labour (rRNA sequence determinations; Yeung *et al*., 2004). *Lactobacillus* and *Leuconostoc* are also highly

heterogeneous giving low correlation between the phylogenetic relationship and rDNA sequencing (Khalid, 2011).

2.2 Sources and effect of microbial contaminants associated with cheese spoilage

The wide array of fermented dairy products in the world market offers challenges to food technologists in order to establish the best ways to prevent microbial contamination, destroy organisms (and their enzymes) already present in the food, and prevent growth and activity of those that escape the processing treatments. The most common spoilage microbial groups in ripened cheese include aerobic psychrotrophic Gram-negative bacteria such as *Pseudomonas*, yeasts, moulds, heterofermentative lactobacilli and spore-forming bacteria (Ledenbach and Marshall, 2009). Psychrotrophic bacteria often produce heat resistant extracellular hydrolytic enzymes that cause taints and rancid off-flavours and the extent of recontamination of cheese with these bacteria is a major determinant of its shelf life (Champagne *et al*., 1994). Fungal spoilage is manifested by the presence of a wide variety of metabolic by-products causing off-odours and off-flavours, in addition to visible changes in colour and texture (Filtenborg *et al*., 1996). Coliforms, yeasts, heterofermentative LAB and spore-forming bacteria can all cause gassing defects in ripened cheese (Ledenbach and Marshall, 2009). The rate of cheese spoilage can be slowed by lactic acid production, introduction of desirable microflora that prevent growth of undesirable microorganisms and addition of salt to reduce water activity (Beresford *et al*., 2001). Raw milk is the major source of microorganisms associated with dairy products. However, in cheeses made from pasteurised milk (such as Stilton), microbial contamination can occur from other sources (Fig. 2.1).

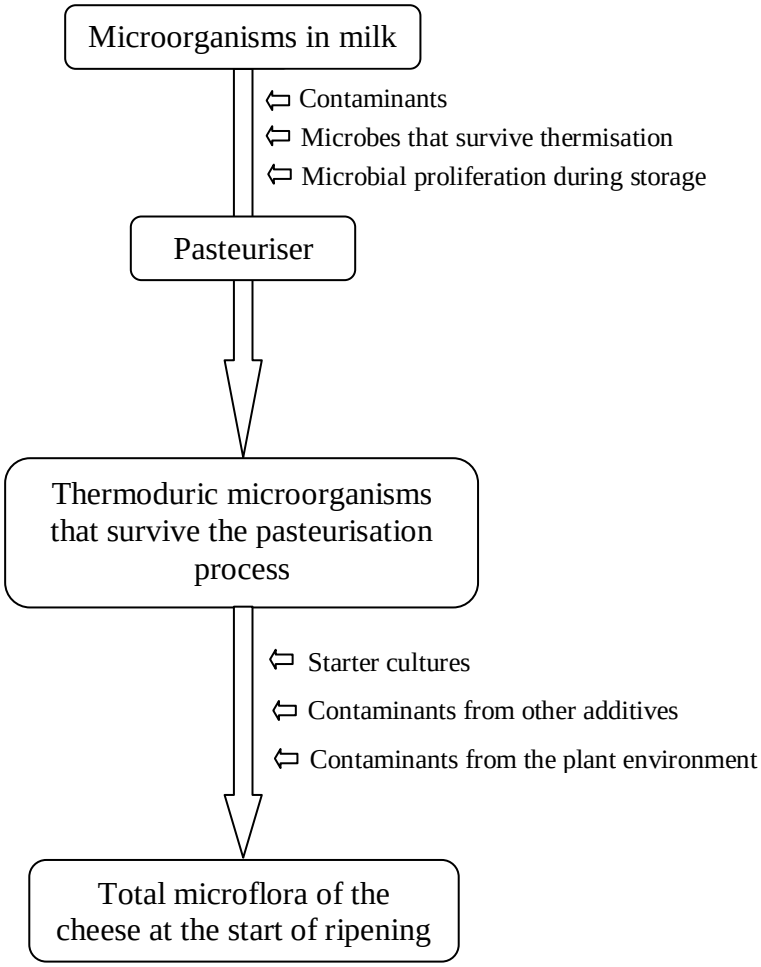


Figure 2.1 Potential sources of microbial contamination during cheese manufacture. Adapted from Martley and Crow (1993)

Raw milk for cheese manufacture may be contaminated with different microorganisms from the infected cows' udder (Mubarack *et al*., 2010). Environmental contaminants also represent a significant percentage of the cheese spoilage microflora. Spoilage microorganisms are ubiquitous in the environment from which they contaminate the feeds, cows, equipment, water and the milkers' hands (Torkar and Teger, 2008). Bacteria that re-contaminate pasteurised milk originate primarily from the resident microflora of water and equipment or from the immediate surroundings (Martley and Crow, 1993). Little *et al*. (2005) examined the microbiological quality of cheeses made from pasteurised milk in the UK. Their results indicated that whereas 98% of the 2,636 samples had satisfactory microbiological quality, 2% were unsatisfactory with *Staphylococcus aureus* (>10^3 CFU/g), *Escherichia coli* (>10^3 CFU/g) *Listeria* spp. (<20 CFU/g) and *L. monocytogenes* (detected in 0.2% of the samples) being the major organisms. The

incidence of these organisms was higher in semi-hard cheeses, and in the products imported from Poland (12.5% of 8 samples) and Italy (5.5% of 201 samples). Insufficient control systems, poor hygiene and storage at temperatures above 8^{o}C were the major contributory factors for contamination of the final products.

Washed curd cheese types are particularly susceptible to coliforms from contaminated water. In smear type cheeses, *Saccharomyces cerevisiae*, *Candida famata*, *Torulaspora delbrueckii* and *Pichia membranaefaciens* and other microorganisms are often introduced from milk, brine, air in ripening rooms, ripening shelves, human skin, wooden tables used for dry salting of cheese blocks and processing equipment (Pacheco and Galindo, 2010). Many mould species are well adapted to the cheese-making environment and can be difficult to eradicate from the production facility. *Cladosporium cladosporioides*, *Penicillium commune*, *Cl. herbarum*, *Pen. glabrum* and *Phoma* spp. often cause a "thread mould" defect in Cheddar cheese and are frequently isolated from the cheese factory environment, on the equipment, in air, and in the curd and whey providing a wide range of potential sources of contamination with these organisms (Hocking and Faedo, 1992).

A major cause of failure of processing and packaging systems in the dairy industry is the development of biofilms on equipment surfaces. These communities of microorganisms develop when nutrients and water remain on surfaces between times of cleaning and reuse. Bacteria in biofilms are more resistant to chemical sanitisers (Kumar and Anand, 1998). This is why bacteriocins and enzymes are gaining importance due to their effectiveness in bio-control and removal of biofilms (Mills *et al*., 2011). Therefore, it is possible that chemical sanitisers which are often used in cheese production industries may be rendered ineffective by biofilms. This could leave viable bacteria to be dislodged into the cheese. NSLAB which survive the pasteurisation process, and those which contaminate the cheese from the ripening room environment (air, floors, drains, equipment surfaces, etc) usually proliferate during ripening to dominate the bacterial flora of the mature cheeses made from pasteurised milk (Banks and Williams, 2004). Although secondary microflora are believed to have a positive contribution on cheese flavour properties, their presence also introduces variability in the cheese-making process due to differences in their respective populations in individual cheese batches and production plants. Floors, packaging materials, brine and starter cultures used in cheese manufacture

are also major sources of contamination of enterococci, psychrophilic bacteria and coagulase-positive staphylococci whereas the cheese vat, cheese cloth, curd cutting knife and production room air frequently introduce aerobic mesophilic bacteria, yeast and moulds (Temelli *et al*., 2006).

2.2.1 Psychrotrophs and coliforms

Psychrotrophic organisms have the ability to grow at low temperature (3-7^{o}C) and to hydrolyse and use proteins and lipids for growth. However, coliforms usually do not grow at these temperatures. Ledenbach and Marshall (2009) have summarised that the psychrotrophs commonly associated with raw milk and dairy products include members of the genera *Bacillus, Micrococcus, Aerococcus*, and *Lactococcus*, and of the family Enterobacteriaceae. In unsalted cottage cheese, pseudomonads and coliforms reduce the diacetyl content which leads to yogurt-like flavour due to imbalance of the diacetyl-acetaldehyde ratio. In salted ripened cheese however, low pH and a_W levels limit the growth and metabolic activity of these organisms and undermine their impact in the final products. Slow lactic acid production by starter cultures favours the growth and production of gas by coliform bacteria. In soft and semi-soft mould-ripened cheeses such as Stilton, there is a pH increase during ripening (Gkatzionis *et al*., 2009), which increases the potential of coliform bacteria to grow and cause spoilage defects in the cheese. However, there is a low incidence of pseudomonads and coliforms/ Enterobacteriaceae in Stilton and other pasteurised milk cheeses, as the organisms are heat sensitive.

2.3 Origin and production of blue cheeses

Blue cheeses have been produced for a long time either deliberately or by accident before they were described in writing. Gorgonzola was the first blue-veined cheese to be mentioned in the literature in 879 (Fernández *et al*., 2009), while Roquefort was described in customs papers in 1070. Stilton cheese was not mentioned until the Seventeenth Century. In Denmark, production of Danablu and Mycella blue cheeses from cows' milk started in the 1870s. In 1916, homogenisation was employed to improve the ripening process of Danablu by accelerating lipolysis (Fernández *et al*., 2009).

Different strains of *Pen. roqueforti* are inoculated to ripen the cheese giving a variety of products. However, in most English and Italian blue cheeses, the strain *Pen. roqueforti* CECT 2905 is utilised (Fernández *et al*., 2009). The variation in mould strains used in ripening can pose substantial differences in quality characteristics of blue cheese from one producer to another. This is due to differences in types and levels of enzymes and other metabolites produced by the moulds. For example, whereas most industrial strains of *Pen. roqueforti* produce high levels of proteases, others produce moderate or barely detectable levels of these substances (Fernández *et al*., 2009), creating differences in the cheese quality characteristics.

Blue cheese is traditionally produced from high quality pasteurised whole cows' milk containing 3.5% fat and 9% non-fat-solids. The primary starter culture, *Lc. lactis*, is added and milk coagulated with the enzyme rennet. Spores of *Pen. roqueforti* may be added to the cheese milk at the outset of fermentation or sprinkled on the surface after moulding. After fermentation, whey is drained, the curd allowed to dry overnight and then salted with 2.5-5% (w/w) sodium chloride. The salted curd is filled in the mould (or hoops) and turned daily for six days to give it a relatively open texture. The mould is removed and the cheese surface smoothed with a stainless steel knife. It is then punctured to permit entrance of air and ripened at 8-15^{o}C for 8-12 weeks to acquire the characteristic blue veins (Nelson, 1970).

In the case of Stilton cheese, it requires about 10 l of cows' milk to make 1 kg of the product. Briefly, fresh pasteurised cows' milk is fed into an open vat to which *Lc. lactis*, rennet and spores of *Pen. roqueforti* are added. After curd formation, whey is drained overnight and the curd is cut into blocks to allow further drainage before being milled and salted (3.5%, w/w). The salted curd is fed into cylindrical moulds, the moulds placed on boards and turned daily to allow natural drainage for 5-6 days. This ensures even distribution of moisture throughout the cheese so that, as the cheese is never pressed, it creates the flaky, open texture required for blueing. After one week, cylinders are removed and the cheese surface sealed by smoothing or wrapping to prevent entry of air. The cheese is then ripened under carefully controlled temperature (10-20^{o}C) and relative humidity (85%) with regular turning. At six weeks, the cheese is pierced with stainless steel needles to allow entry of air and promote blue venation typical of the cheese. At about nine weeks, the cheese is checked for aroma grade and dispatched for sale.

Sometimes, the product is ripened for about three more weeks to develop a smoother, buttery texture and a more rounded mellow flavour desirable by some consumers. The final product is wrapped in wax paper and stored under refrigeration or freezing (SCMA, 2009). High quality Stilton cheese is usually semi-soft and has blue veins and a crumbly whitish interior. The product has a sharp, tangy flavour and melts quickly (Recipe Goldmine, 2009). SCMA (2009) specified that other legal requirements to use the name Stilton should fulfill the following, the cheese should:

- Be made in Derbyshire, Leicestershire and Nottinghamshire in the UK
- Be made from pasteurised milk obtained from local cows in the above counties
- Be only made in a traditional cylindrical shape
- Be allowed to form its own outer crust
- Be un-pressed
- Have delicate blue veins that radiate from the centre
- Have a taste and aroma typical of Stilton
- Have a fat and protein content of approximately 35 and 23%, respectively

2.3.1 Microflora of Stilton cheese

Production of Stilton cheese involves the addition of starter microorganisms to the milk during manufacture, but the final flora develops during ripening and contains a complex mixture of organisms that are not originally added (secondary flora).

2.3.1.1 Starter cultures used in the manufacture of Stilton cheese

The starter cultures used in manufacture of Stilton cheese include single or a mixture of the primary acid-producing *Lactococcus lactis* strains (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* or *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*), and the mould *Penicillium roqueforti* which is responsible for its blue venation. The homofermentative *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are usually applied to produce lactic acid, whereas *Lc. lactis* subsp. *lactis* var. *diacetylactis* is included to produce diacetyl and acetoin (from citrate metabolism), which are important aroma compounds of ripened cheese (Whitley, 2002). Citrate metabolism in the latter *Lactococcus* occurs at an optimum pH of 5-6.9 (Sanchez *et al*., 2008), implying flavour formation from the latter strain would decrease (due to low pH) as ripening progresses. However, after piercing (~6

weeks), sporulation of *Pen. roqueforti* brings about an increase in pH which could favour diacetyl formation in *Lc. lactis*.

Pen. roqueforti produces conidiophores exhibiting a green/blue colouration. The majority of these organisms produce extracellular proteases and lipases with different strains showing different levels of activity (Floreza *et al*., 2007). In Stilton cheese, strains PV from Visbyvac®, Danisco (Whitley, 2002) and CECT 2905 (Fernández *et al*., 2009) are normally utilised. These strains are moderately proteolytic and lipolytic, have a high salt (1-3% salt is stimulatory for its growth) and acid (pH 4-7) tolerance and grow at 8-25^{o}C (Whitley, 2002). *Pen roqueforti* also produces the mycotoxins roquefortine C, patulin, penicilic acid and mycophenolic acid (Erdogan *et al*., 2003). Whereas the significance of these substances for human health is unclear (Bulleman *et al*., 1981), it has been suggested that their level in ripened cheese is generally low due to biochemical degradation into non-toxic metabolites (Whitley, 2002).

2.3.1.2 Non-starter microorganisms associated with Stilton cheese

Rennet-coagulated cheeses involve ripening for several weeks prior to consumption. The process leads to complex microbiological and biochemical changes resulting in the typical flavours and textures. The microflora of ripened cheese is often dominated by non-starter microorganisms, also referred to as secondary flora. In Stilton cheese, the secondary microflora is dominated by non-starter bacteria, yeasts and moulds, and largely comes from the processing plant environment (Gkatzionis, 2010). There is a need to understand the role these organisms play in the development of the cheese flavour, and how this can be harnessed for improved quality stability and reliability of Stilton cheese.

Published literature on the microbiology of Stilton cheese is limited. Gkatzionis (2010) examined the microbial flora of different sections (outer crust, blue veins and white core) of the cheese focussing on the impact of fungal flora on the distribution of aroma volatiles in these sections. The author employed classical microbiology and molecular methods (denaturing gradient gel electrophoresis-DGGE, restriction fragment length polymorphism-RFLP and terminal RFLP) to screen the local fungal communities in the cheese. Fungal communities were different for each of the cheese sections. The only mould detected was *Pen. roqueforti* and was mainly isolated from the blue veins, whereas yeasts were found in all the cheese sections. The yeast communities detected included

Kluyveromyces lactis, *Yarrowia lipolytica*, *Candida catenulata*, *Trichosporon ovoides* and *Debaromyces hansenii*. *Kl. lactis* and *Deb. hansenii* mainly colonised the blue veins and outer crust respectively, whereas *Can. catenulata* and *Yarr. lipolytica* were predominantly found in the outer crust and white core.

The above findings were similar to an earlier account by Whitley (2002). The author employed culture-based methods to compare the microflora of low quality (poor blueing) Stilton cheese batches with that of good quality samples. The most occurring yeasts were *Kl. lactis* and *Deb. hansenii*, with the latter species occurring in both the good and poor quality cheeses. In the same study, the bacterial flora was found to be different with *Lb. plantarum* dominating in good quality cheeses. Presence of the latter alone or its co-presence with other lactobacilli including *Lb. curvatus*, *Lb. casei* (homofermentative LAB) or *Lb. brevis* was correlated with high quality Stilton, whereas dominance of the heterofermentative *Lb. brevis* was associated with the poor quality samples. Whitley (2002) pointed out that *Lactobacillus* constitutes the dominant non-starter LAB in Stilton cheese at the end of ripening. This account was supported by other studies on Stilton cheese including Sharpe and Brindley (1956), Fitzpatrick (1971) and Hiscox *et al*. (2008). A similar observation was made for other blue cheese varieties including Cabrales from Spain (Florez and Mayo, 2006) and Gorgonzola from Italy (Fontana *et al*., 2010).

The most comprehensive study on the bacterial diversity of Stilton cheese was reported by Ercolini *et al*. (2003). These authors applied 16S rDNA analysis with PCR-DGGE of DNA extracted directly from the cheese and from bulk cells from culture media. The dominant species were found to be close relatives of *Lc. lactis*, *Enterococcus faecalis*, *Lb. plantarum*, *Lb. curvatus*, *Leuconostoc mesenteroides*, *Staphylococcus equorum* and *Staphylococcus* sp. When different sections of the cheese matrix were examined using fluorescence in situ hybridisation (FISH) with probes developed to detect *Lc. lactis*, *Lb. plantarum* and *Leu. mesenteroides*, lactococci were found in the blue veins as mixed colonies, and as single colonies within the white core. *Lb. plantarum* was detected only underneath the outer crust, while *Leuconostoc* microcolonies were homogeneously distributed in all parts the cheese. FISH demonstrated differential location and distribution of bacterial species within Stilton cheese suggesting specific ecological reasons for establishment of sites of actual microbial growth in the cheese. This phenomenon can have implications on the quality characteristics of the cheese as different

organisms employ various metabolic routes to generate compounds of significance to aroma and other quality properties. The contribution of yeasts to the aroma profile of Stilton cheese has been established (Gkatzionis *et al*., 2009). However, there are no conclusive studies about the effect of non-starter LAB on the quality properties of the cheese. Given that the microflora of ripened cheese is usually dominated by facultative heterofermentative lactobacilli which are known to promote flavour and texture properties of the cheese (McSweeney, 2004), this aspects needs to be investigated for Stilton in order to identify strains that can be employed to optimise the cheese production process (culture adjuncts) without compromising its traditional nature.

2.3.2 Aroma profile of Stilton cheese

Similar to its microflora, the aroma profile of Stilton cheese has not received extensive research attention. The earlier published studies on flavour of Stilton cheese (made from unpasteurised milk, at the time) were reported by Madkor *et al*. (1987[a,b]) and focussed on proteolysis and lipolysis. The studies monitored the progress of these biochemical activities with time, in two lots of Stilton cheese manufactured under commercial conditions. Thin-layer chromatography was applied to separate and quantify amino acids of the nitrogen fraction of samples obtained at different time points during manufacture. Lipolysis was quantified using high performance liquid chromatography. A decrease in pH during the white stage (pre-mould growth) was recorded, but increased during the active mould growth phase (after 6 weeks) due to production of a wide array of free amino acids (FAA) and other protein derivatives. The concentration of free FAA increased from 5 to 36-fold by the end of ripening with valine, leucine, lysine and glutamic acid accounting for more than 50% of the total FAA occurring throughout ripening. The level of free fatty acids also increased during ripening with long-chain FFA occurring at higher concentrations than short chain fatty acids. Whitley (2002) compared the volatile compounds in good quality Stilton with those present in poor quality cheese using gas chromatography-mass spectrometry (GC-MS). Alcohols, aldehydes and branched ketones (metabolites of protein and lipid metabolism) were the major compounds in good quality Stilton, whereas poor quality cheeses were dominated by acetone and unidentified compounds. Both cheese types also contained methyl ketones, which were attributed to mould growth.

In a subsequent study, Gkatzionis *et al*. (2009) employed solid phase micro-extraction with GC-MS (SPME GC-MS) and direct headspace analysis (atmospheric pressure chemical ionisation with MS, APCI-MS) to determine the aroma profiles of Stilton cheese regarding its three main sections (outer crust, blue veins and white core). As yeasts had been found to constitute the dominant fungal flora of the cheese, the authors further examined (using SPME GC-MS) how the interaction of yeast flora and the starter mould *Pen. roqueforti* could influence the aroma profile of a model UHT milk medium. In the cheese studies, blue veins and the outer crust contained higher levels of ketones while the white core was dominated with alcohols and aldehydes. Co-culture of *Pen. roqueforti* with *Yarr. lipolytica* resulted in an aroma profile more similar to that of blue cheese than when the mould was grown alone. Given that ripened cheeses are often dominated by non-starter microflora (principally, the fungi and NSLAB), the need to examine the effect of the different microbial interactions on the quality properties of Stilton cheese cannot be overemphasised.

2.4 Characteristics of lactic acid bacteria and their metabolism

2.4.1 Classification of lactic acid bacteria

Identification and classification of bacteria and other organisms has always been a hard task. Basically, the process involves arranging the organisms into different taxonomic groups (taxa) on the basis of their similarities or relationships. The groups are assigned names according to taxonomic rules and subsequently determine whether a new isolate belongs to one of the established named taxa as exemplified for *Lactobacillus plantarum* (Table 2.3). In bacterial nomenclature, the lowest official taxonomic rank is the subspecies. This is based on minor but consistent phenotypic variations within the species or on genetically determined clusters of strains within the species that diverge in phenotype (Khalid, 2011).

Table 2.3 Taxonomic classification of *Lactobacillus plantarum*

Taxonomic rank	Nomenclature	Characteristic features	References
Domain	Bacteria	Membranes composed of un-branched fatty acids attached to glycerol by ester bonds	Rogers and Smith (1917)
Lineage	Firmicutes	Most have peptidoglycan in cell wall, rods or cocci, some produce endospores	’’
Class	Bacillus	Gram-positive cells	’’
Order	*Lactobacillales*	Non-sporing, Gram-positive cells, 32-54 %mol G+C	’’
Family	*Lactobacillaceae*	Short or long-slender cells, rods or coco-bacillus, normally occur in chains, non-sporing, Gram-positive, catalase-negative, fermentative mainly producing lactic acid	’’
Genus	*Lactobacillus*	Non-motile, nitrate negative, complex nutritional requirements	’’
Species	*Lb. plantarum*	Facultative heterofermentative	Felis and Dellaglio (2012)

The term ‘lactic acid bacteria’ (LAB) was accepted at the beginning of the 20th century. LAB is a group of Gram-positive, low GC content, acid-tolerant, generally non-sporulating, non-respiring rod or cocci that catabolise sugars to essentially produce lactic and acetic acid. LAB are classified into different genera based on cell morphology, mode of glucose fermentation, growth at different temperatures, and configuration of lactic acid produced, ability to grow at high salt concentrations and acid tolerance. The genus *Vagococcus* are the only known motile organisms in the LAB group (Khalid, 2011). LAB are typically fermentative bacteria with low proteolytic activity. Lactobacilli constitute the major flora of ripened cheese with the production of short chain fatty acids being their best known physiological effect (Collins *et al*., 2003). Klein *et al*. (1998) proposed that the most important genera in the LAB group include *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Weissella*, *Carnobacterium*, *Tetragonococcus* and *Bifidobacterium*.

Lactobacilli are Gram-positive rods, which frequently form chains. Whereas metabolism of these organisms is mainly fermentative, some species are aero-tolerant (Khalid, 2011). Their growth is optimal at pH 5.5-5.8. Lactobacilli have complex nutritional requirements for amino acids, peptides, nucleotides, vitamins, minerals, fatty acids and carbohydrates. The genus *Lactobacillus* is divided into two main groups based on fermentation patterns: Homofermentative lactobacilli, which produce more than 85% lactic acid from glucose, and heterofermentatives that produce only 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide (Axelsson, 2004). In cheese, some of these compounds are important aroma metabolites as well as imparting antimicrobial activity against undesirable microflora, especially under conditions of low redox potential.

2.4.2 Lactic acid bacteria associated with cheese defects

LAB generally grow in nutrient-rich habitats such as milk, meat and vegetables. Lactococci in cold-stored (7^{o}C) cheese produce diacetyl reductase which reduces the diacetyl content resulting in a yogurt-like flavour (Ruas-Madiedo *et al*., 1998). Heterofermentative LAB use the pentose phosphate pathway to metabolise lactose, producing equimolar quantities of lactic acid, ethanol and carbon dioxide (Khalid, 2011). Examples of these organisms include *Leuconostoc* and some lactobacilli such as *Lb. brevis*, and can develop off-flavours and gas in ripened cheese (Doyle, 2007). Their growth is favoured over that of homofermentative LAB when cheese is ripened at 15^{o}C than 8^{o}C (Ledenbach and Marshall, 2009). When the homofermentative LAB fail to metabolise all of the fermentable sugars in the cheese, heterofermentative ones at populations of ~10^{6} CFU/ml would complete the process producing gas and off-flavours (Taskila *et al*., 2009).

Some facultative lactobacilli co-metabolise citric and lactic acid and produce carbon dioxide. Amino acid catabolism in cheese by non-starter lactobacilli, propionibacteria and *Lc. lactis* subsp. *lactis* can produce small amounts of gas (Martley and Crow, 1993). Some strains of *Streptococcus thermophilus* and *Lb. helveticus* can form carbon dioxide and 4-aminobutyric acid from decarboxylation of glutamic acid leading to cracks in the cheese due to excess gas formation (White *et al*., 2003). Ledenbach and Marshall (2009) provided details for some cheese defects caused by LAB. These authors reported that oxygen-dependent tyrosine metabolism by certain lactobacilli causes a pink-brown discoloration on the surface of some ripened cheeses.

The racemic mixture of L(+) and D(-) lactic acid that usually forms an (undesirable) white crystalline material on the surface of Cheddar and Colby cheeses is produced by the combined growth of starter culture lactococci and NSLAB producers. In Swiss cheese, the white spot defect is normally caused by *Enterococcus faecalis* subsp. *liquefaciens* which survives pasteurisation. The bacterium inhibits the growth of propionibacteria and *Lb. fermentum* starter cultures resulting in poor ‘‘eye’’ development and lack of flavour. The above factors highlight the need for application of starter culture adjuncts not only for the control of spoilage LAB and other microorganisms, but also to promote faster and reliable cheese ripening process. In this context, homofermentative lactobacilli such as *Lb. casei* and *Lb. helveticus* are used as adjunct cultures to control spoilage, reduce bitterness, and improve flavour and/or to accelerate ripening in Cheddar, Emmental and Mozzarella cheeses (Mullan, 2001).

2.4.3 Metabolism of lactic acid bacteria

LAB lack the ability to synthesise cytochromes and porphyrins which are major components of the respiratory chain. Therefore, they do not generate chemical energy (adenosine tri-phosphate, ATP) by creation of a proton gradient. These organisms obtain ATP by fermentation, usually of sugars. However, LAB are aero-tolerant organisms. They are protected from oxygen by-product production such as hydrogen peroxide produced by peroxidases (King *et al*., 2000). The genera *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* are most important in food fermentation. Axelsson (2004) summarised that there are two main sugar fermentation pathways through which LAB generate energy: the glycolytic (Embden-Meyerhof-Parnas) and 6-phosphogluconate-phosphoketolase (6-PG/PK) pathways. Homofermentative LAB employ the glycolytic pathway to produce almost exclusively lactic acid as the end product (Fig 2.2), whereas heterofermentatives use the 6-PG/PK pathway for fermentation of most carbohydrates producing other end products such as ethanol, acetate, diacetyl, carbon dioxide and other metabolites (Fig. 2.3), in addition to lactic acid. Various growth conditions may alter end product formation due to altered pyruvate metabolism. For example, in a slightly aerated environment, *Leuconostoc* preferentially produce lactic acid and ethanol, but mainly produce lactic and acetic acid in a more aerated medium. In cheese and other fermented foods, this would have a positive effect on flavour by inducing the formation of acetic acid in higher concentrations. Formation of acetic acid in aerated cultures is due to increased activity of the enzyme NADH oxidase (NOX), which allows the organism to

redirect the conversion of acetyl phosphate from ethanol to acetic acid (Adler-Nissen and Demain, 1994). Although it appears that LAB possess simpler metabolism resulting in few end products, these organisms can exhibit diverse metabolic capacities enabling them to adapt to a variety of conditions (Hugenholtz and Kleerebezem, 1999).

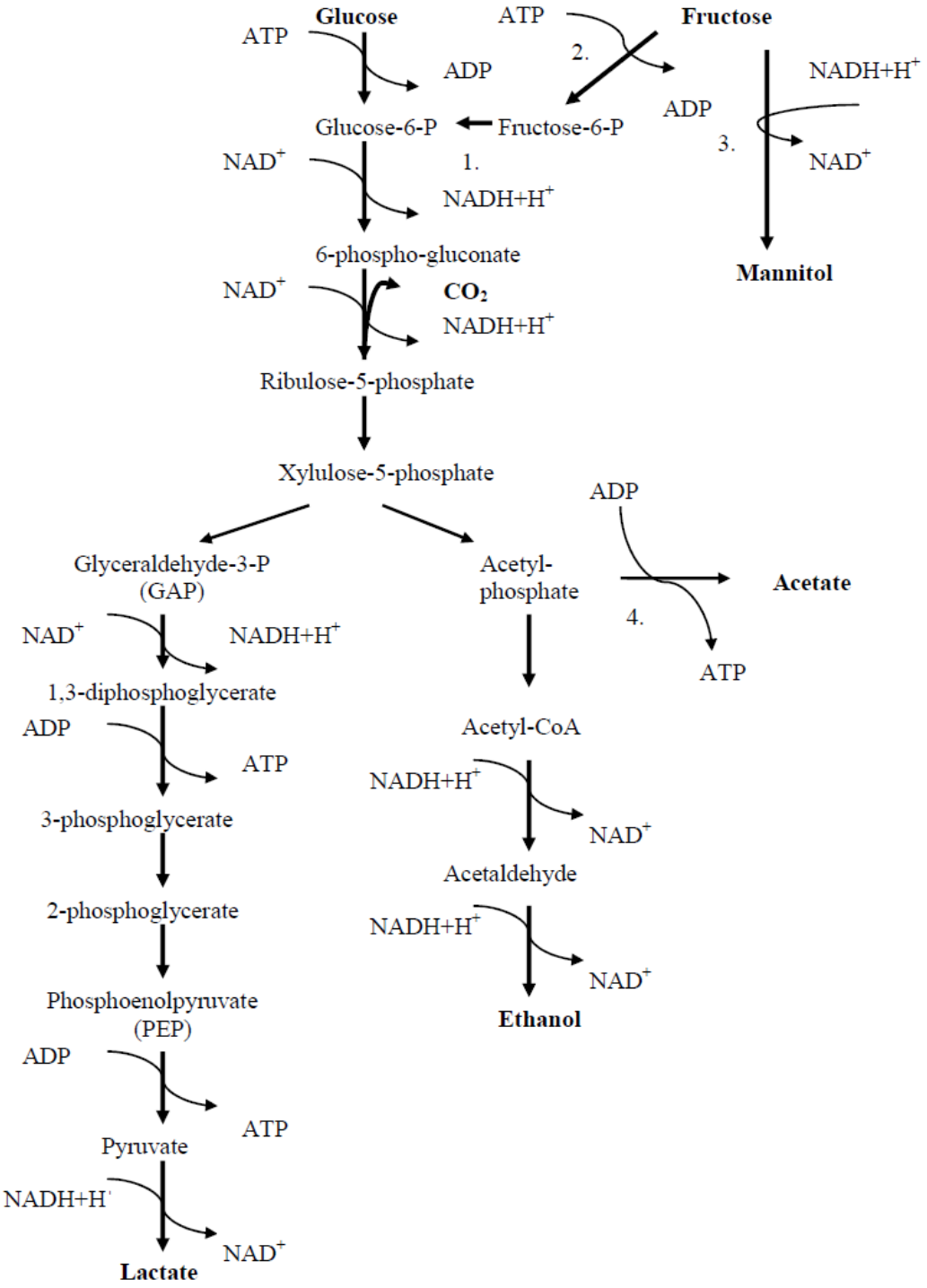


Figure 2.2 Glucose and fructose metabolism of LAB through the glycolytic pathway. Enzymes: (1) phosphoglucoisomerase, (2) fructokinase, (3) mannitol dehydrogenase and (4) acetate kinase. Adapted from Maicas *et al*. (2002) and Aarnikunnas (2006).

The homofermentative pathway involves glycolysis of hexose sugars to form the terminal electron acceptor pyruvate which is subsequently reduced to lactic acid (Fig. 2.2). In heterofermentative metabolism, xylulose 5-phosphate obtained from glycolysis is cleaved into acetyl phosphate and glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate is then metabolised into lactic acid following the homofermentative pathway, whereas acetyl phosphate is either reduced into ethanol (Fig. 2.2), or it is converted to acetic acid, depending on environmental conditions. Pyruvate is an essential electron acceptor in metabolism of LAB as it enables regeneration of nicotinamide adenine dinucleotide (NAD+). Depending on media conditions, pyruvate is utilised in alternative pathways (Fig. 2.3), a phenomenon which is strain-specific (Liu, 2003).

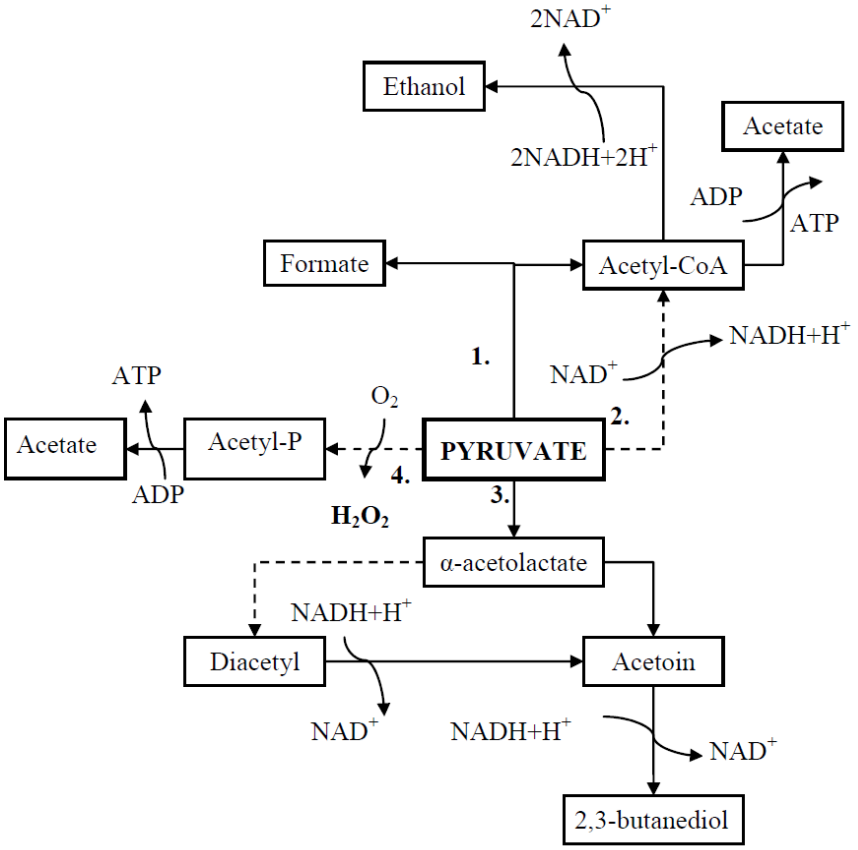


Figure 2.3 Alternative metabolic pathways of pyruvate in LAB. (1) pyruvate-formate lyase pathway, (2) pyruvate dehydrogenase pathway, (3) diacetyl-acetoin pathway, (4) pyruvate oxidase pathway. Dashed lines illustrate pathways that are favoured by presence of oxygen. Adapted from Ott *et al*. (2000) and Aarnikunnas (2006).

2.4.3.1 Diacetyl-acetoin pathway

In presence of surplus amounts of pyruvate (relative to the need for NAD+ regeneration) in cells, the compound is shunted into the diacetyl-acetoin pathway and transformed into diacetyl (DA) and acetoin as the end products (Fig. 2.3). This normally occurs when there are other sources of pyruvate (other than the fermented sugar) in the growth medium or when other compounds such as oxygen act as the electron acceptors instead of pyruvate (Aarnikunnas, 2006). In general, low sugar concentrations and low pH favour diacetyl/acetoin formation (Axelsson 2004). In *Lb. plantarum*, conversion of pyruvate to acetoin helps to maintain cellular pH homeostasis (Tsau *et al*., 1992). In this pathway, acetolactate is synthesised from pyruvate by acetolactate synthase, and chemically decomposed (in presence of low pH and aeration) to DA following a non-enzymatic reaction.

2.4.3.2 Pyruvate-formate lyase (PFL) pathway

Pyruvate-formate lyase (PFL) catalyses the formation of acetyl CoA and formate from pyruvate and CoA (Fig. 2.3). Acetyl CoA then either serves as an electron acceptor resulting in ethanol formation or as a precursor of ATP leading to acetate production. PFL system is oxygen sensitive and is inactivated in aerobic conditions. In some LAB such as *Lb. casei* and *Lc. lactis*, PFL system is usually inactivated under conditions of substrate limitation resulting in a change from homolactic to heterolactic fermentation to form lactate, acetate, formate and ethanol as the metabolic end-products (Axelsson, 2004) (Fig. 2.3).

2.4.3.3 Pyruvate oxidase pathway and NADH oxidases

In LAB, oxygen has a profound effect on re-routing of pyruvate, being mediated directly by pyruvate oxidase (POX) or indirectly by NADH oxidase (NOX) (Aarnikunnas, 2006). POX uses oxygen to convert pyruvate into acetyl phosphate, carbon dioxide and hydrogen peroxide (H_2O_2) (Fig. 2.3). In *Lb. plantarum*, this enzyme plays a major role in aerobic formation of acetic acid (Sedewitz *et al*., 1984). POX activity in *Lb. plantarum* is enhanced by presence of oxygen or hydrogen peroxide and is reduced by glucose (Aarnikunnas, 2006).

2.4.3.4 Pyruvate dehydrogenase pathway (PDP)

Under aerobic conditions, pyruvate dehydrogenase has an anabolic role in some LAB producing acetyl CoA used for lipid synthesis (Axelsson, 2004). This enzyme complex can also have a catabolic role similar to PFL, producing acetyl CoA but only under aerobic conditions (Fig. 2.3). In this case, acetyl CoA is metabolised further to acetate with concomitant formation of ATP and pyruvate. Excess NADH formed in PDP is re-oxidised by NOX to form H_2O_2 or water (Aarnikunnas, 2006). The impact of NSLAB on the production and distribution of the afore-mentioned (Section 2.5) compounds in Stilton cheese has not been widely investigated. Indeed, several lactobacilli including *Lb. plantarum* have been found to produce esters, FFA, alcohols and carbonyls which are important for blue cheese flavour (Vítová *et al*., 2006).

2.5 Cheese flavour development

Cheese flavour is derived from metabolic pathways controlled by rennet and other enzymes produced by microorganisms in the cheese matrix as illustrated by Fig. 2.4. Cheese aroma primarily originates from glycolysis, lipolysis and proteolysis.

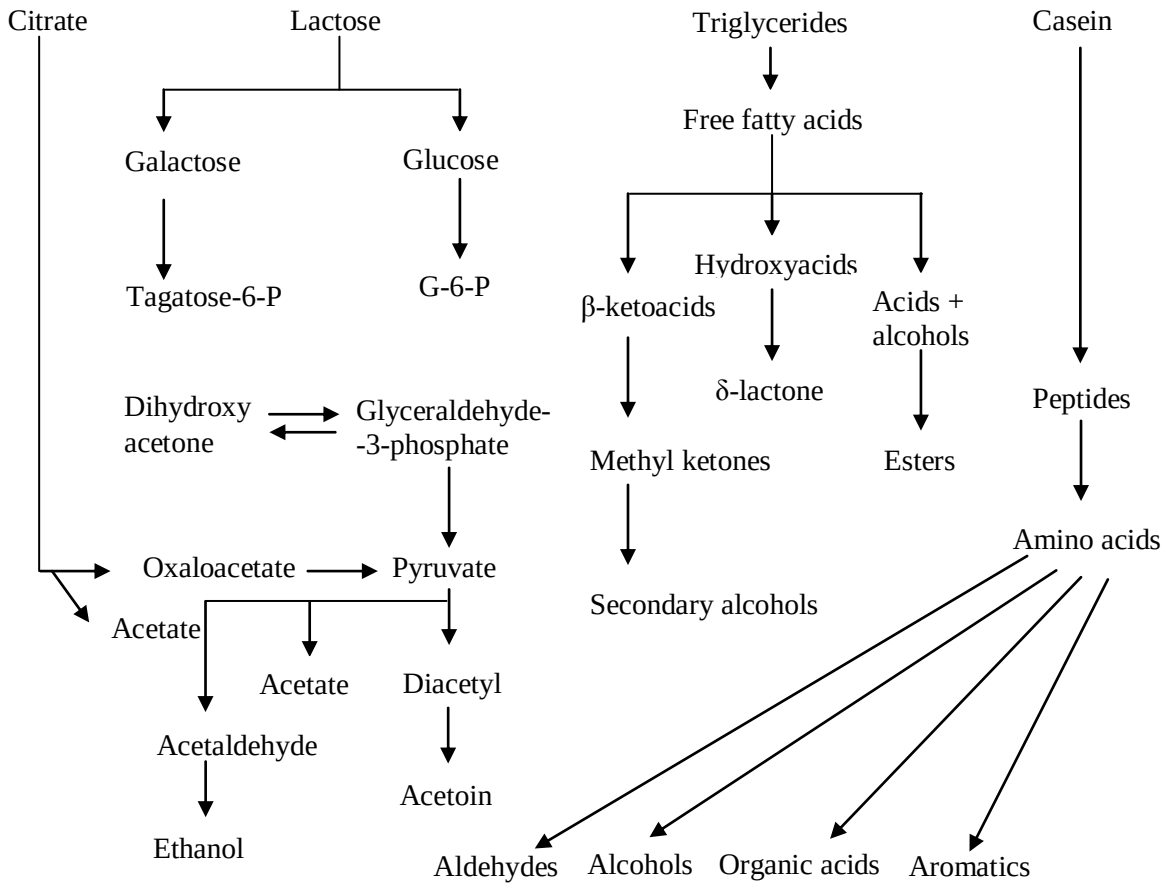


Figure 2.4 Metabolic pathways of cheese flavour formation. Source: McSweeney (2004).

2.5.1 Glycolysis and citrate metabolism

During fermentation, LAB catabolise sugars to produce lactic acid by homo- or heterofermentative pathways as already discussed in Section 2.4. These organisms also metabolise citrate to produce carbon dioxide, diacetyl, acetoin and butanediol (Fig. 2.4). Diacetyl and acetoin have aromatic properties in ripened cheese whereas carbon dioxide contributes to the formation of "eyes" (holes) in some cheese varieties but can have a detrimental effect by causing cracks in most other varieties. Essentially, citrate is metabolised to oxaloacetate which is then decarboxylated to pyruvate (Sing *et al.*, 2003). Pyruvate can be converted to lactic acid through the glycolytic pathway or ethanol, acetic

acid, acetoin/diacetyl and other metabolites via the phosphate pentose pathway (Section 2.4.3).

2.5.2 Lipolysis

Carboxylic acids or FFA originate from lipolysis or from degradation of lactose and amino acids (Sing *et al*., 2003). Organic acids can also be derived from oxidation of ketones, esters and aldehydes. Long chain (>12 carbon atoms) FFA play a minor role in cheese flavour owing to their relatively high perception thresholds. However, short (e.g. acetic acid), moderate chain and even-numbered FFA such as butanoic, hexanoic and octanoic acid have much lower perception thresholds and impart characteristic vinegar or sour notes, but higher concentrations can cause rancid off-flavours. It has been previously demonstrated that *Pen. roqueforti* secretes lipases in cheese which participate in synthesis of butanoic and hexanoic acid that are highly correlated with 'mouldy' flavours (Lawlor *et al*., 2003). FFA can also serve as precursors for synthesis of lactones and methyl or ethyl esters which contribute to fruity, creamy, buttery or sweet flavours (Longo and Sanromán, 2006). The level of FFA in Stilton cheese increases slightly during the first 28 days followed by rapid increase up to the end of ripening (Madkor *et al*., 1987[b]). Long chain FFA usually present at higher concentrations than low molecular weight counterparts are important precursors of the high molecular weight (C9 and above) aroma volatiles (Madkor *et al*., 1987[b]). It has been suggested that odd carbon (C3-C15, inclusive) and some even-numbered carbon chain methyl ketones comprise the principal compounds responsible for the unique flavour of blue cheeses (Lawlor *et al*., 2003). Methyl ketones are normally derived from β-oxidation of FFA or from β-ketoacids and are primary contributors of typical fruity, floral, mushroom or musty notes in ripened cheese (Gkatzionis *et al*., 2009).

2.5.3 Proteolysis

For most ripened cheese varieties, proteolysis, which results in the accumulation of free amino acids, is the major source of flavour development. Amino acids, particularly the sulphur-containing, aromatic and branched types, are the key substrates for the development of more complex flavour and aroma compounds in cheese (Tavaria *et al*., 2002). Starter culture lactococci contain a range of catabolic enzymes which facilitate the conversion of amino acids to potential flavour compounds. Several non-starter lactobacilli

including *Lb. paracasei* (Beresford and Cogan, 2000), *Lb. casei* and *Lb. plantarum* (Milesi *et al*., 2010) have also been shown to produce similar enzymes.

Amino transferase (AT) is the enzyme responsible for initiating amino acid conversion to flavour compounds during cheese ripening. Indeed several mesophilic non-starter lactobacilli normally show AT activity with valine, isoleucine, leucine, tyrosine, tryptophan, phenylalanine, methionine and other amino acids as substrates (Tavaria *et al*., 2002). AT activity varies from one *Lactobacillus* species to another and also depends on the prevailing conditions. For instance, in *Lb. paracasei*, AT has optimum activity towards valine, leucine and isoleucine near neutral pH but activity is reduced in presence of 5% salt (Beresford and Cogan, 2000). Therefore, inclusion of *Lb. plantarum* and other lactobacilli can be associated with an increased content of total free amino acids and higher concentration of aroma compounds in cheese (Milesi *et al*., 2010).

The immediate products of amino acid catabolism are α-ketoacids (oxoacids) such as acetolactate, pyruvic acid and oxaloacetate. Oxoacids are important intermediates in aroma development and can be subsequently converted to α-hydroxyacids, acetyl-CoA and aldehydes (Fig. 2.4). Aldehydes may be subsequently reduced to alcohols and carboxylic acids (Steele *et al*., 2013). Acetyl CoA can be converted to its derivatives including ethanol, diacetyl, acetoin, acetic acid or formate depending on the environmental conditions. For example, low pH and lack of glucose in the medium favours formation of acetoin. Co-presence of mesophilic lactobacilli such as *Lb. plantarum* with the starter culture lactococci has been shown to enhance the cheese aroma due to degradation of amino acids, particularly glutamate, to produce α-ketoglutarate and hydroxyl acids which are subsequently converted to carboxylic acids by the *Lc. lactis* (Kieronczyk *et al*., 2003).

2.5.4 Role of starter lysis in cheese flavour development

Steele *et al*. (2013) have surmised that lysis of starter bacteria affects cheese flavour development through release of intracellular enzymes such as peptidases, and substrates for NSLAB growth. Lysis occurs via the activity of cell wall-degrading enzymes of the cells or through induction of a prophage (Lortal and Chapot-Chartier, 2005). Starter culture lysis is enhanced by constructing or selecting for fast-lysing strains, using bacteriocin-producing LAB as adjunct cultures, or by employing bacteriophage lysins

either alone or in combination with the corresponding holin (Meijer *et al*., 1998; Lortal and Chapot-Chartier, 2005), all of which have been found to enhance cheese flavour. The above pathways are critical (beneficial or detrimental) to flavour development in cheese. Therefore, understanding role NSLAB play and the factors that determine the fluxes through these competing pathways is essential for consistent and rapid generation of cheese-specific flavours.

2.6 Objectives of the study

2.6.1 To characterise the non-starter bacterial isolates obtained from different sites (outer crust, blue veins and white core) of a Stilton cheese using phenotypic and genotypic methods.

2.6.2 To determine the tolerance of different genotypes of the dominant NSLAB to some stress conditions (heat, acid, salt and desiccation) typical of the microenvironments in the cheese from where they were isolated.

2.6.3 To determine the potential of the isolates for application as bio-preservatives on the basis of their antimicrobial properties.

2.6.4 To determine whether or not the interactions between selected genotypes of the dominant NSLAB obtained from the sampled sites and *Lc. lactis* alone or in combination, affect the profiles of aroma compounds produced in the model medium.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Microbial isolation and growth conditions

The 123 microbial isolates (Table 3.1) for this study had been obtained from the outer crust, blue veins and white core of a commercial Stilton cheese sample using Rogosa (CM0627, Oxoid, UK) and M17 agar (CM0785, Oxoid) (Gkatzionis, 2010). Briefly, an 8 kg commercial sample of blue Stilton cheese at the end of ripening (~8 weeks) was precisely partitioned into outer crust, blue veins and white core. Microbiological analysis was performed on each of the cheese sections by aseptically scrapping 130-190 mg micro-samples into sterile o-ringed micro-centrifuge vials (Biospec Products, UK). The samples were mixed with nine parts of maximum recovery diluent (MRD; CM0733, Oxoid) and four glass beads (2 mm, acid washed, Biospec Products, UK), and homogenised using a Mini Beadbeater-1 (Biospec Products) at 2500 rpm for 2 x 40 s, cooling on ice between each treatment. Samples of the same cheese section were pooled and 1 ml volumes used for further 10-fold serial dilutions and subsequently plated on Rogosa (CM0627, Oxoid) and M17 (CM0785, Oxoid) agar, and incubated aerobically/anaerobically for 24-48 h at 30-37 ^{o}C. After incubation, three to five colonies with different morphologies were randomly selected and streaked twice on Rogosa and M17 agar for purification. The strains were stored in brain heart infusion (BHI) broth (CM1135, Oxoid) with the addition of 20% (v/v) glycerol (G/0650/17, Fisher Scientific) at -80^{o}C in a freezer (U570, New Brunswick Scientific, England).

The isolates were resuscitated from -20^{o}C by growth in brain heart infusion (BHI; CM1135, Oxoid) and de Man, Rogosa, Sharpe (MRS; CM0359, Oxoid) broth at 30-37^{o}C (Yavuz *et al*., 2004). All isolates from the frozen stocks were sequentially sub-cultured twice on BHI and MRS agar by streaking on the media and separately incubating the plates under aerobic or anaerobic conditions for 24-48 h at 30-37^{o}C.

Table 3.1 Microbial isolates used in the study and their site of isolation

Strain ID	Site of isolation	Media used for isolation
R1-R15	Outer crust	Rogosa agar
R16-R30	Blue veins	Rogosa agar
R31-R45	White core	Rogosa agar
B1-B30	Blue veins	M17 agar
W1-W30	White core	M17 agar

Source of isolates: Gkatzionis (2010)

3.2 Biochemical characteristics

3.2.1 Gram staining

Gram staining was carried out according to the method described by Rollins and Joseph (2004). A loopful of maximum recovery diluent (MRD; CM0733, Oxoid) was transferred to the surface of a clean 1-1.2 mm glass slide (BS7011/2, Thermofisher Scientific). One colony grown on BHI agar (Oxoid) was dispersed in the MRD and spread over a small area using a 10 µl sterile disposable loop (731171, Greiner Bio-One, Hungary). The film was allowed to dry for about 15 min at ambient temperature (~20^{o}C) and heat fixed by briefly passing the slide through a Bunsen flame three to five times, exposing the dried film directly to the flame. The slide was flooded with the primary stain, crystal violet (PL8000, Pro-Lab Diagnostics), for 1 min, followed by washing with tap water and then treated for 30 s with the mordant, Lugol's iodine (PL8010, Pro-Lab Diagnostics). Excess iodine was washed off with water, the slide decolourised for 1 min with general purpose methylated spirit (M/4450/17, Fisher Scientific) and the excess washed off. Finally, the slide was counter stained for 30 s with carbol fuschin (PL8004, Pro-Lab Diagnostics), the excess rinsed off with water, and the slide allowed to dry for ~15 min at ambient temperature. Gram reaction and cell morphologies were examined using oil immersion microscopy (PBA Microscopes T250, England) at a magnification of X1000. Gram-negative cells stained pink while Gram-positive ones were purple. *Escherichia coli* and *Pediococcus pentosaceus* (Table 3.2) were used as the Gram-negative and Gram-positive controls, respectively.

3.2.2 Catalase test

Using a sterile disposable loop, 2-3 colonies from a 24 h MRS agar plate culture were placed on a clean Petri-dish. One drop of 30% (v/v) hydrogen peroxide (H1009, Sigma, UK) was added with a sterile Pasteur pipette and the test observed for bubbling within 10 s. Vigorous bubbling was taken as positive while no bubbling was considered to be a negative result. A hand lens (10X) was used to observe very slight catalase production. *Ped. pentosaceus* and *Pseudomonas fluorescens* (Table 3.2) were used as the negative and positive controls, respectively.

3.2.3 Oxidase test

Production of cytochrome oxidase was detected using oxidase identification sticks (BR0064A, Oxoid). The stick tip was completely rolled on 5-10 colonies of the cultures grown aerobically on BHI agar for 24 h at 30-37^{o}C. A positive result was indicated by a colour change from pink to purplish-black after 30-180 s of contact between the stick and cells. *Ped. pentosaceus* and *Ps. fluorescens* (Table 3.2) were used as the negative and positive controls, respectively.

3.2.4 Coagulase test

The Staphytect plus kit (DR0850M, Oxoid) was used to identify coagulase producing staphylococci. All the isolates tested were first confirmed as Gram-positive catalase-positive cocci according to the manufacturer's instructions. The latex reagents were brought to ambient temperature and mixed vigorously by shaking for ~10 s. One drop of each of the test (DR0851, Oxoid) and control (DR0852, Oxoid) reagents was dispensed onto separate circles on the reaction card (L990, Oxoid). Using a sterile disposable loop, 3-5 colonies from a culture grown aerobically on BHI agar (Oxoid) for 24 h at 30-37^{o}C were separately introduced onto the control and test circles and mixed with the reagents to cover the circles. The card was rocked gently; agglutination within 20 s was taken to indicate a positive result. *Staph. epidermidis* and *Staph. aureus* (Table 3.2) were used as the negative and positive controls, respectively.

3.2.5 Lancefield test

The latex agglutination (Lancefield) test was used for preliminary identification of the presumptive enterococci isolate (Collins *et al.*, 1989). The isolate tested was first confirmed as Gram-positive catalase-negative cocci. Prior to the test, the isolate was

grown on Columbia blood agar base (CM0331, Oxoid) supplemented with 5% defibrinated sheep blood (SR0051B, Oxoid) for 24 h at 37^{o}C and examined for haemolytic activity. The *Streptococcus* extraction enzyme (DR593, Oxoid) was re-constituted to 1X with sterile deionised water (SDW) and 0.4 ml of the solution dispensed into a sterile bijou bottle. Five colonies were emulsified in the enzyme preparation using a sterile disposable loop and incubated for 10 min at 37^{o}C. During incubation, the mixture was shaken vigorously after 5 min for 2-3 s and then continued with the incubation. The latex reagents were shaken vigorously, and together with the extract, brought to room temperature (~20 min). Then, a drop from each of the six latex reagents was dispensed onto individual circular rings on the reaction card (DR500, Oxoid) and one drop of the extract added to each of the six rings using a sterile Pasteur pipette. The mixture was spread over the entire area of the ring using a sterile wooden mixing stick and the card rocked gently to enhance agglutination within 30 s, which was indicative of the positive test.

Table 3.2 Bacterial strains used as test controls

Control strain	Test	Source	Reference
Ped. pentosaceus	Gram-positive, catalase and oxidase-negative	UNFCC	Collins *et al*. (1989)
E. coli JM 109	Gram-negative	"	"
Ps. fluorescens SM06	Catalase and oxidase-positive	"	"
Staph. aureus NCTC 1803	Coagulase-positive	"	"
Staph. epidermidis (wild type)	Coagulase-negative	"	"
Staph. aureus NCTC 10652	Enterotoxin genes A & D	NCTC	Sharma *et al*. (2000)
Staph. aureus NCTC 10654	Enterotoxin gene B	NCTC	"
Staph. aureus NCTC 10655	Enterotoxin gene C	UNFCC	"
Staph. epidermidis NCTC 12100	Enterotoxin gene C	UNFCC	"

UNFCC - University of Nottingham Food Sciences Laboratory Culture Collection, UK

NCTC - National Collection of Type Cultures, UK

3.2.6 Biochemical profiling

3.2.6.1 API 20 Strep

The API 20 Strep kit (20600, BioMerieux) was used to assess the substrate assimilation profile of the presumptive enterococci identified as group D *Streptococcus* by the Lancefield test (§3.2.5) following the manufacturer's instructions. To the incubation box, 5 ml SDW was added to create a humid atmosphere and the strip placed in it. Then, a dense bacterial suspension with a turbidity equivalent of McFarland standard-4 was prepared by harvesting cells grown on Columbia blood agar as in §3.2.5. The cells were homogenised in 2 ml normal physiological saline (0.85% sodium chloride, pH 7.4±0.2). The first half of the strip, i.e. tests VP (sodium pyruvate, 1.9 mg/cupule) to LAP (L-leucine-β-naphthalamide, 0.0256 mg/cup) was inoculated by distributing the suspension into the tubes and cupules, avoiding the formation of air bubbles. All tubes on the second part of the strip, i.e. tests ADH (L-arginine, 1.9 mg/tube) to GLY (glycogen, 1.28 mg/tube) were filled with GP medium inoculated with the culture suspension and overlaid with sterile mineral oil. The box was closed and incubated for 4 h at 37^{o}C to obtain the first reading and then 24 h for the second reading. The results were read by referring to the interpretation table according to the manufacturer's instructions and species identification obtained using the API software v.3.2.2 (BioMerieux, France).

3.2.6.2 API 50 CHL

The API 50 CHL kit (50300, BioMerieux) was used to assess the presumptive *Lactobacillus* isolates for substrate assimilation profiles in order to preliminarily identify the species. To the API incubation box, 10 ml SDW was added to create a humid atmosphere and the strips placed in it. Five to ten colonies were picked from a 24 h culture grown on MRS agar at 30^{o}C and dispersed in 2 ml physiological saline using a sterile cotton swab (FB61001, Fisher Scientific) to make a heavy suspension (S1). Fifteen drops of S1 were transferred into 5 ml physiological saline in a sterile universal bottle to obtain a second suspension (S2) with a turbidity equivalent of McFarland standard-2. Fifteen drops of S2 were inoculated into API 50 CHL medium (50410, BioMerieux) using a 1 ml graduated Pasteur pipette (LW4005, Alpha Laboratories, UK). The inoculated medium was filled into the tubes on the strip, overlaid with sterile mineral oil and incubated at 30^{o}C. Readings were taken after 24 and 48 h by referring to the colour chart according to the manufacturer's instructions. A positive test was revealed by the bromocresol purple indicator contained in the medium changing to yellow due to

acidification. For the Esculin test (tube 25), a colour change from purple to black was taken as the positive result. Species identification was obtained using the API software (BioMerieux).

3.2.6.3 API 20 NE

The API 20 NE kit (20050, BioMerieux) was used for preliminary identification of the Gram-negative, catalase-positive, oxidase-positive cocco-bacillus isolates. Three to five colonies were collected from a 24 h BHI agar plate grown at 30^{o}C and suspended in 2 ml physiological saline to form a bacterial suspension with a turbidity equivalent of McFarland standard-0.5. The first part of the strip i.e. tests NO_3 (potassium nitrate) to PNPG (4-nitrophenyl-β-D-galactopyranoside) was inoculated by distributing the saline suspension into the tubes using a Pasteur pipette. The cupules GLU (glucose), ADH (L-arginine) and URE (urea) were overlaid with sterile mineral oil. Then, 200 µl of the remaining saline suspension was inoculated into AUX medium (08037, BioMerieux) and the medium was filled into tubes and cupules on the second part of the strip i.e. tests GLU (D-glucose) to PAC (phenyl acetic acid). The box incubated for 24 h at 30^{o}C and the strip read according to manufacturer's guidelines. Species identification was obtained using the API software (BioMerieux) as previously.

3.3 DNA-based molecular methods

3.3.1 Extraction of genomic DNA

Genomic DNA for amplification of 16S rDNA (§3.3.3.1) and class IIb bacteriocin (plantaricin) genes (§3.3.3.3) was extracted using the guanidinium EDTA sarcosyl (GES) method as described by Pitcher *et al*. (1989). The presumptive lactobacilli isolates were grown in 10 ml MRS broth for 24 h at 30^{o}C (100 rpm; Obi-Safe TS, Gallenkamp, England). All the other bacterial isolates were grown in 10 ml BHI broth (Oxoid) for 24 h at 30-37^{o}C (100 rpm; Gallenkamp). Cells from 2-3 ml of the culture were harvested by centrifugation (13000 *g*; Eppendorf 5415R, Germany) for 60 s. The cell pellet was re-suspended and washed twice by spinning (13000 *g*; Eppendorf) at 4^{o}C with 1 ml ice cold lysis buffer [25 mM Tris-HCl, 10 mM EDTA, 50 mM sucrose (S/8560/53, Fisher Scientific) pH 8]. The cell pellet was re-suspended in 100 µl of the same lysis buffer supplemented with 50 mg/ml lysozyme and incubated for 30 min at 37^{o}C. Three hundred microlitres of GES buffer [5 M guanidine thiocyanate (B21250, Alfa Aesar), 100 mM EDTA, 5% (w/v) N-lauryl sodium sarcosyl salt, pH 8] was added and the mixture

incubated for 10-15 min at room temperature until it became clear. The lysate was incubated on ice for 2 min and 250 µl ice cold 7.5 M ammonium acetate (A/3440/53, Fisher Scientific) added. The mixture was vortexed (Clifton Cyclone74405, England) briefly, incubated on ice for 10 min and 500 µl 24:1 chloroform (C/4920/17, Fisher Scientific): isoamyl alcohol (1105, Fisons Scientific) added. This was mixed and spun (9000 *g*; Eppendorf) for 10 min. The upper phase supernatant (850 µl) was transferred into a clean microcentrifuge tube. Exactly 0.54 volumes (459 µl) of ice cold isopropanol (propan-2-ol) (P/7490/PB17, Fisher Scientific) was added and gently mixed for 1 min to precipitate the DNA. Genomic DNA was recovered by spinning (9000 *g*; Eppendorf) for 5 min at 4^{o}C. Then, the pellet was washed three times in 500 µl of 70% analytical grade ethanol (E/0650DF/P17, Fisher Scientific), air dried for ~30 min at room temperature, re-suspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and stored at -20^{o}C.

3.3.2 Extraction of chromosomal DNA

Chromosomal DNA used in the multiplex amplification of staphylococcal enterotoxin (SE) genes (§3.3.3.2) was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Ausubel *et al.*, 1992; William and Feil, 2004). Presumptive staphylococci isolates were grown aerobically in 100 ml BHI broth (Oxoid) for 24 h at 30^{o}C. The control strains: *Staph. aureus* NCTC 10652 (SE A&D), *Staph. aureus* NCTC 10654 (SE B), *Staph. aureus* NCTC 10655 (SE C) and *Staph. epidermidis* (SE C; Table 3.2) were grown at 37^{o}C under the same conditions. Cells from 100 ml culture broth were harvested by centrifugation (5500 *g*; Beckman J2-21, England) for 10 min and re-suspended and washed twice by spinning (5500 *g*; Beckman) in 10 ml of wash buffer (2.5 M NaCl, 10 mM EDTA, pH 7.5). The cells were washed twice in 10 ml lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 7.5) and re-suspended in 9.25 ml of the same lysis buffer supplemented with 25 µg/ml lysostaphin (L7386, Sigma). The mixture was incubated (100 rpm; Gallenkamp) for 1 h at 37^{o}C; and 500 µl of 10% sodium dodecyl sulphate (SDS; S/5200/53, Fisher Scientific) solution was added followed by proteinase K (final concentration, 0.3 mg/ml). The mixture was vortexed briefly and incubated (100 rpm; Gallenkamp) for 1 h at 37^{o}C until lysis occurred forming a clear solution. Sodium chloride (5 M, 1.8 ml) and 1.5 ml CTAB solution [0.7 M NaCl and 0.275 M CTAB (52365, Sigma) pre-warmed to 65^{o}C] were added and mixed thoroughly. The mixture was incubated for 20 min at 65^{o}C and an equal volume (13.5 ml) of 24:1 chloroform: isoamyl

alcohol added. This was mixed and spun (5500 *g*; Beckman) for 10 min. The upper phase supernatant (10-12 ml) was taken out and put into a clean 50 ml conical tube, and exactly 0.6 volumes (6-7.2 ml) of ice cold isopropanol added and gently mixed for 1 min to precipitate the DNA. Chromosomal DNA was recovered by spinning (3500 *g*; Beckman) for 10 min at 4^{o}C. The DNA pellet was washed twice with 1 ml 70% ethanol and air dried for ~30 min at room temperature. DNA was finally re-suspended in 5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and stored at -20^{o}C until use.

3.3.3 Measurement of DNA concentration

The concentration of the DNA extracts obtained was measured by spectrophotometry. A 1 µl sample was pipetted onto the pre-cleaned end of the lower measurement pedestal (receiving fibre) of the NanoDrop spectrophotometer (ND1000, England) whilst the second optic cable (sampling fibre) was open. The sampling arm was closed and spectral measurement performed using NanoDrop software v.3 (NanoDrop Technologies Inc., England). Sample carryover between successive measurements was minimised by wiping the sample from the pedestals using a Whatman lens cleaning tissue (2105918, Whatman, England). TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used as the blank.

DNA extracts were diluted with SDW to a working concentration (10-200 ng/µl). Aliquots of DNA extracts (5 µl) were checked for quality and size by electrophoresis on a 1% (w/v) agarose (MB1200, Melford) gel prepared with 1X TAE buffer [40 mM Tris base, 20 mM glacial acetic acid (A/0400/PB17, Fisher Scientific) 1 mM EDTA, 0.2 µg/ml ethidium bromide, pH 8]. TAE (1X) was used as the running buffer for ~40 min at 90 V. A 1 kbp lambda DNA ladder (G571, Promega) was used as the molecular size marker.

3.3.4 Polymerase chain reaction (PCR)

3.3.4.1 PCR amplification of 16S rDNA

The primers used to amplify 16S rDNA are listed in Table 3.3. Primers Lac1 and Lac2 were used to amplify the variable V6-V8 regions, while the V3F and V3R primers amplified the variable V3 region of the gene giving PCR products of ~428 and 200 bp, respectively. The reaction mixture (final volume, 50 µl) for the variable V6-V8 region amplification contained 5 µl of 10X PCR buffer IV (AB0289, Fisher Scientific, 10 mM Tris-HCl, pH 8.5; 50 mM KCl); 2.5 mM $MgCl_2$; 0.2 mM (each) of the deoxynucleotide triphosphates, dATP (U120D, Promega), dCTP (U122D, Promega), dGTP (U121A,

Promega) and dTTP (U123D, Promega); 0.2 pmol/µl (each) forward and reverse primers; 1.25 U of *Taq* DNA polymerase (AB0351, Fisher Scientific); and 3 µl of template DNA (150 ng). The samples were amplified in a programmable thermocycler (Techne TC512, USA) as follows: initial denaturation at 94°C, 5 min; 35 cycles of 94°C, 30 s; 52°C, 1 min; 68°C, 1 min; and 68°C, 7 min.

The reaction mixture for amplification of the variable V3 region was constituted as above with minor modifications; 2.5 mM (each) of the dinucleotide triphosphates (dNTPs) and different primers (V3F and V3R; Table 3.3) included in the mixture. DNA was denatured for 5 min at 94^{o}C. Then, a touchdown PCR was performed as follows: initial annealing temperature was 66°C, and this was decreased 1°C every cycle for 10 cycles; finally, 20 cycles were performed at 56°C. The extension for each cycle was carried out at 72°C, 3 min, while the final extension was at 72°C, 10 min.

Table 3.3 Primers and their target sequences in the PCR reactions

Primer name	Sequences	Gene location	Target	Reference
Lac1	5'AGCAGTAGGGAATCTTCCA-3'	364-382	V6-V8 region of 16S rDNA of lactobacilli (forward primer)	Lopez *et al*. (2003)
Lac2	5'-ATTTCACCGCTACACATG-3'	690-707	V6-V8 region of 16S rDNA of lactobacilli (reverse primer)	"
V3F	5'-CCTACGGGAGGCAGCAG-3'	341-357	V3 region of 16S rDNA (universal forward primer)	Muyzer *et al*. (1993)
V3R	5'-ATTACCGCGGCTGCTCG-3'	518-534	V3 region of 16S rDNA (universal reverse primer)	"
SA-U	5'- TGTATGTATGGAGGTGTAAC-3'	-	SE (A-E) genes (universal forward primer)	Sharma *et al*. (2000)
SA-A	5'- ATTAACCGAAGGTTCTGT-3'	639-657	SE A gene (reverse primer)	"
SA-B	5'- ATAGTGACGAGTTAGGTA-3'	564-582	SE B gene (reverse primer)	"
SA-C	5'- AAGTACATTTTGTAAGTTCC-3'	457-477	SE C gene (reverse primer)	"
SA-D	5'- TTCGGGAAAATCACCCTTAA-3'	676-696	SE D (reverse primer)	"
SA-E	5'- GCCAAAGCTGTCTGAG-3'	584-600	SE E (reverse primer)	"
plnEF-F	5'-GGCATAGTTAAAATTCCCCCC-3'	3-23	Plantaricin EF operon (forward primer)*	Yi *et al*. (2010)
plnEF-R	5'-CAGGTTGCCGC AAAAAAAG-3'	100-118	Plantaricin EF operon (reverse primer)	

				"
plnJK-F	5'-ACGGGGTTGTTGGGGGAGGC-3'	74-93	Plantaricin JK operon (forward primer)	Cho *et al*. (2010)
plnJK-R	5'-TTATAATCCCTTGAACCACC-3'	148-168	Plantaricin JK operon (reverse primer)	
				"
plnN-F	5'-GGGTTAGGTATCGAAATGG-3'	25-43	Plantaricin N operon (forward primer)	
				"
plnN-R	5'-CTAATAGCTGTTATTTTTAACC-3'	163-184	Plantaricin N operon (reverse primer)	
				"

*Beginning of coding sequence of plnI

3.3.4.2 Multiplex PCR for amplification of staphylococcal enterotoxin genes

The primers used to amplify sequences of the SE A, SE B, SE C, SE D and SE E genes (Table 3.3) in a multiplex PCR reaction were as described by Sharma *et al*. (2000). The forward primer (SA-U) was universal to all the SE genes, and five reverse primers amplified a site in the variable regions for each of the specific SE genes.

PCR was performed using the method of Sharma *et al*. (2000). The reaction mixture (final volume, 50 µl) contained 25 µl of 2X Dream*Taq* buffer (K1081, Fermentas; 10 mM Tris-HCl, pH 9; 50 mM KCl; 4 mM $MgCl_2$); 5 µl (2 ng/µl) of template chromosomal DNA; 0.6 pmol/µl (each) of the primers SA-U, SA-A, SA-B, SA-C, SA-D and SA-E; 0.2 mM (each) of dATP, dTTP, dGTP, dCTP; and 1 U of *Taq* DNA polymerase. DNA amplification was carried out in a programmable thermal cycler (Techne) as follows: initial denaturation at 94°C, 5 min, 25 cycles of 94°C, 30 s, 50°C, 30 s, 72°C, 30 s, and 72°C, 2 min.

3.3.4.3 PCR amplification of the class IIb bacteriocin (plantaricin) operons

The primers used to amplify the various plantaricin (pln) operons are listed in Table 3.3. The primer sequences and PCR conditions were adopted from Cho *et al*. (2010) and Yi *et al*. (2010). The reaction mixture was constituted as the one for amplification of the V6-V8 regions of 16S rDNA (§3.3.4.1). The samples were amplified in a programmable thermocycler (Techne). Amplification conditions for plantaricin EF operon were as follows: initial denaturation at 94°C, 5 min; 30 cycles of 94°C, 1 min; 51°C, 40 s; 72°C, 3 min; and 72°C for 10 min. In the case of plnJK and plnN, amplification conditions were as follows: initial denaturation step at 94°C, 3 min; followed by 32 cycles of 94°C, 1 min; primer annealing conditions were 50°C, 1 min and 56°C, 1 min for plnN and plnJK respectively; and 72°C, 30 s extension; followed by a final extension step at 72°C, 6 min.

3.3.4.4 Gel electrophoresis of PCR amplicons

PCR product (10 µl) was mixed with 2 µl of 6X loading dye (G190A, Promega) and run on 1-2% (w/v) agarose (Melford) gels containing ethidium bromide (0.2 µg/ml) in 1X TAE running buffer for 2 h at 75 V to check the product size. A 100 bp lambda DNA ladder (G210A, Promega) was used as the molecular size marker. The gel was visualised on a UV transilluminator (Bio-Rad), images recorded with Quantity one Gel Doc

software v.4.6.3 (Bio-Rad) and the bands of PCR products excised from the agarose gel with sterile scalpel blades and purified (§3.3.4.5) prior to sequencing (§ 3.3.4.6).

3.3.4.5 Purification of PCR products

PCR products were purified using the Zymoclean Gel DNA Recovery Kit (D4002, Zymo Research). Five hundred micro-litres of ADB buffer (D4001, Zymo Research, UK) was added into each gel sample and the tubes placed into a heating block (Stuart Scientific SHT 1D, England) for 5-10 min at 55-60^{o}C until solubilised. The molten agarose solution was poured into the Zymo-spin column, placed into a collection tube and centrifuged (9000 *g*; Eppendorf) for 30 s. Then, DNA was washed thrice by adding 200 µl wash buffer (D4003, Zymo Research), centrifuging (9000 *g*; Eppendorf) for 30 s for each wash cycle. Finally, 10 µl SDW was added directly onto the column matrix and purified DNA eluted by spinning (9000 *g*; Eppendorf) for 30 s. The purified PCR products were kept at -20^{o}C until use.

3.3.4.6 DNA sequencing and database search

The eluted purified DNA was diluted to 2-5 ng/µl with SDW and sent for sequencing (MWG Eurofins, Ebersberg, Germany). To determine the closest known relatives of the partial DNA sequences obtained, searches were performed in public data libraries (GenBank) using the BLAST programme. Percent similarity values of the most closely related identities were retrieved from NCBI chromosome gene bank using the NC accession numbers.

3.3.5 Pulse-field gel electrophoresis (PFGE)

3.3.5.1 Preparation of genomic DNA

Genomic DNA was prepared *in situ* in agarose blocks as described by Moore and Datta (1994) and Yeung *et al*. (2004). For each of the *Lactobacillus* isolates, a colony was inoculated in 10 ml MRS broth (Oxoid) and incubated for 16 h at 30^{o}C under moderate agitation (100 rpm; Gallenkamp). Cells from 1.5-3 ml of the culture were harvested by centrifugation for 60 s in a micro-centrifuge (Eppendorf) at 13000 *g*. The cells were re-suspended and washed twice at 4^{o}C with 1 ml sterile cell wash buffer [1 M NaCl (S/316/60, Fisher Scientific), 10 mM Tris-HCl (T6066, Sigma)] at pH 7.6. The cell pellet was re-suspended in 300 µl of the same cell wash buffer, warmed to 55^{o}C and mixed with 300 µl of 1% (w/v) pulse-field certified agarose (161-3109, Bio-Rad) solution in the cell

wash buffer maintained at 55^{o}C in a water bath (Grant JB1A08578, England). The suspension mixture (400 µl) was poured into a CHEF plug mould (Bio-Rad) and allowed to solidify for 15-20 min at room temperature. The plugs were transferred to 5 ml sterile bijou bottles and lysed *in situ* with 4 ml lysis buffer [6 mM Tris-HCl, 0.1 mM EDTA (D/0700/53, Fisher Scientific), 1% (w/v) N-lauroyl-sarcosine sodium salt (L9150, Sigma), 10 mg/ml lysozyme (L6876, Sigma) and 2 U/ml mutanolysin (M4782, Sigma)] at pH 7.6 for 16 h at 37^{o}C (MIR262, Sanyo, Japan) to remove cell walls, membranes and RNA. The lysis reagents were drained and and the plugs washed thrice with 3 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) for 30 min at room temperature. The plugs were incubated in 4 ml proteinase-K buffer [0.5 M EDTA, 1% (w/v) N-lauroyl-sarcosine sodium salt and 1 mg/ml proteinase-K (EO0491, Fermentas)] at pH 8.5 for 24 h at 55^{o}C (100 rpm; Gallenkamp) to hydrolyse the cellular proteins. Subsequently, the plugs were treated twice for 2 h with 4 ml of 1 mM phenylmethylsulphonyl fluoride (PMSF; P7626, Sigma) in TE1 buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) at 55^{o}C (100 rpm; Gallenkamp) to inactivate proteinase-K. The plugs were finally rinsed thrice with TE2 buffer (10 mM Tris- HCl, 50 mM EDTA, pH 8) for 30 min at room temperature, and stored at 4^{o}C in TE1 buffer until use.

3.3.5.2 Digestion with *Not*I

The plug was placed in a sterile Petri-dish and using a sterile scalpel blade, it was sliced into three pieces each measuring ~2 mm. The plug slices were rinsed in 500 µl SDW for 20-30 min at room temperature and rinsed again in 200 µl restriction buffer-D (R004A, Promega, USA; 6 mM Tris-HCl, 6 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, pH 7.9) for 30 min at room temperature. Then, the plug slices were incubated for 4 h at 37^{o}C with 40 U of *Not*I GQ (R643A, Promega) in a 200 µl solution consisting of the enzyme buffer (20 µl) and 0.1 mg/ml bovine serum albumin (BSA; R396D, Promega) following the manufacturer's recommendations for concentrations of each reagent. The reaction reagents were removed and digestion stopped by addition of 400 µl of 0.5X TBE buffer [44.5 mM Tris-HCl, 44.5 mM boric acid (B7901, Sigma), 1.25 mM EDTA] at pH 8 and kept at 4^{o}C until use (Vancanneyt *et al*., 2006).

3.3.5.3 Pulse-field gel electrophoresis

The electrophoresis conditions were adopted from Obodai (2006) with minor modifications. Plug slices were loaded directly into wells of 1% (w/v) PFGE certified agarose (Bio-Rad) gel prepared in 0.5X TBE buffer containing 100 µM thiourea (88810, Sigma). Electrophoresis was performed in 2 l of 0.5X TBE buffer containing 100 µM thiourea in a contour-clamped homogenous electrophoresis cell (CHEF) DRII, (Bio-Rad) at 14^{o}C for 21 h at 6 V/cm and pulse times ramping linearly from 4 to 45 s at a switching angle of 120^{o} and pump pressure of 80 rpm. A 50-1000 kbp DNA ladder (Sigma D-2416, USA) was used as a molecular size marker. For each run, ~2 mm of the pulse marker was placed in the first, middle and last lanes to allow alignment of the gel during subsequent analysis of the gel images. After electrophoresis, the gel was stained in 100 ml of SDW containing 10 µl (10 mg/ml) of ethidium bromide (Fisher Scientific) for 1 h at room temperature, and subsequently de-stained in 100 ml of SDW for 30 min at room temperature. Images were visualised on a UV trans-illuminator (Bio-Rad) and recorded with Quantity one Gel Doc software (Bio-Rad).

3.3.5.4 Cluster analysis

PFGE images were converted and patterns normalized with background subtraction and further processed using FP Quest software (Bio-Rad) to generate the dendrogram. Clustering of isolates was calculated using the un-weighted pair group method with arithmetic averages (UPGMA) and comparison of the combined PFGE patterns done using the band-based Dice similarity coefficient (Zapparoli *et al*., 1998).

Clusters in the dendrogram were defined at a selected similarity level of 52%. This similarity threshold and significance of clusters were tested using analysis of molecular variance (AMOVA) as described by Excoffier *et al*. (1992). The significance of clustering was examined by calculating the PhiPT value (Φ_{PT}), a measure of sub-population (cluster) genetic differentiation that suppresses intra-individual variation. The null hypothesis (H_0; $\Phi_{PT} = 0$) implied that there was no genetic difference amongst the *Lactobacillus* sub-populations, whereas the alternative hypothesis (H_1; $\Phi_{PT} > 0$) implied genetic differences existed between the sub-populations and their component demes. Calculation of Φ_{PT} was performed using GenAlEx v.6.5 software according to Peakall and Smouse (2006).

3.4 Stress tolerance response of *Lactobacillus*

3.4.1 Heat injury of cells

Tolerance of *Lactobacillus* isolates to heat stress was ascertained in sterile full cream cows' milk (Drinks Brokers Ltd., Norfolk, England) as the heating matrix. An MRS broth (Oxoid) culture (1 ml) grown for 24 h at 30^{o}C was centrifuged (9000 *g*; Eppendorf) for 1 min and cells washed in phosphate buffered saline (PBS; BR0014G, Oxoid) and re-suspended in 1 ml PBS. Before being used in the experiment, the suspension was vortexed (Clifton Cyclone) twice for 1 min to de-clump the cells, and spiked into milk to a final concentration of ~8 $\log_{10}$ CFU/ml. Inoculated milk was vortexed briefly and dispensed in 500 µl aliquots into 1.5 ml Wheaton vials (NJ 08332-2092, Wheaton Scientific Products, USA). The vials were immersed in a water bath (Grant, UK) pre-heated to 72±1^{o}C, and samples withdrawn at different time points for enumeration of the survivor counts by plating 0.1 ml samples of 10-fold serial dilutions in MRD (Oxoid) on BHI agar in triplicate. All plates were incubated under anaerobic conditions for 48 h at 30^{o}C and enumeration performed on plates with 30-300 colonies. To determine the recovery of heat-injured cells, samples of the heat-treated milk were kept in a cold room for 24-48 h at 4^{o}C and thereafter viable counts enumerated on MRS and BHI agar as before.

3.4.2 Acid and salt tolerance response

Acid and salt tolerance of *Lactobacillus* isolates was assayed in MRS broth (Oxoid) as described by Santos *et al*. (2003), Succi *et al*. (2005) and Pelinescu *et al*. (2009). To examine acid tolerance, an overnight MRS broth culture (~10^{9} CFU/ml) grown as in §3.5.1 was serial diluted 10-fold (three times) and 1 ml transferred into 10 ml MRS broth (Oxoid) acidified to pH 3, 3.5, 4, 4.5 and 5 using lactic acid (L/0100/PB08, Fisher Scientific). The broths were incubated at 30^{o}C (100 rpm; Gallenkamp), and 1 ml aliquots withdrawn at different time intervals for enumeration of the viable counts. Salt tolerance was examined under the same conditions by inoculating the strains in MRS broth containing 3.5, 5, 8 and 10% (w/v) sodium chloride (S/3160/60Fisher Scientific). Treated cells were enumerated on BHI agar as in §3.5.1.

3.4.3 Tolerance to desiccation

A modified method based on Pedersen *et al*. (2008) was employed to examine the effect of relative humidity (RH) levels on the viability of *Lactobacillus* isolates. Cells from 1 ml of an overnight MRS broth culture grown as in §3.4.1 were harvested by centrifugation (9000 *g*; Eppendorf) for 1 min at 4^{o}C. The pellet was washed and re-suspended in 1 ml MRD and vortexed for 2 min to de-clump the cells. Fifty micro-litre aliquots were aseptically added to two sets of U-shaped wells in 86 x 128 mm micro-titre plates (Nalge Nunc, USA) taking care to inoculate central wells of the tray. All un-inoculated wells were filled with 50 µl of sterile MRD (blank) to minimise non-uniform drying due to edge effects. The plates were placed in an incubator (Sanyo) for 24 h at 30^{o}C to dry the cells onto the surface, and subsequently transferred to separate desiccators with RH levels established and maintained at 33±1 and 54±1% humidity by the presence of saturated solutions of magnesium chloride hexahydrate (C12, Acros Organics, USA) and magnesium nitrate hexahydrate (M5296, Sigma), respectively. A calibrated digital thermo-hygrometer (DT625, ATP, UK) was used to ascertain the RH values at the corresponding temperature. The desiccators were incubated (Sanyo) at 20±1^{o}C for the remainder of the study (7 days) and viable counts enumerated at different time points as in §3.5.1. Desiccated cells were rehydrated for 30 min by adding 0.1 ml MRD, and then mixed by pipetting prior to plating on BHI agar. In order to ascertain the behaviour of the organism in absence of nutrients, the experiment was repeated by suspending the cells in SDW (R0581, Fermentas) prior to drying and desiccation.

3.5 Screening for bacteriocin producing isolates

3.5.1 Antimicrobial activity using plate agar overlay method

Preliminary screening for bacteriocin producing lactobacilli was carried out against a series of selected indicator bacteria (Table 3.4) using the agar layer overlay method described by Powell *et al*. (2007) and Suwanjinda (2007). For each of the *Lactobacillus* isolates tested, 10-fold serial dilutions of an overnight MRS broth culture were prepared as in §3.4.1 and plated on MRS agar and incubated for 24 h at 30^{o}C to obtain 10-100 colonies per plate. The colonies were carefully overlaid with 0.7% (w/v) BHI agar, seeded with 10^{6} CFU/ml of the indicator strain. *Lb. pentosus* was seeded in 0.7% (w/v) MRS agar. The plates were allowed to solidify for ~20 min in a cold room, incubated for 24 h at 37^{o}C (Sanyo) and examined for distinct colonies surrounded by a clear halo zone. Colonies with the largest zones of growth inhibition were isolated, inoculated into MRS

broth and incubated for 24 h at 30°C. Pure cultures were obtained by streaking onto MRS agar and confirmation of antimicrobial activity was assessed using the disc diffusion assay on the most sensitive indicator strains as described in Section 3.5.2. *Ped. acidilactici* NCIMB 700993 (UNFCC) was used as the bacteriocin producing control strain.

Table 3.4 Strains used for testing the antimicrobial activity of *Lactobacillus* isolates

Indicator strain	Source
Staph. aureus	Gkatzionis (2010)
Salmonella Typhimurium	UNFCC
Listeria monocytogenes NCTC 11944	"
E. 0157H-stx	"
Staph. aureus NCTC 12100	"
Clostridium sporogenes	"
Pseudomonas aeruginosa glaxo 3	"
Lc. lactis NCIMB 9918	"
Lb. pentosus NCIMB 8026	"

UNFCC - University of Nottingham Food Sciences Laboratory Culture Collection, UK
NCTC - National Collection of Type Cultures, UK
NCIMB - National Collections of Industrial, Marine and Food Bacteria, UK

3.5.2 Antimicrobial activity using the paper disc diffusion assay

Using a method described by Albano *et al*. (2007) and Buntin *et al*. (2008), antimicrobial activity of cell-free supernatants (CFS) and treated CFS of *Lactobacillus* isolates was assayed on *Ps. aeruginosa*, *E. coli*, *L. monocytogenes* and *Lb. pentosus* which had demonstrated the highest level of sensitivity to *Lactobacillus* isolates (§6.3.1).

3.5.2.1 Antimicrobial activity of cell-free supernatants

Lactobacillus isolates were grown in 10 ml MRS broth for 24 h at 30°C, 100 rpm. The culture was centrifuged (3400 *g*; Megafuge 40R, Thermo Fisher Scientific, Germany) for 15 min at 4°C to obtain the CFS, which was used to screen isolates for antimicrobial activity. CFS was sterilised by membrane filtration (0.2 μm, Ministart AG37070, Sartorius) and stored at 4°C until use. *Lb. pentosus* was grown in 10 ml MRS broth for 24 h at 30°C whereas *Ps. aeruginosa*, *E. coli* and *L. monocytogenes* were cultured in 10 ml BHI broth for 24 h at 30-37°C, depending on optimum temperature of the indicator strain.

For each of the latter three strains, Petri dishes filled with 20 ml of 1.5% (w/v) MRS agar were overlaid with 10 ml of BHI agar (0.7% agar) inoculated with 50 µl of the indicator strain (final concentration, 10^6 CFU/ml). Conversely for *Lb. pentosus*, cells (final concentration, 10^6 CFU/ml) were suspended in 10 ml MRS agar (0.7% agar) and overlaid on BHI agar in a Petri dish. All agar overlays were allowed to solidify for 15-20 min in a cold room. Thirteen milli metre sterile filter paper discs (Whatman AA, Fisher Scientific) were soaked in the supernatants for 30-60 min. The discs were applied to the seeded plates in duplicate and allowed to dry for 30 min at room temperature. All plates were incubated for 24 h at 37°C. The diameter (mm) of the resulting zone of inhibition was measured with a 100 mm ruler as the distance from the edge of the paper disc to the edge of the zone of clearing. Clear zones extending for 0.5 mm or more were considered as positive for inhibition (Litopoulou-Tzanetaki *et al*., 1989). *Ped. acidilactici* NCIMB 700993 (UNFCC) was used as the bacteriocin-producing positive control strain.

3.5.2.2 Antimicrobial activity of treated CFS

The CFS from *Lactobacillus* isolates that gave positive results in the CFS assays were subdivided and treated as follows: (i) cell-free supernatant without any treatment (CFS), (ii) neutralised cell-free supernatant adjusted to pH 6.5-7 using 1 N NaOH (CFS-N), (iii) cell-free supernatant treated with 500 U/ml catalase (C3155, Sigma) (CFS-C), (iv) cell-free supernatant treated with 1 mg/ml proteinase K (EO0491, Fermentas) (CFS-P), (v) cell-free supernatant adjusted to pH 6.5-7 and treated with catalase (CFS-N-C). Enzyme-treated supernatants were incubated for 30 min at 37°C and thereafter the enzymes inactivated by boiling in water for 2 min (Bromberg *et al*., 2004). The treated CFS were used to determine the nature of antimicrobial activity as in §3.5.2.1. Paper discs treated with sterile MRS broth and 100 µg/ml chloramphenicol (C0378, Sigma) in MRS broth were used as the negative and positive controls, respectively, and in the CFS disc tests.

3.6 Dynamics of viable microbial population changes

3.6.1 Culture conditions

Mixed culture studies of lactobacilli and *Lc. lactis* was ascertained in full cream UHT milk (ASDA Stores Limited, Leeds, LS11 5AD, UK) at 18 and 30°C according to the method of Martin-Platero *et al.* (2008). One hundred millilitres of the milk in a 250 ml sterile Duran bottle was inoculated with one *Lactobacillus* isolate and one *Lactococcus* strain (final concentration, 3 $\log_{10}$ CFU/ml for each species; Table 3.5) grown at 30°C as

in §3.4.1. Single strain cultures were used as controls. The inoculated samples were incubated for 48 h at 30°C (100 rpm; Gallenkamp) and changes in pH (§3.6.2) and viable counts enumerated by plating on Rogosa and MRS agar (§3.6.3). Subsequently, each of the fermented milk samples was divided into two portions. Salt (3.5%, w/v, Fisher Scientific) was added to one portion whilst the second portion (control) was not salted. All samples were further incubated for 12 weeks at 18°C and microbial populations and pH changes further monitored at different time intervals.

Table 3.5 Bacterial strains used in mixed culture studies

Strain	Source (site of isolation)
Lb. plantarum strain R2	Gkatzionis (2010) (outer crust)
Lb. plantarum strain B30	Gkatzionis (2010) (blue veins)
Lb. plantarum strain W8	Gkatzionis (2010) (white core)
Lc. lactis NCIMB 6681	UNFCC
Lc. lactis subsp. *lactis*	"
Lc. lactis NCIMB 9918	"

UNFCC - University of Nottingham Food Sciences Laboratory Culture Collection, UK
NCTC - National Collection of Type Cultures, UK
NCIMB - National Collections of Industrial, Marine and Food Bacteria, UK

3.6.2 pH determination

The pH of the samples was determined by potentiometry. The pH meter glass probe (pH 212, Hanna Instruments, Japan) was first calibrated using standard buffers (J/2820/15, Fisher Scientific) at pH 4 and 7 before being used to measure the sample pH. Sample pH was measured by submerging the tip of the probe into the sample for ~2 min until a stable reading was registered on the pH meter scale.

3.6.3 Selective microbial enumeration

One milli-litre samples from §3.6.1 were aseptically collected from the flasks into 9 ml sterile MRD (Oxoid) and used for bacterial enumeration as in §3.4.1. The mixed LAB cultures were differentially enumerated by incubating anaerobically for 48 h at 30°C on different selective media. Rogosa agar (Oxoid) was used for enumeration of *Lb. plantarum* whereas *Lc. lactis* was enumerated from MRS agar (Harris *et al*., 1992). MRS agar plates used for enumeration of lactococci in mixed culture (A) were replica plated on

Rogosa agar to ascertain lactobacilli counts (B) and then worked out the difference (A-B) to obtain the result for lactococci.

3.7 Screening for proteolytic and lipolytic activity

Production and activity of proteolytic enzymes for *Lactobacillus* isolates was assessed using the casein agar diffusion method as described by van den Tempel and Jakobsen (2000). Five grams of casein (acid hydrolysate; LP0041, Oxoid) was mixed with water (final volume, 50 ml) and autoclaved for 10 min at 110^{o}C to obtain casein hydrolysate solution (CHS). The agar base medium [calcium hydroxide (21918-100, Fisher Scientific), 0.3 g/L; calcium chloride (C/1400/62, Fisher Scientific), 0.2 g/L; and bacteriological agar No. 1 (LP0011, Oxoid), 15 g/L; pH 5.8] was autoclaved for 15 min at 121^{o}C. CHS (50 ml) was added to 450 ml of the agar base medium to obtain a final concentration of 1% casein in the medium, which was gently mixed and aseptically distributed into sterile 20 ml Petri dishes. The medium was allowed to dry and kept at 4^{o}C in a cold room until use.

Isolates were resuscitated from -80^{o}C and purified by streaking twice on MRS agar and incubated anaerobically for 48 h at 30^{o}C. For each isolate, a colony was inoculated in MRS broth (Oxoid) and grown for 24 h at 30^{o}C (~9 $\log_{10}$ CFU/ml), subjected to three 10-fold dilutions in MRD and 20 µl spotted on casein agar in triplicate. Replicate plates were allowed to dry, and plates separately incubated aerobically and anaerobically for 1-3 weeks at 18, 30 and 37^{o}C, and examined for clear halo zones.

Lipolytic activity was evaluated on tributyrin agar (TA; PM0004C, Oxoid) using the same approach. TA was supplied as a prepared homogenate of nutrient agar containing 1% (v/v) tributyrin (glyceryl tributyrate) and pH 7.5. The medium was liquefied in hot water for ~10 min, aseptically distributed into sterile 20 ml Petri dishes and allowed to solidify prior to use. *Yarrowia lipolytica* strain Y1 (Gkatzionis, 2010) was used as the lipolytic and proteolytic positive control, whereas *E. coli* NCTC 86 (UNFCC) was the negative control. *Yarr. lipolytica* was grown and maintained on Rose Bengal chloramphenicol (RBC) agar base (CM0549, Oxoid) supplemented with 100 mg/l chloramphenicol (SR0078, Oxoid). The strain was cultured in potato dextrose broth (P6685, Sigma) for 2 days at 25^{o}C, cells enumerated using a counting chamber (Weber Scientific, UK), diluted to 10^{6} CFU/ml and 20 µl spotted on the media as described above. *E. coli* was grown in

BHI broth for 24 h at 37^{o}C (~9 $\log_{10}$ CFU/ml), subjected to three 10-fold dilutions in MRD and spotted on the media as above.

3.8 Quantification of lactic and acetic acid production

3.8.1 Culture conditions and preparation of the cell-free supernatants

Lb. plantarum isolates and *Ped. acidilactici* NCIMB 700993 were grown in 10 ml MRS broth for 24 h at 30^{o}C. The culture was serial diluted in sterile MRS broth (Oxoid), and 0.1 ml corresponding with ~6 $\log_{10}$ CFU/ml inoculated into 30 ml of MRS broth in a 50 ml sterile conical tube and incubated at 30^{o}C (100 rpm; Gallenkamp). Five millilitre aliquots were withdrawn at different time points, transferred into 15 ml conical tubes and centrifuged (3500 *g*; Megafuge) for 10 min at 4^{o}C to remove the cells. The cell-free supernatants (CFS) were sterilised by membrane filtration (Ministart) and stored at -20^{o}C until use.

3.8.2 Sample preparation for HPLC injection

Organic acids in the CFS were extracted according to the method of Fernandez-Garcia and McGregor (1994). The hydrophobic impurities in the samples were removed using 30 mg Strata-X polymeric reverse solid phase extraction (SPE) cartridges (8B-S100-TAK, Phenomenex, Macclesfield, UK).

Using a vacuum pump, the cartridge column was conditioned by slowly passing 1 ml HPLC grade methanol (M/4062/17, Fisher Scientific) through it. It was then allowed 2 min to drain completely. Methanol was washed off by slowly passing 1 ml of HPLC grade water under vacuum and allowed to equilibrate for 5 min at room temperature. Finally, 2 ml of the sample (CFS) was loaded into the cartridge and slowly (1-2 min) forced though the column using a 10 ml disposable syringe with the first few drops (~500 µl) discarded and collecting the remainder (~1 ml) into a 2 ml glass vial (Chromocol VGA050-511C, Fisher Scientific). All samples were stored at -20^{o}C prior to injection into HPLC.

3.8.3 Preparation of standards

Standard solutions with a range of concentrations of lactic and acetic acid were prepared (Table 3.6). HPLC grade DL lactic acid (69785, Sigma; ~90%, 73.6 µl) and glacial acetic acid (A/0407/PB08, Fisher Scientific; 99.7%, 38.25 µl) were mixed with HPLC grade water (final volume, 4 ml) to give the stock standard solutions containing 10 and 20 g/L acetic and lactic acid, respectively. Subsequently, 1 in 2 serial dilutions were prepared by mixing 1 ml of the solution with 1 ml of water and repeating the procedure until the lowest dilution was obtained.

Table 3.6 Concentration (g/L) of lactic and acetic acid in the standard solution

	Concentration of lactic acid	Concentration of acetic acid
1	20	10
2	10	5
3	5	2.5
4	2.5	1.25
5	1.25	0.62
6	0.62	0.31
7	0.31	0.15

3.8.4 HPLC system and chromatographic conditions

The HPLC system used for analysis comprised an auto-sampler (Jasco AS2055, Japan) and a pump (Jasco PU1580, Japan). Organic acids were separated on an ion-exclusion Rezex ROA organic acid H^+ column phase (5 µm, 7.8x300 mm) (Phenomenex) operated at ambient temperature with 0.005 N H_2SO_4 as the mobile phase flowing at 0.5 ml/min, at a pressure of 50±5 kg/cm^2. This low pH eluent prevented organic acids from undergoing dissociation. The sample (10 µl) was injected into the HPLC system using an auto-sampler and organic acids detected using a refractive index (RI) detector (Jasco RI2031, Japan). The retention time (Rt) for lactic and acetic acid was 16 and 19 min, respectively. Analysis was completed in 25 min. Standard solutions covering a range of concentrations for lactic and acetic acid (Table 3.6) were run with the samples. Identification of the organic acids in sample extracts was based on matching Rt of standards. Quantification was carried out by linear integration of the data based on peak areas using Azur (1999-

2005) software v.4.6 (Jasco). Mean values (g/L) were computed for individual organic acids after triplicate independent injections.

3.9 Determination of volatile production

Changes in the profile of aroma compounds of milk due to growth of LAB isolates was determined by solid phase micro-extraction gas chromatography-mass spectrometry (SPME GC-MS).

3.9.1 Culture conditions and sample preparation

Aliquots (5 ml) from §3.6.1 were taken at different time points for aroma analysis. The samples were transferred into 20 ml headspace vials (22.5 x 75.5 mm, Grace Alltech, UK). The vials were immediately sealed with a PTFE-Silicone lined magnetic cap (20 mm diameter, 5 mm centre, Grace Alltech) and stored at -20^{o}C until analysis. Samples were defrosted overnight at 4^{o}C and allowed to equilibrate at room temperature for 30 min prior to analysis. Un-inoculated milk was included as a control. Three independent replicates were prepared for all samples.

3.9.2 Headspace analysis using SPME GC-MS

A 1 cm Stableflex 50/30 μm SPME fibre with divinylbenzene-carboxen on polydimethylsilicone bonded to a flexible fused silica core (Supelco 5951, USA) was used for extraction of volatile compounds from the headspace of sample vials prepared in §3.9.1. The fibre was first conditioned for 60 min in the injection port at 230^{o}C. The SPME needle was then introduced into the septum in the lid of the vial using a PAL auto-sampler (CTC Analytics, Switzerland) and the fibre exposed to the headspace for 20 min at room temperature. The temperature of the injection port was 230^{o}C.

Chromatography was carried out with a Trace GC Ultra gas chromatograph (Thermo Electron Corporation, UK) using a 30 m Zebron ZB-5 capillary GC column (internal diameter 0.25 mm, film thickness 1 μm; Phenomenex). Helium gas was employed as the carrier gas at a constant pressure of 18 psi. The GC oven temperature programme was as follows: initial temperature was 40^{o}C maintained for 2 min and increased at a rate of 8^{o}C per min to a final temperature of 220^{o}C. The transfer line from GC to MS was held at 250^{o}C. MS was performed with a DSQ mass spectrometer (Thermo Electron Corporation) operating in positive ionisation electron impact mode (EI+) at 70 eV. The detector was

operated in scan mode (2 scans/s) scanning from m/z 20-250. Source temperature was 200^{o}C and pressure 39 mtorr. Identification was based on linear retention indices and mass spectra matches with those published in the National Institute of Standards and Technology (NIST) mass spectral library (2008). Data were processed with Xcalibur software v.14 (Thermo Fisher Scientific).

3.10 Statistical analysis

All microbial counts were normalised by conversion to $\log_{10}$ CFU/ml and parametric statistics (means ± standard errors) and analysis of variance (ANOVA) computed using the Predictive Analytical Software (PASW), v.19. Pair-wise comparison of the data was performed using XLSTAT v.2013.4.05. The relationship between *Lactobacillus* isolates from different cheese sites (outer crust, blue veins and white core) and production of volatile compounds in presence or absence of *Lc. lactis* and salt was evaluated by principal component analysis (PCA) using the Unscrambler software v.9.0 (Camo Process AS., Norway). PCA and other techniques such as factor analysis and partial least squares regression (PLSR) are regarded as appropriate methods for determination of relationships between volatile compounds in dynamic or static headspace and their aroma and/or flavour attributes during food consumption (Lawlor *et al.*, 2003).

CHAPTER 4

DIVERSITY OF NON-STARTER BACTERIAL FLORA OF STILTON CHEESE

4.1 Introduction

Numerous processing parameters influence the microbiological profile of ripened cheese. Consequently, it is often difficult to predict the microbial content of cheese and to determine the impact of its biochemical properties on the quality of the product. This is because the contribution of microorganisms to the ripening process depends on the availability of substrates, pH, salt concentration, temperature and ripening time, all of which can vary within a single cheese product and between different manufacturers (Martley and Crow, 1993).

The indigenous microflora of cheeses made from heat treated milk is often over shadowed by the activity of starter culture organisms whose primary function is to produce lactic acid which, together with rennet, promotes milk coagulation. In the majority of ripened cheeses, secondary flora is generally composed of complex mixtures of bacteria, yeasts and moulds and may include non-starter culture adjuncts as well as adventitious NSLAB present in the cheese as contaminants (Martley and Crow, 1993; Beresford *et al*., 2001). Although non-starter bacteria are often implicated in the production of enzymes involved in flavour generation in cheese (Beresford *et al*., 2001; Coolbear *et al*., 2008), the contribution of these organisms to the quality characteristics of Stilton cheese has not been widely studied.

Stilton cheese has heterogeneous microenvironments with pronounced gradients of pH, salt, a_w and redox potential (Fernández *et al*., 2009). The piercing step (after 6 weeks) causes considerable structural changes and influences the level and distribution of oxygen and carbon dioxide within the cheese. These factors influence the growth and biochemical activity of the resident microflora and impacts on the quality characteristics of the final product.

The initial aim of the study was to employ a polyphasic taxonomic approach based on phenotypic, biochemical and genotypic methods, to obtain reliable identification of the cultured microbial isolates obtained from different sites (outer crust, blue veins and white core) of Stilton cheese and to determine their contribution to the cheese quality and safety. Polyphasic taxonomy is appropriate for delineation of bacterial taxa at all levels of classification (Vandamme *et al*., 1996). The approach has been previously applied for studies on microbial composition of complex ecosystems such as ripened cheese (Mounier *et al*., 2005; Bora *et al*., 2007), marine (Park *et al*., 2009) and the human gut microflora (Turroni *et al*., 2009), and so was found appropriate for this work. The 123 microbial strains which had been isolated from the outer crust, blue veins and white core of Stilton cheese were identified on the basis of their phenotypic, biochemical and genotypic characteristics as described in Sections 3.2-3.4.

4.2 Results

4.2.1 Preliminary identification

Each of the microbial isolates from the three sampled sites of Stilton cheese was grown on MRS and BHI agar at 30-37^{o}C. Ideally, the selective MRS agar should support the growth of all LAB species regardless of their individual growth requirements. BHI agar was used for preliminary examination of all microbial isolates that could not be recovered on MRS agar. On the basis of cell morphologies after Gram staining, 80 of the 123 isolates were found to be bacteria while 43 were yeasts. As the yeast species had been studied extensively in a previous study (Gkatzionis, 2010), only the bacterial isolates were taken forward for identification. The bacterial isolates were examined for colony morphologies, cell shape and biochemical characteristics and classified into five groups (*Group 1-5*) (Table 4.1). *Groups 1* & *5* grew on MRS and BHI agar and therefore were substantively identified as LAB. *Groups 2-4* only grew on the non-selective BHI agar and therefore were regarded as non-LAB.

Table 4.1 Phenotypic and biochemical characteristics of bacterial groups isolated from Stilton cheese

Group	Site of isolation	Catalase	Oxidase	Gram stain	Cell morphology	Number (%) of isolates
1	Outer crust	-	-	+	cocci	1 (1.25)
2	Outer crust	+	-	+	cocci	5 (6.25)
3	Outer crust	+	+	-	cocco-bacillus	14 (17.5)
4	White core	+	-	-	cocco-bacillus	1 (1.25)
5	Outer crust, blue veins, white core	-	-	+	rods	59 (73.8)

4.2.2 Species identification using 16S rDNA sequence analysis

Following the grouping of the isolates according to their macroscopic, microscopic and biochemical characteristics, 16S rDNA PCR was applied for molecular analysis in order to obtain a species identification. The universal V3 primers were used to amplify the variable V3 region of the partial 16S rDNA of *Group 1-4* isolates whereas lactobacilli-specific V6-V8 primers were applied for *Group 5* isolates. PCR produced a single discrete band for each strain of the expected size, depending on the primers used (Fig. 4.1a-b). The intensity of individual bands varied among the isolates. Lanes without bands as in the case of *Group 5* strains R22 and R36 (Fig. 4.1b) are suggestive of products below the detection limit of PCR due to low levels of genomic DNA (Jordan *et al*., 1999). However, most of these were successfully amplified by doubling the amount of lysozyme in lysis buffer and extending the incubation time for cell lysis from 30 to 60 min at 37^{o}C.

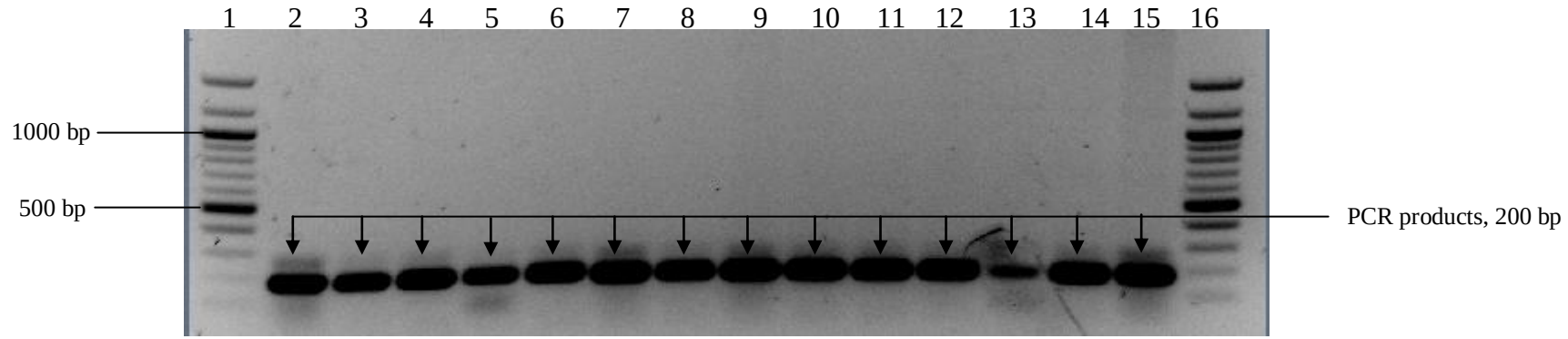


Figure 4.1a 16S rDNA PCR analysis of Stilton cheese bacterial isolates amplified with V3 primers giving expected product size of 200 bp. Lane 2, *Group 1* isolate Ou9; lanes 3-6, *Group 2* isolates Ou8, Ou10, Ou25 and Ou30; lanes 7-10, *Group 3* isolates Ou7, Ou15, Ou22 and Ou23; lane 11, *Group 4* isolate W22; lanes 12-15, *Group 5* isolates R5 (outer crust), R20, R27 (blue veins), R40 (white core); lanes 1 & 16, 100 bp ladder. The samples were run on 1% (w/v) agarose gel in 1X TAE buffer for 2 h at 70 V.

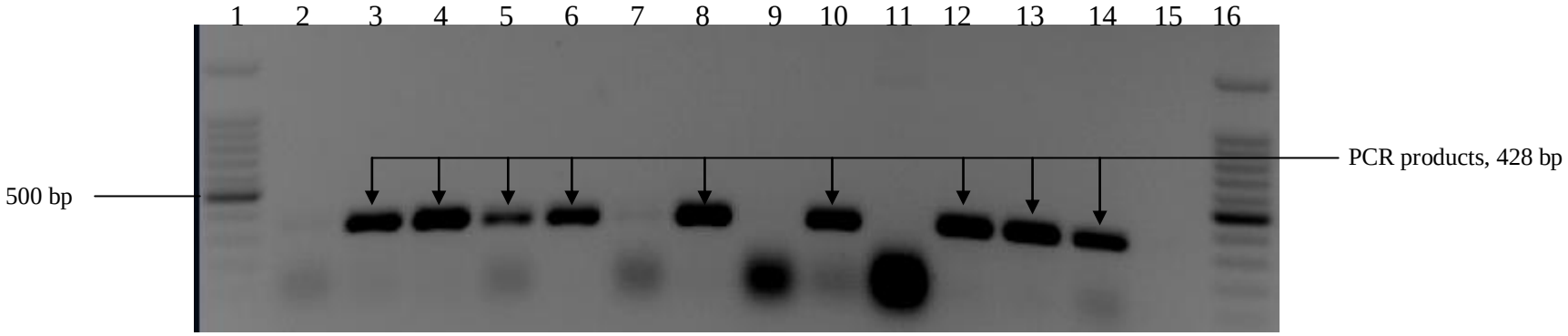


Figure 4.1b 16S rDNA PCR analysis of *Group 5* Stilton cheese isolates amplified with V6-V8 primers giving expected product size of 428 bp. Lanes 2-6, isolates R1, R2, R3, R4, R15 (outer crust); lanes 7-10, isolates R17, R19, R22, R24 (blue veins); lanes 11-14, isoalates R36, R37, R38, R39 (white core); lane 15, negative control; lanes 1 & 16, 100 bp ladder. The samples were run on 1% (w/v) agarose gel in 1X TAE buffer for 2 h at 70 V.

The bands corresponding to the isolates (*Group 1*, n=1 of 1; *Group 2*, n=5 of 5; *Group 3*, n= 7 of 14; *Group 4*, n=1 of 1; *Group 5*, n=59 of 59) were excised from the agarose gels; DNA in these bands was extracted using the Zymoclean Gel DNA Recovery Kit as described in §3.3.4.5 and sent for sequencing. To obtain species identification, sequences were compared using the BLAST programme to query the National Centre for Biotechnology Information (NCBI) database and the results are given in Table 4.2. Results of database search demonstrated that most strains that gave visible bands with the partial 16S rDNA gene produced identifiable sequences. The discrimination of isolates belonging to *Group 1-4* was good given the high %ID (95-98%) and low E values (2^{-47} to 3^{-72}) obtained. Similarly, good identification was obtained for *Group 5* isolates as demonstrated by the high %ID (99-100) and low E values (2^{-41} to 4^{-156}). Some *Group 5* strains such as R36 (white core), R7 (outer crust) and R25 (blue veins) were only able to produce bands with identifiable sequences when genomic DNA was amplified with V3 primers (gel not shown).

For each of the bacterial groups isolated from Stilton cheese, nucleotide sequences which gave similar species and percent identity (%ID) levels with BLAST were aligned to establish possible gene mismatches (Appendix 4.1). Minimal base shifts were observed when percent identity was 100% but more mismatches were noted as %ID decreased to 98% and below. Variations (mismatches) generally ranged from single base pair change, substitutions, insertions or an omission, which implies that the strains involved could be genetically distant relatives (Jordan *et al*., 1999). However, the slight variation in base sequences may also be attributed to mutations, random variations or PCR errors and need further investigation.

Table 4.2 BLAST matches of the sequences from PCR amplification of genomic DNA from *Group 1-5* isolates. Identification was based on variable V3 and V6-V8 regions of 16S rDNA

Isolates	Primer	Amplicon size (bp)	Closest relative	% ID	E-value	Matching sequence length (nt)	Genbank accession Number
Group 1	V3	200	*En. faecalis*	99	2^{-47}	111	NC004668
Group 2	V3	200	*Staph. aureus*	97	8^{-46} - 4^{-65}	155-157*	NC013450
Group 3	V3	200	*Ps. cryohalolentis*	95-96*	2^{-57} - 8^{-57}	94-145*	NC007969
Group 4	V3	200	*Ac. baumanii*	95	4^{-55}	144	NC011586
Group 5a	V6-V8	428	*Lb. plantarum*	99-100*	3^{-72} - 4^{-156}	165-311*	NC012984
Group 5b	V6-V8	428	*Lb. brevis*	99-100*	2^{-41} - 1^{-112}	98-231*	NC008497

*Results for a range of isolates: *Group 2*, 3 isolates; *Group 3*, 7 isolates; and *Group 5*, 32 isolates.

4.2.3 Phenotypic, biochemical and molecular characteristics of the isolates

4.2.3.1 *Group 1*

Group 1 isolate was found to be a Gram-positive, catalase and oxidase-negative coccus. The isolate formed large cream-whitish colonies on MRS and BHI agar. Under oil immersion microscopy, cells appeared slightly elongated and in short chains or pairs (Fig. 4.2a). From the above characteristics, the isolate was presumptively identified as *Streptococcus*, *Lactococcus* or *Enterococcus*. As most streptococci would not grow on MRS agar, the genus *Streptococcus* was eliminated at this stage whereas the latter two were examined further in order to discriminate them. On sheep blood agar, the isolate formed small grey, non-haemolytic colonies confirming it was not streptococci as these exhibit either alpha or beta haemolysis. On the basis of this observation, the organism was presumptively classified as *Enterococcus* or *Lactococcus* as the former organisms may be haemolytic or not, while the latter are non-haemolytic (Collins *et al*., 1989). The Lancefield latex agglutination test categorised it as group D streptococci (*Enterococcus*) due to the ability to react with group D antisera on the reaction card. The isolate was assessed on KFSA and formed small reddish colonies typical of *Enterococcus* spp. (Fig. 4.2a) and further identified as *En. faecalis* (%ID=99.7, t=0.66) using an API 20 Strep kit.

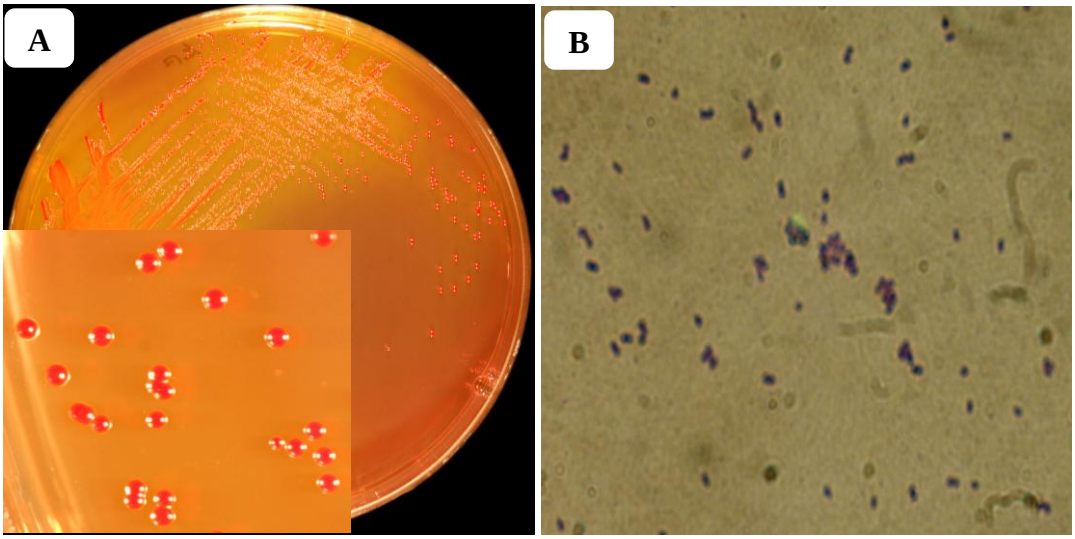


Figure 4.2a Colony (A) and cell (B) morphology of the *Group 1* Stilton cheese isolate on KFSA. The small reddish colonies obtained were typical of enterococci.

On the API strip, the isolate produced acetoin, β-glucosidase, arginine dehydrolase, leucine arylamidase, pyrrolidonylarylamidase as well as the enzymes that hydrolyse hippurate. The isolate also assimilated ribose, mannitol, sorbitol, lactose and trehalose, which were the basis for its identification. The API t-index was relatively low suggesting identification was not typical of the species obtained. However, API speciation was confirmed with 16S rDNA sequence analysis which identified the isolate as closest relatives of *Enterococcus faecalis* (Table 4.2).

4.2.3.2 *Group 2*

The five Gram-positive, catalase-positive cocci in this group were presumptively identified as *Staphylococcus*. Single, tetrad or grape-like clustered cells were observed under light microscopy (Fig. 4.2b). The colonies were round and cream-yellowish on BHI agar. On MSA, small pink-yellowish colonies surrounded by yellow agar resulting from acid production due to mannitol fermentation were observed (Fig. 4.2b), which is usually associated with coagulase-positive species. Further examination on BPA gave black, shiny and convex colonies that lacked the narrow, white edges surrounded by clear zones or opaque rings within clear zones (Fig. 4.2b), suggesting they were coagulase-negative staphylococci, and so were tested for coagulase production using the Staphytect agglutination card. On the reaction card, the isolates exhibited variable reactions, whereby four isolates gave a weak positive reaction, whereas one gave a negative result implying possible strain differences. Analysis of 16S rDNA sequences revealed that all the tested strains (n=5) were closest relatives of *Staphylococcus aureus* (Table 4.2). As pathogenic strains of these organisms are usually coagulase-positive and produce staphylococcal enterotoxins (SE), all the five Stilton cheese isolates were assessed for prevalence of the various SE encoding genes using multiplex PCR (Section 4.2.3.2.1), since they exhibited some (slight) level of coagulase activity.

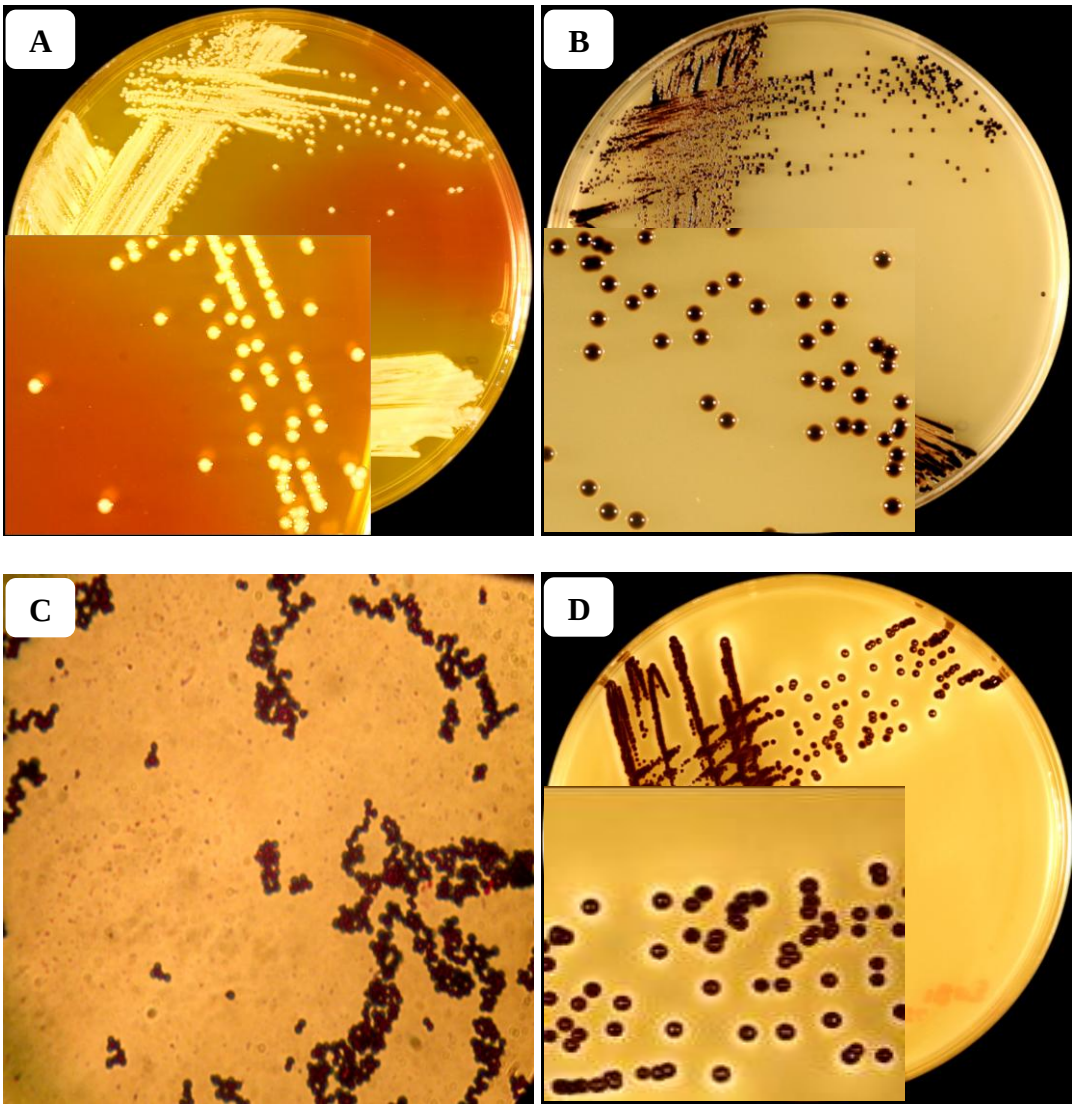


Figure 4.2b Colony (A) and cell (C) morphology of *Group 2* Stilton cheese isolate on mannitol salt agar. Colony morphology of the isolate (B) compared with the control strain, *Staph. aureus* NCTC 1803 (D) on Baird Parker agar.

4.2.3.2.1 Prevalence of SE encoding genes among staphylococcal isolates

From the sequencing results, it was concluded that these isolates might be *Staph. aureus* which are potentially pathogenic (Collins *et al*., 1989) despite the poor coagulase test and atypical results on BPA. There was the need to establish the profile of possible SEs produced by the strains in order to evaluate their potential virulence. The first step was to screen the isolates for prevalence of SE encoding genes which would provide the baseline data for further assessment of gene expression *in vitro*. The genes encoding SE A, SE B, SE C and SE E are the most frequently encountered in most food products (Lamprell *et al*., 2004) and were therefore the main focus of this part of the study.

Genomic DNA was extracted using CTAB method (Sharma *et al*., 2000) as described in §3.3.2 and used in multiplex PCR reactions. The reaction conditions were optimised to ensure that the target gene sequences were amplified using primers designed to target the non-homologous structural coding regions of the SE genes (Sharma *et al*., 2000). The primers had almost equal annealing temperature (49.1-53.2^{o}C). Therefore, the average temperature used (50^{o}C) reduced the possibility of occurrence of unwanted bands on the agarose gel, which usually originates from non specific amplification (Mehrotra *et al*., 2000).

As expected, DNA from *Staph. aureus* NCTC 10652 (SE A and SE D producer) formed three discrete bands of 270, 306 and 800 bp (Fig. 4.3, lane 2). Another band of 165 bp was obtained from *Staph. aureus* NCTC 10654 (SE B producer). Two close fragments ranging between 60-100 bp were evident from each of the *Staph. aureus* NCTC 10655 (SE C producer) and *Staph. epidermidis* NCTC 12100 (Fig. 4.3, lanes 4 and 5, respectively). The latter strain of *Staph. epidermidis* is a known SE C carrier and was used by Sharma *et al*. (2000) to show the specificity of this multiplex PCR assay. These results corresponded to the predicted sizes (Table 3.2). However, only template DNA extracts from the positive control *Staph. aureus* strains gave PCR amplicons (Fig. 4.3, lanes 2-5). Although the DNA extract was amplifiable as shown from Fig. 4.2a (Section 4.2.2), none of the target genes were amplified in the SE multiplex PCR reactions (Fig. 4.3, lanes 6-10) from Stilton cheese staphylococcal isolates.

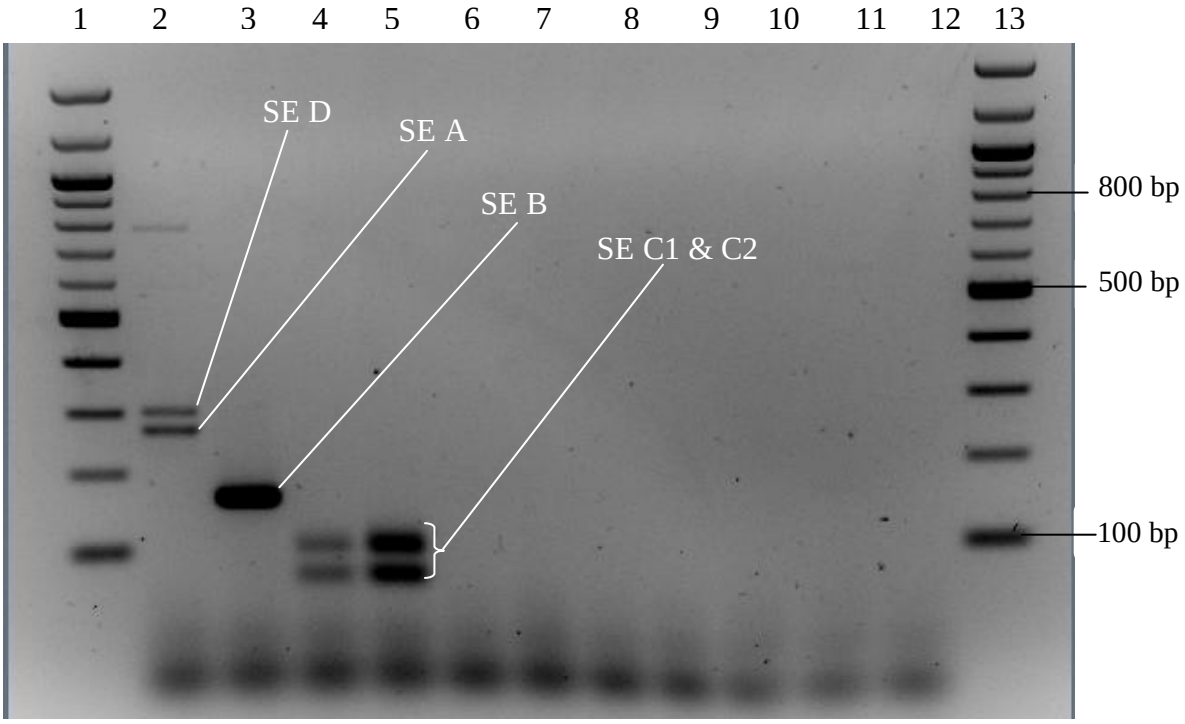


Figure 4.3 Gel analysis showing multiplex PCR amplification products for SE genes. Lanes 2-5, positive control *Staph. aureus* NCTC type strains: 10652 (SE A & D), 10654 (SE B), 10655 (SE C) and *Staph. epidermidis* NCTC 12100 (SE C); lanes 6-10, Stilton staphylococcal isolates: ou8, ou10, ou21, ou25 and ou30; lanes 11-12, negative control; lanes 1 and 13, 100 bp ladder. The samples were run on 2% (w/v) agarose gel in 1X TAE buffer for 2 h at 80 V.

A representative band of two of the known amplicons was excised from the agarose gel. These were sequenced and subjected to BLAST analysis. As expected, the 270 bp amplimer from *Staph. aureus* NCTC 10652 was confirmed as part of the enterotoxin type A encoding gene from closest relatives of *Staph. aureus* (%ID=99%, $E=2^{-55}$, gene accession no., NC002953). Also, the 165 bp amplimer from *Staph. aureus* NCTC 10654 was found to be part of the enterotoxin B encoding gene from closest relatives of *Staph. aureus* (%ID=100%, $E=3^{-52}$, gene accession no., NC002951). Following these results, it was evident that Stilton cheese staphylococcal isolates lacked the SE A to SE E encoding genes. Therefore, it was deemed unnecessary to probe the isolates further for SE gene expression (Schmitz *et al*., 1998) as they lacked the target SE genes.

4.2.3.3 *Groups 3 & 4*

The cells for *Group 3* & *4* isolates were non-motile, catalase-positive and Gram-negative cocco-bacilli (Fig. 4.2c). Neither grew on MRS agar. *Group 3* isolates were oxidase-positive and formed large smooth, circular, cream coloured, convex colonies with a sticky texture on BHI agar. The *Group 4* isolate was oxidase-negative and formed small but thick, elevated off-white colonies on BHI agar. For both groups, under the microscope the cells often appeared single or in pairs (diploforms) with pointed ends. The *Group 4* isolate could not grow on MacConkey agar implying it was sensitive to bile salts and therefore not an Enterobacteriaceae. All isolates in *Group 3* & *4* were subjected to API 20 NE for preliminary identification. From this, the *Group 4* isolate was identified as *Sphingomonas paucimobilis* (%ID=96.8, t=0.26). On the API strip, the isolate assimilated nitrate, urea, aesculin, D-maltose, potassium gluconate, N-acetyl glucosamine, D-glucose and D-mannose. However, the low t-index obtained implies that the identification was not typical for this species. *Group 3* isolates gave no positive reactions on the API strip and could not be identified from phenotypic and biochemical characteristics.

From 16S rDNA sequence analysis, *Group 3 & 4* isolates were respectively identified as closest relatives of *Psychrobacter cryohalolentis* and *Acinetobacter baumanii* (Table 4.2). Molecular identification of *Group 4* isolate was in accord with the observed morphological (Gram-negative, cocco-bacilli) and biochemical (catalase-positive, oxidase-negative) characteristics typical of *Acinetobacter* species (Constantiniu *et al*., 2004). Whereas API speciated the isolate as *Sph. paucimobilis*, the latter organisms are typically oxidase-positive (Ryan and Adley, 2010), which was not the case for the isolated strain tested in this study. On the basis of this, and given the low t-index obtained from API, molecular identification was regarded as definitive for the *Group 4* isolate. Failure to metabolise L-arabinose, trisodium citrate and fatty acids (capric, adipic, malic and phenylacetic acid) on the second part of the API strip led to inappropriate API identification of *Ac. baumanii* leading to a different speciation, i.e. *Sph. paucimobilis*.

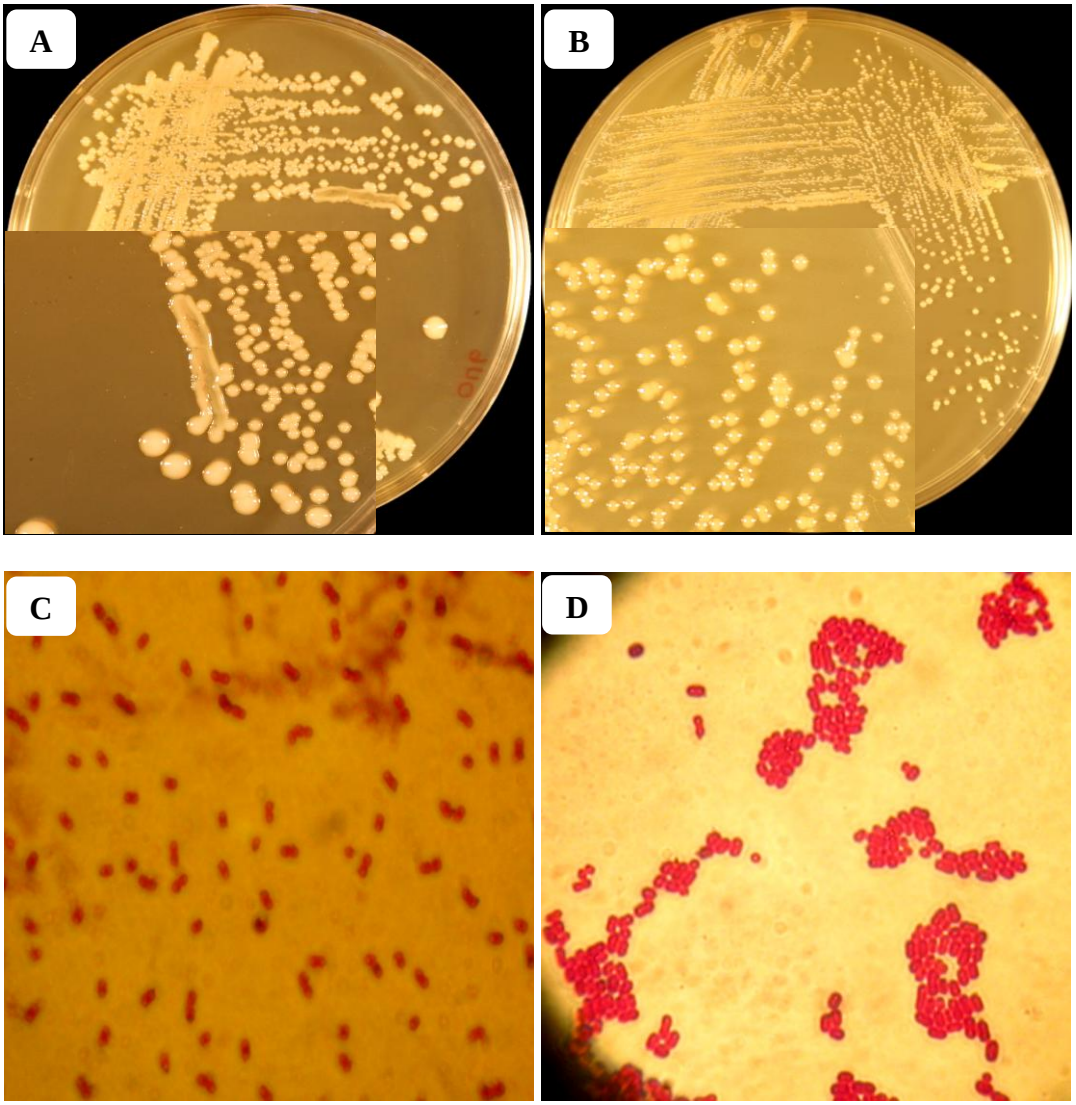


Figure 4.2c Colony and cell morphology of *Group 3* (**A**, **C**) and *Group 4* (**B**, **D**) Stilton cheese isolates on BHI agar.

4.2.3.4 *Group 5*

This group consisted of Gram-positive, catalase and oxidase-negative, non-sporing rods presumptively identified as *Lactobacillus*. The strains formed profuse and small, round (elliptical) off-white colonies on MRS (Fig. 4.2d, A) and BHI agar. Cells appeared slender and short under the microscope (Fig. 4.2d, B). BLAST analysis of *Group 5* sequences demonstrated that the majority (97% of the 32 isolates) were closest relatives of *Lactobacillus plantarum* (*Group 5a*; Table 4.2) and two isolates were identified as closest relatives of *Lactobacillus brevis* (*Group 5b*; Table 4.2). As there were 60 isolates which had all come from a single cheese, they were assessed for substrate assimilation profiles (Section 4.2.3.4.1) and PFGE (Section 4.2.3.4.2) to determine their biochemical profiles and genotypic relatedness, respectively.

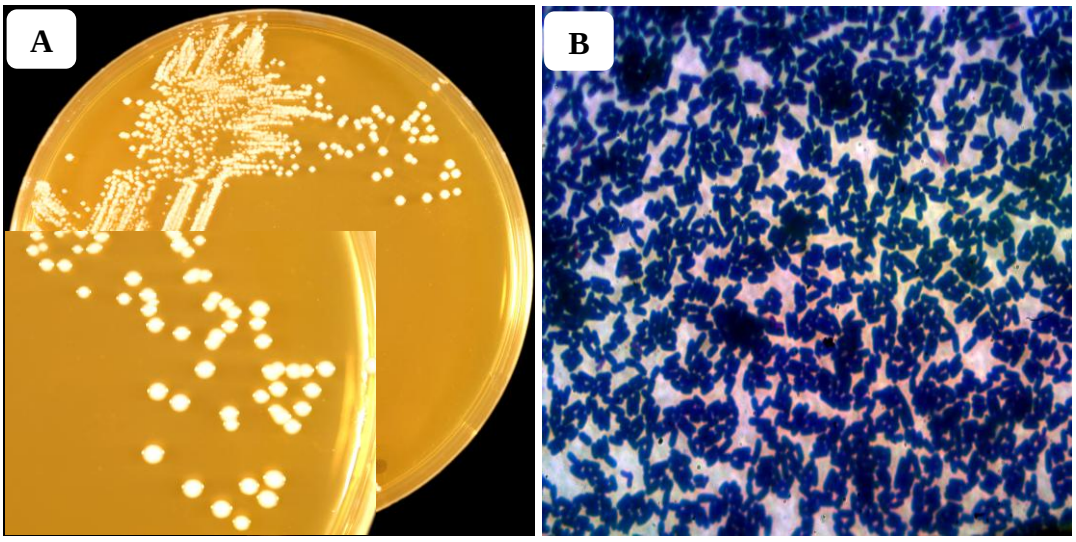


Figure 4.2d Colony (A) and cell (B) morphology of *Group 5* Stilton cheese isolate on MRS agar

4.2.3.4.1 Substrate assimilation profiles for *Lactobacillus* isolates

In order to evaluate the phenotypic relatedness and confirm the identity of the dominant non-starter bacterial flora obtained with 16S rDNA sequence analysis (*Group 5*), carbohydrate fermentation patterns of representative isolates from each sampled site and identified as closest relative of *Lb. brevis* (2 strains, outer crust) and *Lb. plantarum* (outer crust, n=3; blue veins, n=5; white core, n=7) were characterised based on API.

The API identification level for *Lb. plantarum* isolates ranged from 90.4 to 99.9% (t=0.4-0.78) and 95-96.1% (t=0.47) for *Lb. brevis* isolates (Table 4.3). From these results, 80% of the isolates identified as *Lb. plantarum* had similar substrate assimilation profiles but 20% showed a different pattern suggesting a mixed population of *Lb. plantarum* was present in Stilton cheese. The diversity was caused by the differences in the ability to assimilate L-rhamnose, methyl-αD-mannoside and D-melezitose (Table 4.3). In comparison with other *Lb. plantarum* isolates, isolates R4 & R5 (outer crust) and R37 (white core) could assimilate D-melezitose and methyl-αD- mannoside but not L-rhamnose. Also, there was variation in the assimilation profiles for the isolates identified as *Lb. brevis*. The variation was caused by differences in their ability to assimilate D-galactose, D-glucose and α-keto-gluconate (Table 4.3). Isolate R9 from the outer crust could assimilate the above three carbohydrates whereas R15 (outer crust) could not. Compared with *Lb. plantarum* strains, the isolates identified as *Lb. brevis* could not assimilate most of the substrates on the API strip. In fact, *Lb. brevis* strains could only assimilate D-ribose, D-xylose, D-glucose, D-fructose and gluconate out of the 50 substrates examined.

API appropriately assigned the isolates to *Lb. plantarum* and *Lb. brevis* which was concordant with the 16S rDNA sequence identifications presented in Table 4.2. Although API results corresponded with 16S rDNA sequence identification, API t-indices were substantially lower than 1.0 suggesting that the profiles were not absolutely typical of the species. This is probably indicative of the fact that the API database does not contain a large number of wild isolates demonstrating the diversity of lactobacilli isolates selected in the cheese. However, the technique was found to be more sensitive than 16S sequence analysis as it was able to provide some measure of strain/biotype differentiation and gave valuable information on the inherent metabolic properties of *Lactobacillus* species obtained from different sites in Stilton cheese. The strains were subjected to sub-species typing using PFGE (Section 4.3.4) to evaluate their genotypic relatedness.

Table 4.3 Substrate assimilation profiles of *Lactobacillus* isolates. Data obtained from two independent determinations.

Substrate	Assimilation profiles for isolates identified as																
	Lb. brevis		*Lb. plantarum*														
	R9	R15	R2	R4	R5	B14	B27	R17	R23	R30	R36	R37	R40	R44	W10	W24	W26
D-ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactose	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-rhamnose	-	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+
D-mannitol	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-sorbitol	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl- αD-mannoside	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl glucosamine	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin ferric citrate	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Salicin	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-cellobiose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-maltose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-lactose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Sucrose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-melezitose	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-
Gentiobiose	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-arabitol	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Potassium gluconate	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-keto-gluconate	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
%ID (API)	95	96.1	99.9	90.4	99.8	99.9	99.9	99.9	92.4	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9
API t-index	0.47	0.7	0.6	0.72	0.6	0.6	0.6	0.74	0.4	0.6	0.6	0.73	0.6	0.6	0.6	0.6	0.6
%ID (16S rDNA)	96	96	100	99	98	99	99	99	99	100	99	99	99	100	99	ND	99

(+) positive reaction, (-) negative reaction, ND (not done). Isolates were obtained from the: outer crust (R2-R15), blue veins (B14-B27, R17-R30), and white core (R36-R44, W10-W26)

4.2.3.4.2 Species and sub-species typing of the lactobacilli using PFGE

PFGE was carried out on the *Lactobacillus* isolates to determine their genetic relatedness. The restriction enzyme *Not*I was employed to digest genomic DNA from the 59 strains. This enzyme with the recognition sequence 5'-GC$^{\blacktriangledown}$GGCCGC-3' had been previously successfully applied to cut genomic DNA producing suitable PFGE patterns for lactobacilli compared with other enzymes such as *Sma*I, *Xba*I, etc (Obodai, 2006; Vancanneyt *et al*., 2006). This is partly because these organisms have a relatively low guanine and cytosine content (35-47 G+C mol %) in their genome (Yeung *et al*., 2004). Therefore, *Not*I which recognises G+C rich sequences cuts the DNA less frequently giving fewer fragments which aids separation and visual evaluation of the resulting PFGE patterns (typical results shown in Fig. 4.4).

Electrophoresis was performed for 21 h at 6 V/cm, ramping linearly from 4 to 45 s, temperature of running buffer, 14^{o}C (§3.3.5.3), to obtain visually informative patterns from all isolates. Five to sixteen bands were produced with the highest number recorded from isolate W23 obtained from the white core whereas the lowest was recorded from isolate R6 obtained from the outer crust (gel not shown) suggesting differences in genomic composition of the lactobacilli isolates obtained from different sites in Stilton cheese. The size of resolvable fragments ranged from approximately 50 to ~700 kbp. Although a high degree of similarity was generally evident in PFGE band patterns above ~700 kbp, the region between 50 to ~600 kbp delineated a relatively variable and heterogeneous band region. Another relatively constant and similar band pattern occurred below 50 kbp for all the strains.

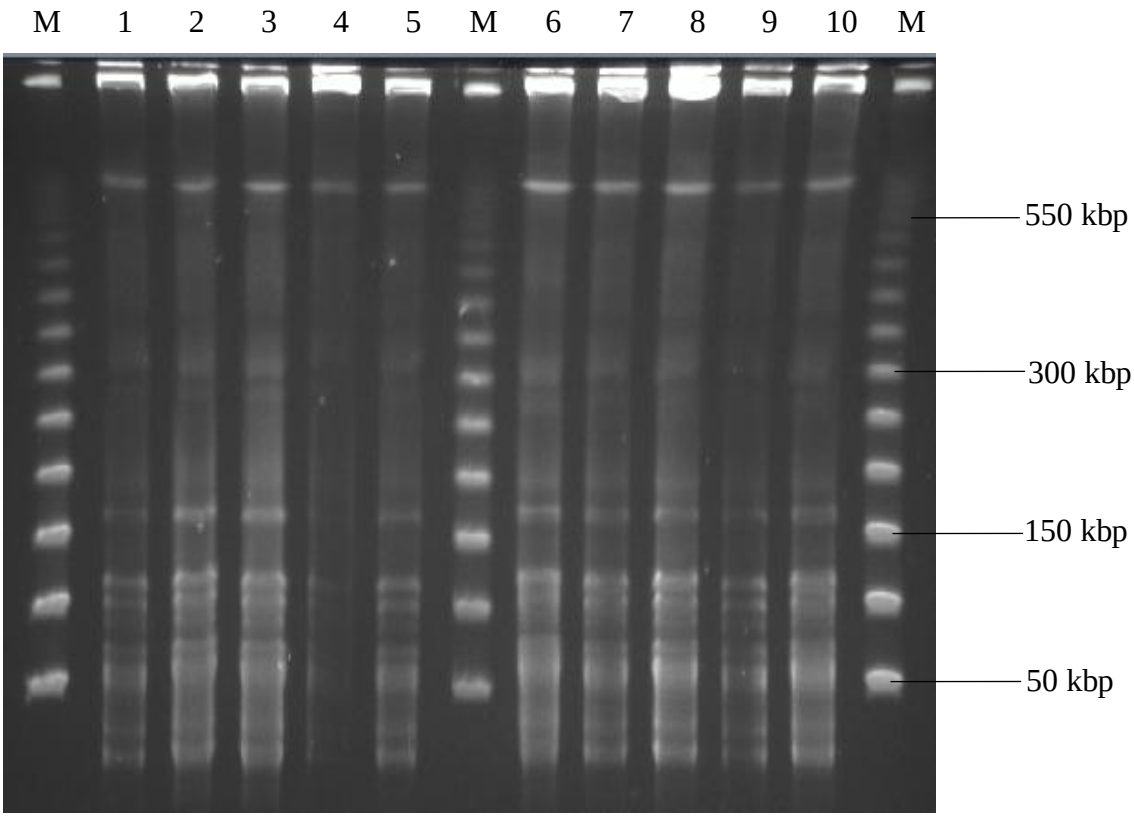


Figure 4.4 PFGE patterns of *Lactobacillus plantarum* isolates obtained from Stilton cheese. Genomic DNA was cut with *Not*I and fragments separated by PFGE on 1% (w/v) pulse-field certified agarose gel at 6 V/cm with switch time ramped from 4 to 45 s for 21 h at 14°C. Lanes 1-10, isolates from the white core: R36, R37, R38, R39, R40, R41, R42, R43, R44 and R45. Lane M, 50-1000 kbp marker.

4.2.3.4.3 Cluster analysis

Cluster analysis was conducted to establish the degree of genomic relatedness of the 59 *Lactobacillus* isolates typed by PFGE. The dendrogram established by the average linkage method (UPGMA) of the PFGE patterns based on a Dice similarity coefficient with a 1.5% band position tolerance demonstrated a range of similarity levels between the lactobacilli as shown in Fig. 4.5. Five major geno-groups (clusters) were delineated at a similarity level of 52% (Φ_{PT}=0.34; p<0.01). There was significant difference in molecular variance among the clusters (Φ_{PT}=0.01-0.56, p<0.01; Appendix 4.3). All clusters (except III and IV) had heterogeneous PFGE profiles and only contained isolates from the blue veins and white core, which implies possible clonal relationship. Cluster I was the largest comprising 38 isolates whereas clusters II and IV were the smallest and comprised two isolates each. Interestingly, all isolates from the outer crust were grouped in clusters III

and IV suggesting they entered into the cheese from a common source. Isolate R24 was shown to be an outlier to geno-group II. Isolates R20 and R37 were also shown to be outliers with R20 being an outlier of geno-group V. In cluster I, isolates B27 & B28 (blue veins) and W9, W12 & W13 (white core) exhibited indistinguishable *Not*I macro-restriction profiles producing 100% identity levels independent of their source of isolation and so were considered to be identical. Surprisingly, although *Lb. brevis* isolates (R9 and R15 from the outer crust) were aligned next to each other and clustered with *Lb. plantarum* from the outer crust (cluster III), the dendrogram did not indicate *Lb. brevis* as an outlier even though it is belongs to a different species. This suggests a high level of homology in genomic DNA of these LAB species although both had been earlier shown to exhibit different API profiles. As *Lb. plantarum* isolates with similar API phenotypes (R4 and R5, outer crust) were clustered close to each other (clusters III and IV), this demonstrated good sensitivity of PFGE.

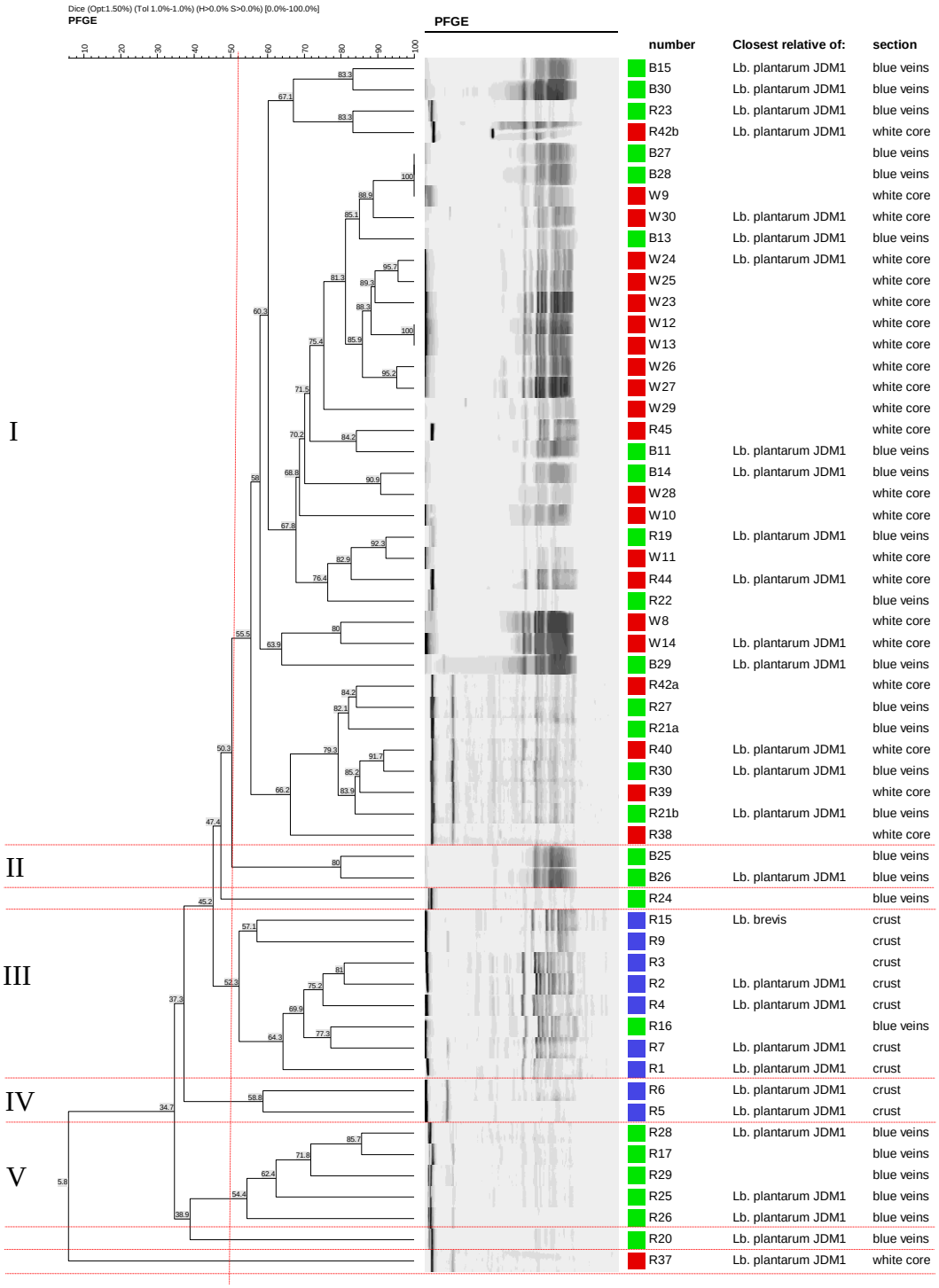


Figure 4.5 Dendrogram showing the clustering of 59 *Lactobacillus* isolates obtained from Stilton cheese. Similarity values were obtained by UPGMA and the Dice coefficient methods with a 1.5% band position tolerance. Strains from: (■) outer crust, (■) blue veins, and (■) white core. All isolates were *Lb. plantarum* except for *Lb. brevis* (strains R9 and R15, cluster III).

4.2.4 Distribution of bacterial species obtained from different sites in Stilton cheese

Although molecular sequence identifications were more accurate as demonstrated by the higher %ID and very low E-values obtained for all isolates, the results of 16S rDNA sequence analysis were congruent with the presumptive identifications obtained using the classical microbiological methods and API. On the basis of these results, the bacterial community structure of the outer crust was found to be more diverse and comprised five of the six species recorded (Fig. 4.6). In comparison, blue veins had the lowest (1 out of 6) number of species isolated and only comprised *Lb. plantarum*.

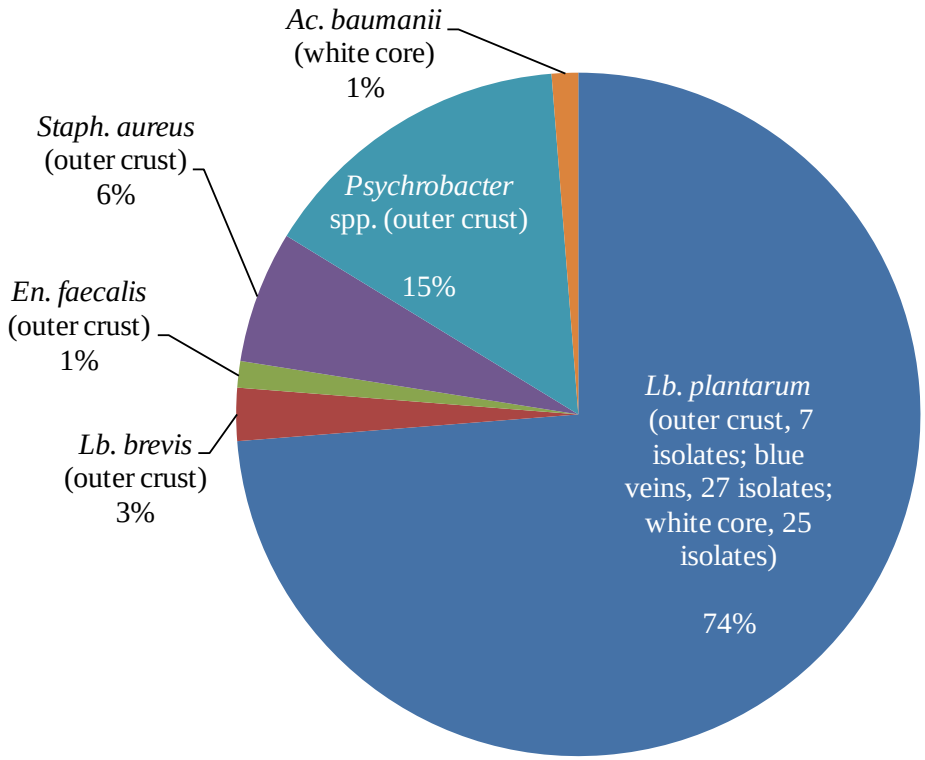


Figure 4.6 Diversity of the non-starter bacterial isolates obtained from different sites (outer crust, blue veins and white core) in Stilton cheese.

4.3 Discussion

The main goal for this part of the study was to identify isolates of the bacterial flora from Stilton cheese. While Stilton cheese is made from pasteurised milk and should, therefore, be free from undesirable bacteria, the final product was dominated by secondary flora. *Lc. lactis* is normally added as a starter culture but was not detected in the community profile of the strains isolated. *Lb. plantarum* was the dominant species spatially distributed in the outer crust, blue veins and white core suggesting it was selected during the ripening process. The dominance of lactobacilli and the absence of lactococci in Stilton cheese showed good comparison with previous studies (Fitzpatrick, 1971; Whitley, 2002; Ercolini *et al*., 2003) implying the latter had been replaced by the more acid-tolerant lactobacilli from the environment (Hiscox *et al*., 2008). This has potential implications on the aroma and other quality characteristics of the ripened product.

The NSLAB species isolated from Stilton cheese included *Enterococcus faecalis, Lb. plantarum* and *Lb. brevis*. Cogan *et al*. (2007) have summarised the NSLAB commonly identified in more than 50 varieties of ripened cheese. In most cheeses, regardless of whether they are made from raw or pasteurised milk, the dominant organisms are usually the facultative homofermentative lactobacilli (FHL) that produce predominantly lactic acid as the end product of carbohydrate fermentation. These commonly include *Lb. casei, Lb. plantarum* and *Lb. curvatus*, all of which normally grow from levels of 10^2-10^4 to ~10^8 CFU/g by the end of cheese ripening. Stilton cheese is made from pasteurised milk. Therefore, the presence of these organisms in the product may be attributed to inadequate heat treatment and/or post pasteurisation contamination from subsequent processing steps and the production plant environment (Robinson, 1990). Given that *Lb. plantarum* isolates were obtained from all the sampled sections of Stilton cheese, this suggests that these FHL could have been introduced into the cheese from various routes: strains introduced from equipment during curd milling prior to moulding, during the piercing step, or strains which could have been introduced to the cheese outer crust during ripening from the processing plant environment and handlers.

Obligate heterofermentative lactobacilli (OHL) such as *Lb. brevis* on the other hand can produce alcohol or lactic acid from sugar fermentation (Mikelsaar *et al*., 2002; Ganzle *et al*., 2007). These are often found in low numbers in mould ripened cheeses (Fitzsimons *et al*., 2001; Gala *et al*., 2008), which is similar to this study. FHL normally dominate the

bacterial flora of high quality blue cheese and they are considered to enhance its flavour development and production of antimicrobial compounds (Whitley, 2002; Ercolini *et al*., 2003). OHL particularly *Lb. brevis* sometimes interact with *Lb. curvatus* and produce metabolites such as acetic acid, carbon dioxide, ethanol and hydrogen peroxide that interfere with the cheese sensory properties (Patil *et al*., 2007). In Stilton cheese, *Lb. brevis* is considered undesirable as it is thought to reduce blueing and generates bad flavour notes in the cheese (Whitley, 2002). *Lb. plantarum* isolated from silage was shown to produce antifungal compounds including 3-phenyllactate and 2-cyclic dipeptides (Cogan *et al*., 2007) and participates in the release of free amino acids and fatty acids that contribute to mature cheese flavours (De Angelis *et al*., 2004).

4.3.1 *Enterococcus* isolate

Enterococci, particularly *En. faecalis, En. faecium,* and *En. casseliflavus* frequently occur as NSLAB in a variety of artisanal cheeses produced from raw or pasteurised milk, and occasionally in natural milk or whey starter cultures (Cogan *et al*., 2007). They contribute to cheese quality characteristics through proteolysis and lipolysis. These organisms also produce aroma compounds and bacteriocins (enterocins) that are inhibitory against pathogens such as *Listeria monocytogenes*, *Staph. aureus*, *Vibrio cholerae*, *Clostridium* spp. and *Bacillus* spp. (Giraffa, 2003). The presence of *En. faecalis* in the outer crust of Stilton cheese was not surprising as the organism is often isolated from highly acidic and salty dairy samples (Ercolini *et al*., 2003), due to cross-contamination from personnel. However, the occurrence of enterococci in food usually indicates poor bacteriological quality and poor hygiene during manufacture due to faecal contamination as their natural habitat is the mammalian intestinal tract (Franz *et al*., 1999). In fact, *En. faecalis* is often the most frequently occurring *Enterococcus* species in faeces of humans and dairy cows; however, *En. faecium*, *En. hirae*, *En. avium*, and *En. durans* have also been found (Gelsomino *et al*., 2002; Rathnayake *et al*., 2011). These organisms are also found in water, soil, plants, vegetables, and have been previously isolated from Cheddar-type cheeses during manufacture and ripening (Gelsomino *et al*., 2002). Although the role of *En. faecalis* in blue cheese is not well known, it has been suggested that the organism can be added as a starter culture adjunct for some semi-hard cheese varieties (Garde *et al*., 1997).

4.3.2 Staphylococcal isolates

The staphylococcal isolates grew on mannitol salt agar (MSA) giving colonies typical of *Staph. aureus*. As they were identified as *Staph. aureus* using 16S rDNA sequence analysis, the strains formed atypical colonies on Baird-Parker agar (BPA) and exhibited weak coagulase activity. The high salt content (7.5%) of MSA inhibits most other organisms and encourages growth of staphylococci regardless of coagulase status. BPA is selective for all staphylococci and can be used as a diagnostic medium for *Staph. aureus*. The medium contains sodium pyruvate which stimulates the growth of staphylococci. The tellurite additive in the medium is toxic to egg yolk clearing strains other than staphylococci and imparts a black colour to the colonies. Glycine and lithium chloride in BPA are inhibitory to organisms other than *Staph. aureus*. Lecithinase activity creates the clear zones in the egg yolk typical of coagulase-positive species.

Four of the five isolates identified as *Staph. aureus* by 16S rDNA sequence analysis had weak coagulase activity and grew on BPA to form atypical black colonies lacking a zone of clearing (Fig. 4.1b). The presence of atypical colonies on BPA that do not show opacity or zones of clearing are known to occur among *Staph. aureus* isolated from foods of bovine origin (CDSC, 2005), and so this observation should not be the basis for invalidating the results obtained from this study. Staphylococci are facultative anaerobes and grow better in a less acidic environment, which is similar to the outer crust of Stilton cheese (pH ~6) from where they were isolated. The presence of *Staph. aureus* in Stilton cheese and other food products is generally undesirable especially when the count is greater than 10^2 CFU/g. Low numbers of these halotolerant organisms is indicative of poor handling conditions whereas high counts are frequently associated with incidences of food poisoning (CDSC, 2005). *Staph. aureus* normally find their way into dairy products from the raw milk, personnel, animal skins and the environment (Roberson *et al*., 1998).

Use of multiplex PCR to detect the presence of SE genes is an improvement over many other previously described PCR protocols, where individual primers are used to identify the various SE genes (Schmitz *et al*., 1998; Mehrotra *et al*., 2000). The three pairs of multiplex primers used in this study, to target the five structural SE genes were shown to be specific, reliable, and efficient in detection of the genes. Multiplex PCR revealed that the staphylococcal isolates lacked the SE encoding genes targeted. As an internal control,

Staph. epidermidis which only rarely harbours the SE C encoding gene was used in the assay. Whereas the study showed that the staphylococcal isolates lacked SE A-E encoding genes, it could be possible that the strains possessed other SE genes such as F-U (De Freitas *et al*., 2008) which were not targeted in this work. The prevalence of SE G, H, I and J encoding genes in *Staphylococcus* spp. from dairy products manufactured using milk from cows diagnosed with subclinical mastitis is normally high at 80.2% (De Freitas *et al*., 2008). Notably, these genes are often detected in instances where none of the genes encoding the classical SE A-E, toxic shock syndrome toxin 1 (TSST-1), and exfoliate toxins (ET A&B) may not be found (De Freitas *et al*., 2008). This is an aspect which requires further investigation for *Staph. aureus* strains isolated in the current study.

It was evident that the *Staph. aureus* isolates lacked the SE A-E encoding genes. Johnson *et al*. (1991) highlighted that non-pathogenic strains of the same genus or species frequently overgrow the pathogens, and in the process, the latter may readily lose plasmid or phage mediated virulence factors such as enterotoxin genes. Although there is scant information about how frequently this phenomenon occurs, it has been reported that many phage-carrying human and animal staphylococcal strains do not produce enterotoxins (Honeyman *et al*., 2002). Some strains of *Staph. aureus* become able to produce enterotoxins through acquisition of a variable number of mobile genetic elements including transposons, temperate and defective bacteriophage, plasmids, pathogenicity islands and uncharacterised DNA inserts (Honeyman *et al*., 2002; Fusco *et al*., 2011).

In general, the results from this study were accordant with previous findings in which it was reported that ripened cheese is relatively free from toxin producing strains of *Staph. aureus* (CDSC, 2005). Whereas SE A and D are the most commonly encountered enterotoxins (Balaban and Rasooly, 2000; Naffa *et al*., 2006; Fusco *et al*., 2011), the prevalence of SE genes in food and clinical isolates of *Staph. aureus* is relatively rare and varies with geographical region and, ecological origin of the organism (food, humans and animals) (Naffa *et al*., 2006). Considering the ability to amplify the DNA as an internal positive control (Section 4.2.2), provided assurance against false negative results and gave the confidence to conclude that the *Staph. aureus* growing in this Stilton cheese may not pose health hazards related with the presence of staphylococcal enterotoxins A-E.

The foods that are most often involved in staphylococcal food poisoning differ widely from one country to another. In the UK, 53% of the staphylococcal food poisonings reported between 1969 and 1990 were due to meat products, meat-based dishes, and especially ham; 22% of the cases were due to poultry and poultry-based meals, 8% were due to milk products implying Stilton cheese and other dairy products are relatively free from SE producing staphylococci (Le Loir et *al*., 2003).

4.3.3 *Psychrobacter* isolates

Psychrobacter cryohalolentis was only isolated from the outer crust of Stilton cheese where redox potential (Eh) is positive due to presence of air suggesting its potential to contribute to the microbial succession dynamics in this part of the cheese matrix. This lipolytic, strictly oxidative, psychrotolerant and moderately halotolerant organism usually lives in extremely cold habitats and mainly contaminates refrigerated poultry, seafood and meat products during storage under aerobic conditions (Pacova *et al*., 2001; Bakermans *et al*., 2006). Milk fermentation and the cheese ripening process are accompanied by reduction of oxygen to water (Beresford *et al*., 2001). As a consequence, the cheese interior essentially becomes more anaerobic (negative Eh) which excludes most obligate aerobes including *Psychrobacter* spp., but supports the growth of obligatory or facultative anaerobic microorganisms such as LAB (Beresford *et al*., 2001) as observed in the study. Although the genus *Psychrobacter* has a ubiquitous distribution in foods (Pacova *et al*., 2001), Bakermans *et al*. (2006) reported that sea water, soil and sediment, as well as marine foods constitute its typical natural habitats. Other sources of *Psychrobacter* spp. include pigeon faeces, fish, dairy products and clinical sources. Although the development of these bacteria in cheese and their contribution to its aroma during ripening has not been widely studied, growth of lipolytic and proteolytic strains in a synthetic cheese medium has been found to produce pronounced 'cheesy' notes attributed to volatile sulphur compounds (Deetae *et al*., 2009).

4.3.4 *Acinetobacter* isolate

The sole Gram-negative cocco-bacillus isolate from the white core of Stilton cheese was identified as *Acinetobacter baumannii*. Some of these organisms are lipolytic (Pacova *et al*., 2001) but pathogenic strains are normally resistant to antibiotics and usually associated with nosocomial pneumonia especially among persons with compromised immune systems such as the wounded, elderly and children (Rello, 1999). *Ac. baumanii* is

non-fermentative and exhibits resistance to antimicrobial compounds but the mechanism of regulating this expression is not yet well defined (Hood *et al.*, 2010). Sodium chloride is thought to enhance tolerance of the organism to aminoglycosides, carbapenems, quinolones, colistin and other antibiotics by inducing genes that encode for putative efflux transporters which are up-regulated in response to the salt (Hood *et al.*, 2010). The major factors that may be responsible for presence of *Acinetobacter* spp. in cheese and other food products include their ability to attach and persist on food contact surfaces, availability of essential nutrients such as iron and subsequent adhesion to epithelial cells of personnel working in the processing plants (Rello, 1999).

The presence of *En. faecalis*, *Staph. aureus* and *Psychrobacter* spp. in the outer crust may be attributed to the oxidative nature of this part of the cheese and its higher pH (~6) which enhance their survival. The pH of the outer crust is elevated relative to that of the blue veins and white core due to metabolic activity of moulds, yeasts and coryneform bacteria that degrade lactic acid and liberate ammonia from amino acids (Zeuthen and Bùgh-Sùrensen, 2003). These factors may also explain the presence of staphylococci in this part of the cheese sample because the organisms are known to be susceptible to acid (Ercolini *et al*., 2003).

4.3.5 PFGE cluster analysis

Based on cluster analysis of PFGE profiles, isolates are considered as belonging to different profiles if differing by five or more bands (Sood *et al*., 2002). The technique is normally used to discriminate strains of the same species based on differences in their genomic DNA. Although not applied in this study, the discriminatory index has been reported as an alternative for PFGE typing comparison and grouping. The index represents the probability that two unrelated strains are characterised as being of different types by a given typing system and is widely used in PFGE-RAPD for *E. coli* (Sood *et al*., 2002).

Lactobacilli isolated from the outer crust were clustered together implying close clonal relationship. These isolates are likely to influence the quality properties of this cheese section in the same way (Gkatzionis *et al*., 2009) as evidenced by the majority (two out of the three tested strains) of the isolates having similar API characteristics. It was surprising that PFGE clustering could not clearly differentiate between *Lb. plantarum* and *Lb.*

brevis, whereas both species of these NSLAB isolates were effectively discriminated by API. The 16S rDNA cataloguing system has clustered *Lb. plantarum* and *Lb. brevis* together and closer to other species including *Lc. lactis*, *Lb. acidophilus*, *Lb. helveticus*, *Lb. rumis*, *Lb. casei*, *Lb. bifermentans*, *Lb. fermentum* and *Ped. pentosaceus* (IDF, 1988). However, PFGE was expected to show high sensitivity in discriminating *Lb. brevis* from *Lb. plantarum* as was the case for API 50 CHL. Moreover, both of these organisms have different chromosomal architectures. There is the need to re-examine this aspect using other typing methods or by application of two or more restriction enzymes in order to determine the reproducibility of these results.

4.4 Conclusions

The study demonstrated that *Lb. plantarum* is the dominant non-starter bacterial species within the outer crust, blue veins and white core of Stilton cheese at the end of ripening. Blue veins and white core had the highest proportion (85% of the 59 *Lactobacillus* isolates), whereas the outer crust only contained 15%. Identification of the five major non-starter bacterial groups was achieved by conventional microbiological and molecular protocols giving generally concordant results except for the isolates identified as *Psychrobacter* spp. which did not give significantly identifiable profiles with API. Species identification with 16S rDNA sequencing was generally more precise, simpler and faster than API. In cases where disagreement occurred, identification obtained with molecular methods was regarded as definitive.

In general, the bacterial community structure was relatively complex and differentially distributed within the cheese sample. Whereas *Lb. plantarum* was present with other bacterial species in all sampled sites, the blue veins contained only this organism whereas the white core contained *Lb. plantarum* along with *Ac. baumanii*, implying the latter were more selective microenvironments than the outer crust. However, there is the possibility that the microbial diversity observed was limited due to the possible presence of other uncultivable microorganisms or having used a narrow spectrum of selective media and incubation conditions to recover the bacterial species identified. For instance, other studies have shown that, in addition to the bacterial species obtained from this study, the microflora of Stilton cheese is composed of *Lb. curvatus* (Whitley, 2002), *Lc. lactis*, *Leu. mesenteroides* and *Staph. equorum* (Ercolini *et al*., 2003), as well as various yeast species

including *Candida catenulata*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Debaromyces hansenii* and *Trichosporon ovoides* (Gkatzionis, 2010).

Lc. lactis and other common Stilton cheese starter culture strains such as *Leuconostoc* spp. could not be isolated despite the fact that they are usually added in the cheese-making process suggesting they were probably on confluent plates and therefore did not produce single colonies to be isolated or they were metabolically impaired or were unable to withstand the high acidity, salt content and low a_W levels characteristic of the ripened cheese. Some of these factors have been examined, the results of which are presented in Chapters 5 and 7.

The presence of *Lactobacillus* in all the sampled sites implies they can grow in a broad spectrum of conditions such as acid, antimicrobial agents, oxygen, etc, and therefore can potentially influence the quality characteristics of the cheese through possible interaction with the other cheese microflora. This may partly arise from differential utilisation of the cheese nutrients due to varying degrees of response to factors such as antimicrobial compounds, pH, a_W and salt content, all of which vary in the different sites of ripened Stilton cheese. The phenomenon is likely to be of significance in relation with the distribution of aroma and other quality attributes within the cheese and has been partly investigated in this study (Chapter 7).

The absence of SE A-E encoding genes among staphylococcal isolates was definitively determined by multiplex PCR. There is the need to verify the lack of toxin production by the Stilton cheese staphylococcal isolates that exhibited some level of coagulase activity. The results obtained in this study cannot be regarded absolute as only one cheese sample taken at a single time point and from a single source was examined.

CHAPTER 5

STRESS TOLERANCE RESPONSE OF *LACTOBACILLUS PLANTARUM* ISOLATES OBTAINED FROM STILTON CHEESE

5.1 Introduction

Adventitious NSLAB introduced into cheese as contaminants from raw milk and the environment usually grow from as low as 2 $\log_{10}$ CFU/g in the fresh curd to dominate the microflora of the mature cheeses (De Angelis *et al*. 2004). The NSLAB that grow in ripened cheese made from pasteurised milk mainly consist of the bacteria that survive the milk pasteurisation process and subsequently become part of its secondary flora (Briggiler-Marco *et al*., 2007). However, the non-starter microorganisms present in cheese are also considered to be part of the resident flora of the processing plant environment and can contaminate the product from the processing steps that follow milk pasteurisation (Briggiler-Marco *et al*., 2007).

Stilton cheese at the beginning of the production process has an average pH of 4.5 and 3.5% (w/w) salt. Piercing at 5-6 weeks and further ripening for 8-12 weeks result in considerable physical and chemical changes creating acid and water activity gradients in the different cheese sections (outer crust, blue veins and white core). By the end of ripening, the pH of the outer crust and blue veins (5-6) is higher than that of the white core (4.5-5) due to mould activity which produces ammonia from oxidative deamination of amino acids and amines (Fox *et al*. 2000). Conversely, the water activity (a_W) of the outer crust is lower than that of the cheese interior due to moisture loss occurring during ripening.

It is, therefore, logical to consider that *Lactobacillus plantarum* growing in Stilton cheese is well adapted to the cheese production process, milk fermentation and to the cheese ripening conditions, becoming the dominant microbiota of the final product after starter culture lactococci decrease. It was anticipated that *Lb. plantarum* isolates obtained from the white core in the current study, could be more acid tolerant as they were able to survive milk fermentation and the cheese ripening processes. The isolates obtained from the outer crust could have been introduced as contaminants from the environment and

introduced into the blue veins during piercing. As the latter lactobacilli were isolated from a relatively low a_W/high pH microenvironment (outer crust) implies they could have been selected to be able to grow at a relatively low a_W/high pH typical of this cheese section.

Isolation and screening of microorganisms from natural sources has always been used as the most powerful approach for obtaining useful and genetically stable microbial strains for use as starter cultures and starter culture adjuncts in the dairy industry (Ibourahema *et al*., 2008). Several members of the *Lactobacillus* genus are frequently applied as culture adjuncts in the manufacture of ripened cheese and other fermented dairy products (Adnan and Tan 2003; Derzelle *et al*., 2003). Lactobacilli mainly improve the quality of ripened cheese through production of lactic acid which acts as a natural preservative as well as a flavour enhancer (Verluyten *et al*., 2004). *Lb. plantarum* is considered to have better potential for development into commercial culture adjuncts than obligate heterofermentative lactobacilli such as *Lb. brevis* (Adnan and Tan, 2003). This is because the organism produces fewer metabolites other than lactic acid, and poses a more predictable and controlled effect on the product quality characteristics. Although the role of *Lb. plantarum* in ripening of Stilton cheese has not yet been resolved, the inclusion of some strains of this organism with the starter culture lactococci is known for positive effects on release of free amino acids and fatty acids that contribute to improved flavour in mature cheeses (De Angelis *et al*., 2004; Spano *et al*., 2004; Briggiler-Marco *et al*., 2007). There is a need to evaluate the potential of *Lb. plantarum* as a culture adjunct for Stilton cheese as the organism dominates its final microflora (Section 4.2).

Viability and survival are the most important parameters for evaluation of the functional properties of bacteria used as starter culture and culture adjuncts (Succi *et al*., 2005). Acid and water activity (a_W) significantly affect the viability of cheese microflora and consequently influence product quality characteristics (Spano *et al*., 2004; Succi *et al*., 2005). Like other blue cheeses, Stilton has heterogeneous microenvironments with pronounced gradients of pH and a_W (Fernández-Salguero *et al*., 1986) due to structural and compositional changes occurring in the cheese during ripening (Fox *et al*., 2000). As already discussed, *Lactobacillus* may be introduced into Stilton cheese from the processing equipment, or post-process contamination to the outer crust from the cheese environment. Subsequently, exposure to different gradients of acid and osmotic stresses may constitute an important selection criterion for presence of the different genotypes of

the organism and consequently influence their behaviour in the cheese. The surviving strains may interact with the starter cultures to modulate the quality characteristics of the final product. The aim of this work was to ascertain whether pasteurisation constituted a sub-lethal heat treatment for *Lb. plantarum* isolates and whether abiotic stresses associated with the microenvironments in Stilton cheese are important in selecting for the presence of different genotypes of the organism in the different sites (outer crust, blue veins and white core) within the ripened product as demonstrated by PFGE clustering (§4.2.3.4.2). *In vitro* methods were employed to determine the survival of genotypically different isolates at different levels of acid, salt and relative humidity.

5.2 Experimental rationale

Different genotypes of *Lb. plantarum* have been observed and discussed in Chapter 4. In preliminary experiments, six isolates (R2 & R4, outer crust; B14 & R25, blue veins; R40 & W30, white core) represented isolates from the three major clusters at (I, III and V) at 52% similarity level of the PFGE genotyping dendrogram (§4.2.3.4.2, Fig. 4.5), and each of the cheese sampled sites. However, as there was little variation, the work presented in this chapter is for stress tolerance response of three isolates, and were evaluated using the approach of Catte *et al.* (1999). Challenge studies involved exposure of the strains to heat treatment, lactic acid, salt and desiccation stresses which comprised the four factors. The three sites of isolation (outer crust, blue veins and white core) constituted the experimental blocks.

As differences in *Lb. plantarum* genotypes have been seen in Stilton cheese whose production involves heat treatment of the milk, the impact of heat on different genotypes of the isolates was examined. The hypothesis was that the strains isolated from the white core could have survived the pasteurisation process and therefore could have higher heat resistance than those found from the outer crust which could have been introduced as contaminants from the environment after pasteurisation. It is known that there are pronounced gradients in pH and water activity (a_W) levels within Stilton cheese (Fernández-Salguero *et al*., 1986) with the outer crust being a high pH/low a_W microenvironment whereas the white core is a low pH/high a_W section. Therefore, it was also hypothesised that *Lb. plantarum* genotypes present in the different cheese sites (outer crust, blue veins and white core) could have been selected to be able to grow in the different microenvironments from which they were isolated.

5.3 Methods

5.3.1 Heat stress tolerance and recovery of injured cells

Thermal tolerance of the isolates (~10^8 CFU/ml) was evaluated at 72^{o}C in cows' milk (§3.4.1), as this is the menstruum that the isolates would be present in during pasteurisation. The thermal kinetics of the heating profile is shown in Appendix 5.1. Samples (0.1 ml) were withdrawn at different time points and immediately transferred into pre-cooled MRD. Each cooled sample was serially diluted in MRD and the dilutions were plated onto BHI agar for enumeration of the viable counts as in §3.5.1. Logarithmic counts of the data ($\log_{10}$ CFU/ml) were plotted against time (s) and the D_{72} values for each strain calculated from the linear portion of the curves using the linear regression method as described by Ahmed *et al*. (1995). The straight lines with correlation coefficients (r^2)>0.90 were regarded as good estimates for estimating the D_{72} values (Jordan and Cogan, 1999). For *Lb. plantarum* isolates obtained from the blue veins (B14) and white core (W30), four values in the straight portion between 10-70 s were used for linear regression analysis. For the isolate obtained from the outer crust (R4), four values in the linear portion between 0-50 s were used as the latter could not be detected after 50 s (limit of detection, LOD, 1 $\log_{10}$ CFU/ml). Recovery from heat stress was assessed by enumeration of the viable counts on MRS and BHI agar after keeping the heat-treated milk samples in a cold room for 48 h at 4^{o}C. Milk inoculated with different levels of each of the isolates (starting cell concentration, 10^2, 10^4 and 10^7 $\log_{10}$ CFU/ml) and kept for 48 h at 4^{o}C was used as a control for the heat recovery experiments.

5.3.2 Acid and salt tolerance

Acid tolerance of an MRS broth overnight culture diluted to 5 $\log_{10}$ CFU/ml was determined at 30^{o}C in MRS broth acidified to pH 3, 3.5, 4, 4.5, 5 and 6 (control) using lactic acid (§3.4.2) according to the method of Succi *et al*. (2005). Salt tolerance was examined using the method of Pelinescu *et al*. (2009) under the same conditions by inoculating the strains in MRS broth containing 0 (control), 3.5, 5, 8 and 10% salt as described in §3.4.2. Acid and salt tolerance was determined by enumerating the survivor viable counts from BHI agar as in §3.5.1.

5.3.3 Desiccation tolerance

Desiccation tolerance was determined at 33 and 54% RH by separately washing and suspending the cells of an overnight MRS broth culture (~9 $\log_{10}$ CFU/ml) in MRD and

SDW prior to desiccation at equilibrated relative humidity (ERH) as described in §3.4.3. Desiccation tolerance was determined by enumerating the survivor viable counts from BHI agar as in §3.5.1.

5.4 Results

5.4.1 Thermal inactivation

Stationary phase cells of three representative *Lb. plantarum* isolates were heat-treated for 70 s at 72°C in cows' milk in order to compare the heat tolerance response of the isolates. The inactivation of the isolates is shown in Fig. 5.1. The linear regression plot of the data used for calculation of the D_{72} value is shown in Appendix 5.2.

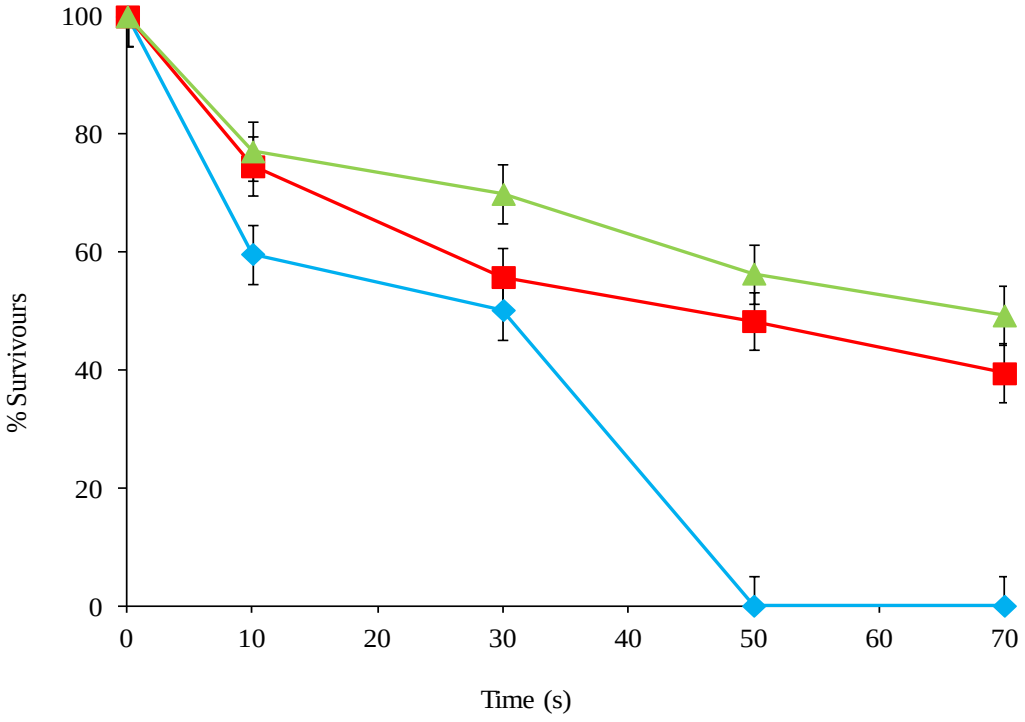


Figure 5.1 Thermal inactivation kinetics of *Lb. plantarum* isolates obtained from Stilton cheese and heated in milk at 72±1°C. Isolates: (▲) W30 white core, (■) B14 blue veins, (♦) R4 outer crust. Counts were taken from BHI agar after incubating anaerobically for 72 h at 30°C. Values are means of six technical replicates obtained from two independent determinations ± standard errors of the means, $p = 0.05$. 100% survival corresponds with 8.21, 8.57 and 8.73 $\log_{10}$ CFU/ml for isolates R4, B14 and W30, respectively.

Incomplete inactivation over 70 s was recorded for isolates B14 (D_{72}, 20.7 s, r^2 = 0.95) and W30 (D_{72}, 23.6 s, r^2 = 0.98; p>0.05) under the time-temperature conditions studied, showing that both strains were more heat tolerant than isolate R4 (D_{72}, 6.9 s, r^2 = 0.92) which only survived for 50 s (p<0.05). The thermal inactivation curves for isolates B14 and W30 were generally concave and showed progressive reduction in percent survival. The isolate from the outer crust showed greater susceptibility to heat rapidly losing 40% viability (0-10 s), followed by relative stability/gradual inactivation (10-30 s) and then rapid cell death to undetectable levels (30-50 s; LOD, 1 log CFU/ml). At 15 s, the reduction in percent survival for isolate R4 (42%) was significantly different from those of B14 (30%) and W30 (25%; p<0.05). However, all isolates could be detected (4.7 to 6.6 $\log_{10}$ CFU/ml) at 15 s, showing they could survive the minimum recommended pasteurisation conditions for milk. These results showed that different genotypes of *Lb. plantarum* present different heat tolerances which may explain why these three isolates were part of lactobacilli as the dominant microflora of Stilton cheese as observed in Chapter 4. *Lactobacillus* isolates in the heat-treated samples were subsequently examined for recovery from the heat injury (Section 5.4.1.1).

5.4.1.1 Recovery of heat-injured cells

After heat treatment for 70 s at 72^{o}C, viable counts of isolate R4 were undetectable (LOD, 1 $\log_{10}$ CFU/ml) whereas isolates B14 and W30 could only grow on the non-selective BHI agar due to heat injury. When the sample preparations were incubated for up to 48 h at 4^{o}C, viable counts were detected on BHI and MRS agar showing recovery from the heat injury. On BHI agar, a significant recovery correlating with increase in viable counts (p<0.05) was mostly observed for *Lactobacillus* isolates R4 and W30 obtained from the outer crust and white core, respectively (Fig. 5.2, p<0.05). At 48 h on this medium, the viable counts of isolates R4, B14 and W30 increased from <1, 3.4 and 4.3 $\log_{10}$ CFU/ml to 2.7, 3.6 and 5.1 $\log_{10}$ CFU/ml, respectively. In the case of isolates R4 and W30, the increase in cell number was due to recovery and not growth as all isolates were shown to give no significant increase in cell numbers over 48 h at 4^{o}C (p>0.05) when incubated in milk as the medium (Appendix 5.3).

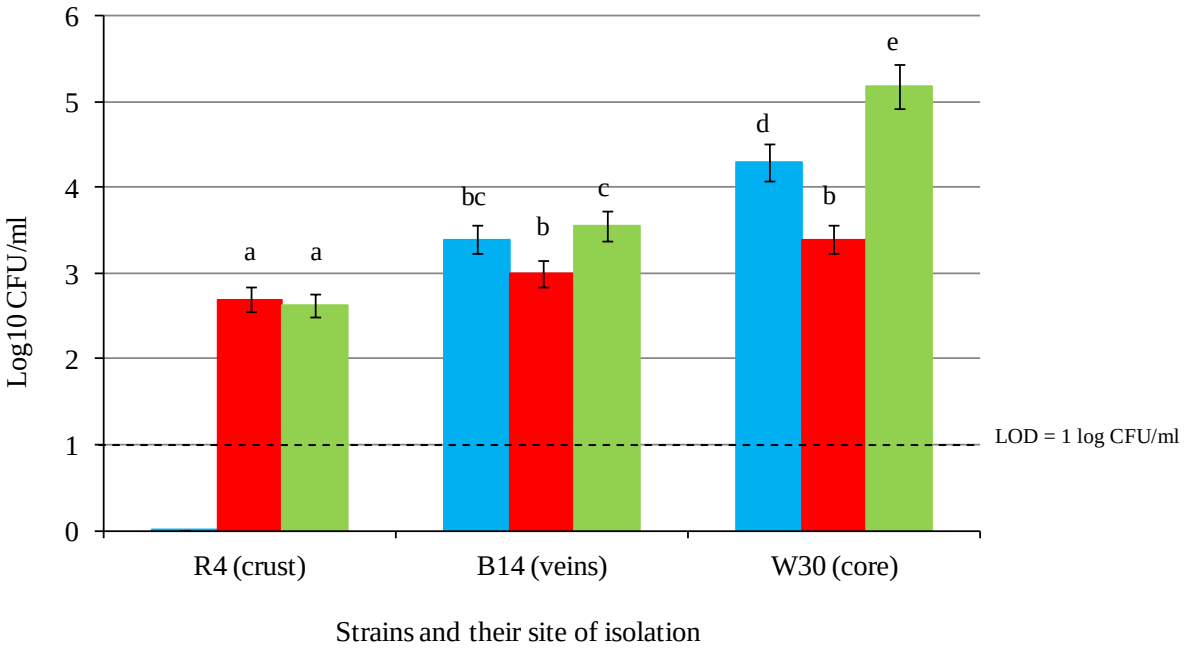


Figure 5.2 Recovery of stationary phase cells of *Lb. plantarum* (initial inoculum, ~9 $\log_{10}$ CFU/ml) heated for 70 s at 72°C and incubated in sterile milk at 4°C for: (■) 0, (■) 24, and (■) 48 h. Counts were taken from BHI agar after incubating anaerobically for 72 h at 30°C. Values are means of six technical replicates obtained from two independent determinations ± standard errors of the means. Bars with different letters are significantly different (p<0.05).

5.4.2 Acid tolerance

Acid tolerance of three *Lb. plantarum* isolates was examined at 30°C in MRS broth acidified to pH 3, 3.5, 4, 4.5, 5 and 6 (control) according to the method of Succi *et al.* (2005) as described in Section 3.4.2. Fig. 5.3 shows the results obtained when three different genotypes of *Lb. plantarum* (R2, outer crust; R25, blue veins; and R40, white core) were examined for growth between pH 3 and 6 in MRS broth acidified with lactic acid. Viable counts for all isolates were undetectable after 2 h at the highest acid treatment (pH 3) (data not shown). The isolates had different sensitivities to acid at pH 3.5 leading to gradual cell death (Fig. 5.3). At 48 h, isolate R2 obtained from the outer crust (Fig. 5.3A) was found to be most sensitive (4.4 $\log_{10}$ CFU/ml reduction), whereas R25 from the blue veins (Fig. 5.3B; 2.9 $\log_{10}$ CFU/ml reduction) and R40 from the white core (Fig. 5.3C; 2.3 $\log_{10}$ CFU/ml reduction) were the most acid-tolerant isolates (p<0.05).

For all isolates, the cells exposed to pH 4 were unable to grow in the medium and remained constant or showed a slight reduction in viable counts (Fig. 5.3A-C). In the pH range 4.5-5, growth of all *Lb. plantarum* isolates was evident and there was no significant difference to growth relative to pH 6 (control) (p>0.05).

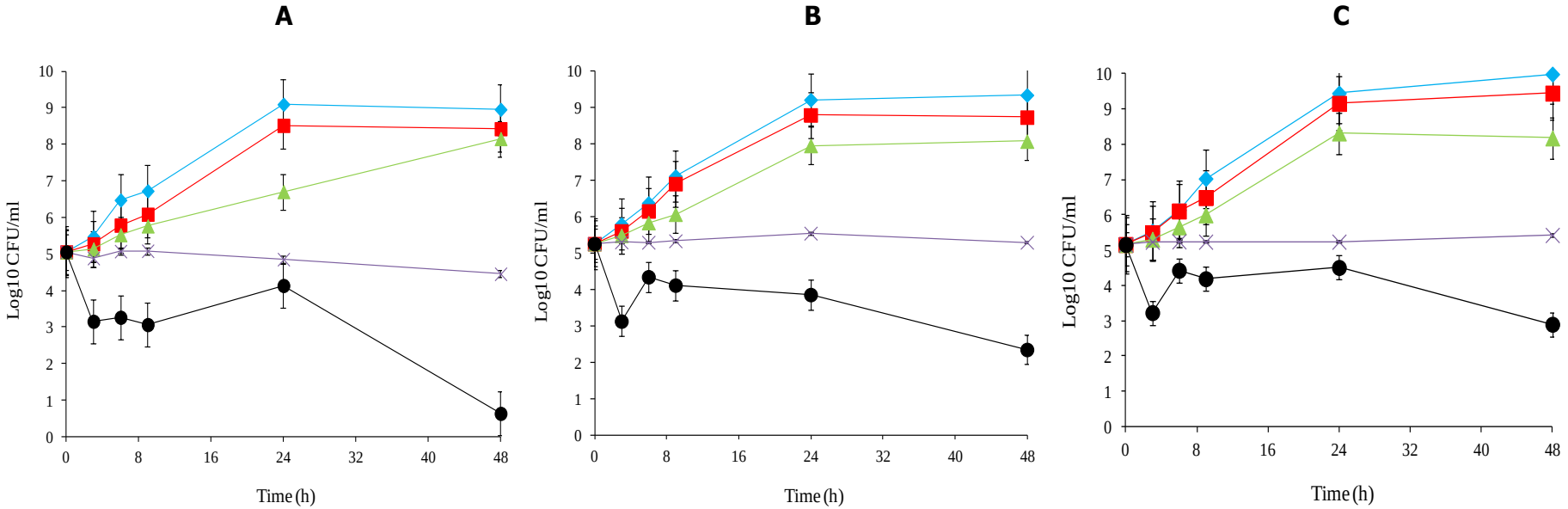


Figure 5.3 Acid tolerance of *Lb. plantarum* isolates obtained from Stilton cheese: (**A**) R2, outer crust; (**B**) R25, blue veins; and (**C**) R40 white core, after incubation at 30°C in MRS broth at pH: (♦) 6, control; (■) 5; (▲) 4.5; (X) 4; and (●) 3.5. Counts were taken from BHI agar after incubating anaerobically for 48 h at 30°C. Values are means of nine technical replicates obtained from three independent determinations ± standard errors of the means, $p = 0.05$.

5.4.3 Salt tolerance response

Salt tolerance of the different genotypes of *Lb. plantarum* (R2, outer crust; B14, blue veins; and W30, white core) was examined at 30^{o}C in MRS broth containing 3.5, 5, 8 and 10% (w/v) sodium chloride according to a method of Pelinescu *et al*. (2009) as detailed in Section 3.4.2. Overall at 48 h, all the tested isolates demonstrated the ability to grow over the salt range 3.5-5% (Fig. 5.4) and showed no significant difference with the unsalted control (p>0.05) indicating their high halotolerance. Exposure to the highest salt concentration (10%) resulted in significant growth suppression (p<0.05) leading to 0.2, 0.9 and 1.1 $\log_{10}$ CFU/ml reductions for isolates R2 (outer crust), W30 (white core) and B14 (blue veins), respectively by 48 h. At the end of this period at 8% salt, the *Lb. plantarum* isolates obtained from the blue veins (Fig. 5.4B) and white core (Fig. 5.4C) had higher sensitivity to salt showing only 0.3 and 0.3 $\log_{10}$ CFU/ml increases to attain 5.7 and 5.9 $\log_{10}$ CFU/ml respectively (p>0.05), compared with the isolate obtained from the outer crust (Fig. 5.4A) which showed a 1.2 $\log_{10}$ CFU/ml increase to a final level of 6.5 $\log_{10}$ CFU/ml. This, therefore shows, as anticipated, that lactobacilli from the outer crust had higher salt tolerance probably due to ecological selection which enabled these isolates to colonise this part of the cheese microecosystem which has a lower a_W level than the cheese interior, as will be discussed later in Section 5.5.3.

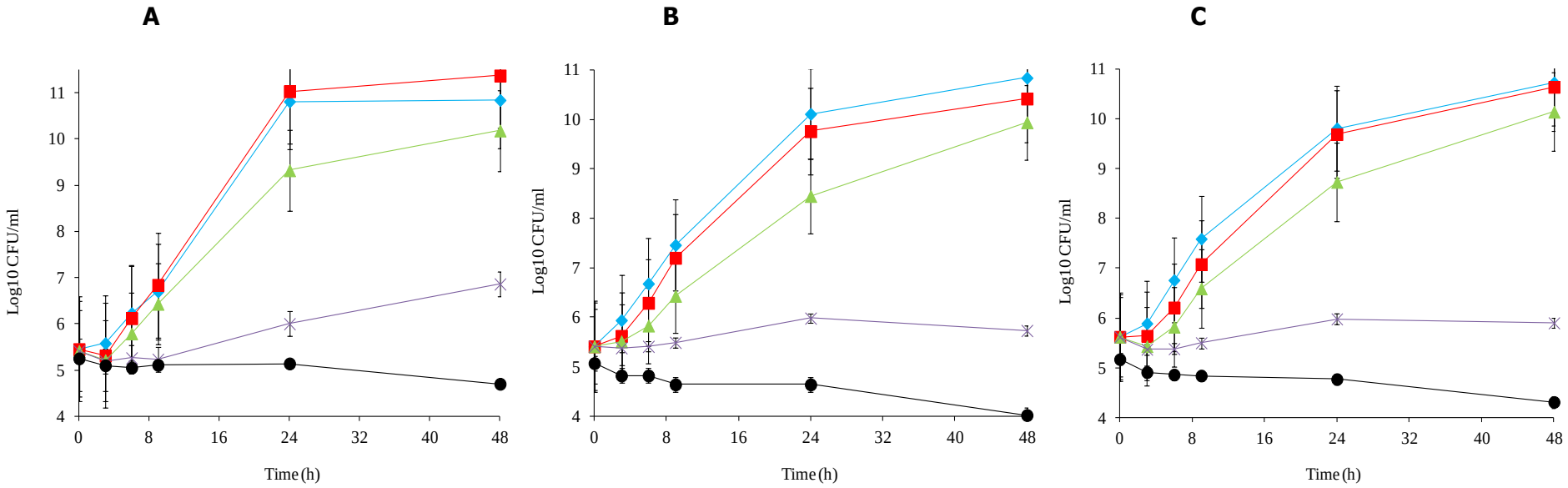


Figure 5.4 Salt tolerance of *Lb. plantarum* isolates obtained from Stilton cheese. Isolates: (**A**) R2, outer crust; (**B**) B14, blue veins; and (**C**) W30, white core. The assay was conducted at 30°C in MRS broth containing different salt concentrations (%, w/v): (♦) 0, control; (■) 3.5; (▲) 5; (X) 8; and (●) 10. Counts were taken from BHI agar after incubating anaerobically for 48 h at 30°C. Values are means of nine technical replicates obtained from three independent determinations ± standard errors of the means, $p = 0.05$.

5.4.4 Desiccation tolerance

Desiccation tolerance of the isolates was performed by drying cell suspensions made from overnight cultures (~9 $\log_{10}$ CFU/ml) suspended in MRD or SDW onto U-shaped Nunc immuno well plates made of highly uniform, immunochemical grade polystyrene. The choice of this surface was based on its recommended application for experimentation demanding uniform cell binding properties from well to well and plate to plate (Nalge Nunc). Cells on the plate surfaces were then exposed to an equilibrated relative humidity (ERH) of 33 or 54% set up with saturated salt solutions as described in §3.4.3. As most bacteria cease to grow at a_w<0.5 corresponding with ≤50% ERH, desiccation tolerance was determined at ERH above and below this humidity. The cells were suspended in SDW in order to avoid salt stress during desiccation (Pedersen *et al*., 2008). MRD was included in order to highlight the protective role of peptone in microbial cells subjected to drying conditions. This would allow evaluation of nutrients as a potential risk factor for persistence and subsequent colonisation of microorganisms in the cheese production environment which may be the source of cross-contamination for *Lactobacillus* isolates obtained from the outer crust into the final product.

The mean concentration of re-suspended cells prior to drying ranged from 8.9±0.1 to 9.3±0.1 $\log_{10}$ CFU/ml (Table 5.1). The strains had variable sensitivities to initial drying (30^{o}C) prior to holding (20^{o}C) at the different ERH levels, and this depended on the medium in which the cells were suspended (Table 5.1). Suspending the cells in MRD caused slight reduction (0.4-0.6 $\log_{10}$ CFU/ml) in viable counts whereas in SDW, higher reductions (0.7-1.1 $\log_{10}$ CFU/ml) were observed (p<0.05). In both suspension media, isolate W30 obtained from the white core was the most tolerant to the initial drying step (4.59-7.15% decrease) compared with isolate B14 from the blue veins (6.27-10.53% reduction) and R2 from the outer crust (6.16-12.22% reduction) (p<0.05).

Table 5.1 Changes in viable counts of different genotypes of *Lb. plantarum* obtained from Stilton cheese after suspending in different media and drying for 24 h at 30°C

Strain	Overnight culture (log_{10} CFU/ml)	After drying (log_{10} CFU/ml)		Reduction (log_{10} CFU/ml)		Percent reduction (%)	
		MRD	SDW	MRD	SDW	MRD	SDW
R2, crust	9.3	8.68	8.12	0.57	1.13	6.16[a]	12.22[a]
B14, veins	8.9	8.37	7.99	0.56	0.94	6.27[a]	10.53[a]
W30, core	9.4	8.94	8.7	0.43	0.67	4.59[b]	7.15[b]

Values in the column with different superscripts are significantly different (p<0.05).

During exposure to a specified humidity, the sensitivity of isolates varied with the RH applied and the medium in which the cells were dried (Fig. 5.5). All strains were sensitive to 33% RH in SDW and became undetectable (LOD, 1 log_{10} CFU/ml) by 5 days of exposure (Fig. 5.5). However, all the isolates survived desiccation at the same RH (33%) in MRD (Fig. 5.5) over 7 days suggesting cellular protection by MRD. Isolates R2 and W30 showed a 3 log_{10} CFU/ml reduction over this period whereas B14 showed a 1 log_{10} CFU/ml lower survival. Survival was more variable at 54% RH; in MRD, all isolates were sensitive to desiccation and died off by 7 days (Fig. 5.5A-C) with the greatest inactivation occurring between 5 and 7 days. In SDW, the *Lb. plantarum* R2 from the outer crust (Fig. 5.5A) was more sensitive and survived for only 5 days. Isolate B14 obtained from the blue veins (Fig. 5.5B) showed a low level of survival (0.6 log_{10} CFU/ml) by 7 days and W30 from the white core (Fig. 5.5C) showed better desiccation tolerance with 4.2 log_{10} CFU/ml survival up to 7 days. Taking into account results of the entire experiment, survival was better at 33% RH in MRD than all other treatments (p<0.05). It was, therefore, evident that survival of *Lb. plantarum* was dependent both on RH and the drying medium and was also strain dependent.

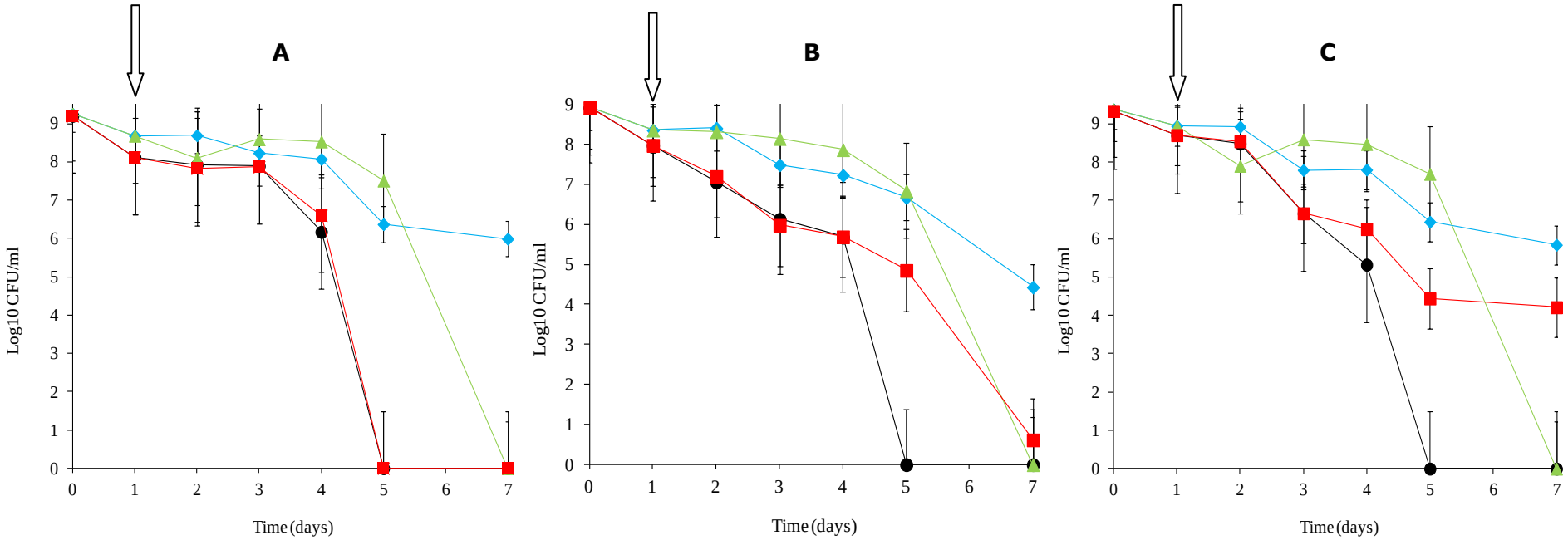


Figure 5.5 Desiccation tolerance of *Lb. plantarum* isolates obtained from Stilton cheese: (**A**) R2, outer crust; (**B**) B14, blue veins; and (**C**) W30, white core. The assay was performed at 20°C in presence of: (♦) 33% RH in MRD; (▲) 54% RH in MRD; (■) 54% RH in SDW; and (●) 33% RH in SDW. Counts were taken from BHI agar after incubating anaerobically for 48 h at 30°C. Values are means of nine technical replicates obtained from three independent determinations ± standard errors of the means, $p = 0.05$. Arrows represent the point at which cells were subjected to equilibrated relative humidity.

5.5 Discussion

The aim of this study was to determine whether stress conditions associated with microenvironments in the different sites (outer crust, blue veins and white core) of Stilton cheese are important in selecting for the presence of different genotypes of *Lb. plantarum* resulting in different sub-populations within the ripened product. The results obtained from Chapter 4 showed that there were genotypically different isolates of *Lb. plantarum* spatially distributed in different sites within the cheese, which could be because the different strains of the organism had been selected during the ripening process or could be due to different routes of contamination.

5.5.1 Thermal tolerance

The use of survivor curves for the study of bacterial resistance to heat stress is a common practice. Jordan and Cogan (1999) and De Angelis *et al*. (2004) applied the logistic/log linear method involving equations of best fit (r^2>0.9) to interpret the survival data of various *Lactobacillus* spp. subjected to heat stress. The major disadvantage of this approach is that it does not account for tailing of the survival curves which occurred after ~10 s in this study. Tailing is normally attributed to cellular clumping during heat treatment (Jordan and Cogan, 1999); and this tends to underestimate microbial heat resistance. McCann *et al*. (2006) proposed that tailing is normally associated with the production of heat shock proteins in a sub-population, which protect cells from denaturation at high temperatures. Microscopic examination of the heat treated samples to reveal this phenomenon is a recommended practice to account for tailing of thermal death curves.

Given the non-linearity of heat inactivation curves, *D*-values are usually derived from the linear portions and used for comparison of microbial heat resistance (Nguyen *et al*., 2010). From this study, the lowest D_{72} value (6.9 s) was obtained from strain R4 obtained from the outer crust. Therefore, following pasteurisation at 72^{o}C for 15 s, there would be ~2 log reduction in cell numbers. Given an initial count of ~3 $\log_{10}$ CFU/ml usually present in cheese milk (Jordan and Cogan, 1999; De Angelis *et al*., 2004), the rate of survival could be greater than 1 CFU/ml of milk, which would be sufficient to result in survival and subsequent growth of the isolate in the cheese. The calculated D_{72} values for isolates B14 (blue veins) and W30 (white core) were 20.7 and 23.6 s, respectively. This implies that there would be a less than 1 $\log_{10}$ reduction in cell numbers for these isolates

during pasteurisation. It should be noted, however, that whereas survival over a given period is dependent on initial microbial levels, *D*-values are not absolute as the values obtained depend on factors such as heating menstruum, pH and a_w, salt content and preservatives (De Angelis *et al*., 2004; Conesa *et al*., 2009).

Pasteurised milk is usually the major source of the heat resistant (thermoduric) NSLAB associated with mature cheeses (Litopoulou-Tzanetaki *et al*., 1989). Strains from the outer crust were more heat sensitive suggesting they may have entered into the cheese post pasteurisation, which is also consistent with their location in the cheese. A study by Jordan and Cogan (1999) on *Lactobacillus* spp. associated with mature Cheddar cheese indicated that the heat resistance of different strains of MRS broth grown cultures of *Lb. plantarum* (8 $\log_{10}$ CFU/ml) at 50-57.5^{o}C was high; with the z-values ranging between 6.2-6.7^{o}C. Milk-grown cultures of the organism showed greater heat resistance than broth grown cultures. A similar observation was reported by De Angelis *et al*. (2004). These researchers observed that stationary phase cells of the organism (7 $\log_{10}$ CFU/ml) had *D* values in sterile milk of 32.9, 14.7, and 7.1 s at 60, 72, and 75^{o}C, respectively. Mid exponential phase cells had lower *D* values than stationary phase cells. This is generally compatible with the data from this study. Therefore, differences in heat tolerances amongst the different strains of *Lb. plantarum* could be innate as this has been found to vary not only with species (Jordan and Cogan, 1999) but also with different strains of the same species (De Angelis *et al*., 2004). This was the case for the results from the current study whereby heat tolerance of the isolates corresponded with the sites from which they were isolated. Isolates B14 and W30 obtained from the blue veins and white core respectively, demonstrated higher heat tolerance implying they could have survived the milk pasteurisation process and adapted to acidification (fermentation) as evident from the results presented in Section 5.4.2, and those to be presented later in Section 7.3.2.

Most cheese production plants employ the energy efficient high temperature short time (HTST) milk pasteurisation process. The system involves thermal regeneration whereby the outgoing hot pasteurised milk is used to pre-warm the incoming cold raw milk before it is fed into the heat exchanger for subsequent heat treatment at 72^{o}C. In this situation, the resident microflora in raw milk is pre-exposed to sub-lethal temperatures which may enhance their survival at subsequently higher temperature treatments. Heat adapted (42^{o}C, 1 h) mid-exponential and stationary phase cells of *Lb. plantarum* exhibit increased

thermal resistance at 72^{o}C (De Angelis *et al*., 2004) due to expression of heat shock genes (Tao *et al*., 2006; Liao *et al*., 2010; Li *et al*., 2011) after induction of the class three stress gene repressor (*CtsR*) regulon. Russo *et al*. (2012) performed a global proteomic analysis of unstressed *Lb. plantarum* WCFS1 and Δ*ctsR* mutant strains grown at optimal temperature (30^{o}C) and those which were grown at 30^{o}C and subsequently heat stressed at 42^{o}C for 30 min. These authors reported a moderate increase (1.25-5.7-fold upon temperature up-shift) of the general stress-induced chaperones GroES, GroEL and DnaK with greater induction in the *ΔctsR* mutant than in the wild-type strain. Levels of the heat shock peptides *Hsp1* and *Hsp3* also drastically increased when the bacteria were submitted to heat stress at 42^{o}C.

In the current study, the *Lb. plantarum* isolates were grown overnight at 30^{o}C and then exposed to heat treatment at 72^{o}C. Under normal growth conditions at 30^{o}C, *Lb. plantarum* expresses a basal level of small heat shock genes such as *hsp*18.5 and *hsp*19.3 (Spano *et al*., 2004) suggesting a housekeeping function for the relative proteins. It is, therefore, possible that *Lb. plantarum* isolates used in this study had developed some level of heat tolerance during the growth phase. Takemoto *et al*. (1993) reported that many major heat shock genes are efficiently expressed in the absence of stresses and such stress proteins are often essential for cell growth. However, expression of the genes increases when the stresses are imposed. Further research is needed to examine the response of the isolates to heat stress after heat shock induction at a higher temperature (42^{o}C). Techniques such as microarray or RNA sequence analysis (Wall *et al*., 2007; Lebeer *et al*., 2008; Li *et al*., 2011) have proven useful to establish gene expression of the *Lb. plantarum* isolates under the conditions applied in the current study.

Thermal tolerance in *Lb. plantarum* may be linked with other stress resistance characteristics which enhance the survival of the organism. For example, the strains used in the experiment were initially grown in MRS broth and caused significant acidification of the medium from pH 6.2 to 3.88-4.67. It could be possible that the organism had developed acid tolerance which could have induced specific groups of genes or regulons to cope with the subsequent heat stress in the challenge experiment as response to these stresses was reported to be linked in most Gram-positive bacteria including lactobacilli (Tao *et al*., 2006). It is well recognised that the heating menstruum affects the thermotolerance for *Lactobacillus* spp. (Jordan and Cogan, 1999). Cows' milk contains a

substantial amount of fat (3.5%) which offers cellular insulation to extreme temperatures due to its low thermal conductivity (Robinson, 1990) and, may have therefore protected the cells against heat stress in the current study.

Microorganisms subjected to sub-lethal stresses undergo metabolic injury, often manifested as the inability to form colonies on selective media on which uninjured cells can survive and grow (Hurst, 1977). The differential in counts between selective and non-selective media is used to determine the degree to which a microbial population is sub-lethally injured. For all the isolates examined in this study, part of the cell population heat treated at 72^{o}C for 70 s recovered from the heat injury during incubation in sterile milk at 4^{o}C for 48 h as manifested by an increased ability to grow on the non-selective BHI agar and to grow on the selective MRS agar when plated after storage. Overall, the isolate obtained from the outer crust showed better recovery than the isolates from the blue veins and white core. Kang and Siragusa (1999) reported similar results for heat stressed (60^{o}C, 90 s) *E. coli* O157:H7 and *Salmonella* Typhimurium (~6 $\log_{10}$ CFU/ml). Both organisms had low recoveries on the selective sorbitol MacConkey and xylose lysine decarboxylase agar respectively; whereas significant viability was observed on the non-selective tryptic soy agar showing that injured cells die on selective agar but grow on the non-selective medium. This study showed that injured cells of *Lb. plantarum* isolates recovered in milk to the level that they can grow on selective media. There are few previous studies available in which thermal tolerance of *Lb. plantarum* has been determined. There is need to determine the physiological mechanisms of response to heat stress for *Lb. plantarum* isolates. Further research in this field would help to provide insights regarding the presence and dominance of these organisms in ripened cheese.

5.5.2 Acid tolerance

It was noticeable from this study that isolates R25 and W30 from the interior of Stilton cheese were more acid tolerant at pH 3.5 than R2 obtained from the outer crust further highlighting the differential selection of the organism in the different ecological niches within the cheese matrix. A similar study was performed by Succi *et al*. (2005) on *Lactobacillus rhamnosus* isolated from Parmigiano Reggiano cheese produced in Italy. Their results indicated that at pH 3, injury due to an acidic environment caused a 2-3 $\log_{10}$ CFU/ml reduction after 2-4 h of incubation whereas at pH 2, a 6-8 $\log_{10}$ CFU/ml was evidenced. Increasing the pH to 7 with sodium bicarbonate did not present an adverse

environment for all the tested strains, showing good survival at high pH. This was congruent with the present study. However, in presence of higher acid levels (pH 3.5) in this study, visible colonies could only be observed when the agar plates were incubated longer (96 h) at 30^{o}C suggesting recovery of the injured cells. All the *Lb. plantarum* isolates exhibited good growth at pH 4.5-6. Given that the pH of the outer crust of Stilton cheese is 5-6 due to mould activity (Gkatzionis *et al*., 2009) compared to that of the white core (4.5-5); suggests that different genotypes of *Lb. plantarum* as observed in Chapter 4 had been selected in the different cheese sites due to acid tolerance differences. Overall, the results highlighted the high acid tolerance of the *Lactobacillus* isolates and suggest their potential to contribute to secondary metabolism during cheese ripening.

The un-dissociated form of lactic acid diffuses across the cell membrane towards the more alkaline cytosol where it dissociates to release hydrogen ions (H^+) that reduce intracellular pH (pHi) (Pan *et al*., 2009). Acid-sensitive cells would fail to buffer hydrogen ions (H^+) from the cell, which leads to a larger intracellular pH gradient (ΔpHi) and consequently cell death as their buffering system becomes depleted (Pieterse *et al*., 2005; Ahmed *et al*., 2009). *Lb. plantarum* tolerates the harmful effect of lactic acid by maintaining a lower ΔpHi (Ahmed *et al*., 2009). However, at high concentration of the acid, there is accumulation of the organic anion (lactate), which leads to end product inhibition, reduced a_W, and failure to regenerate the co-factor NAD, all of which accelerate cell death (Pieterse *et al*., 2005). These factors may partly explain the failure of *Lb. plantarum* isolates to survive the treatment at pH 3-3.5 applied in the current study. Molecular tools should be employed to establish the target genes that show increased expression towards lactic acid for purposes of identifying the most acid resistant isolates.

5.5.3 Salt tolerance

In the present work, *Lb. plantarum* isolates showed optimal growth at 3.5-5% salt and were only sensitive at higher (8-10%) concentrations. The observations concurred with another study whereby growth of the organism in MRS broth was shown to correlate with various salt concentrations (Pelinescu *et al*., 2009). Similar to the findings from this study, these researchers observed that growth of *Lb. plantarum* was optimal at low (0.5-5%) salt levels but higher concentrations (10-12%) were inhibitory for its growth.

Isolate R2 from the outer crust was more halotolerant at 8-10% salt than each of the isolates B14 and W30 from the blue veins and white core respectively, further highlighting differential ecological selection of the organism. Lactobacilli with the ability to grow in presence of more than 6.5% salt usually result from ecological selection during the cheese making process (Sawitzki *et al*., 2009). This is because the salt sensitive bacteria present at the beginning of the process tend to stop growing when the concentration becomes too high by the end of ripening (Sawitzki *et al*., 2009). In Stilton cheese, the initial salt concentration (2.1-3.5%; SCMA, 2009) in the dried curd is relatively low but by the end of ripening, the final concentration may increase up to 4.5-6% (Sawitzki *et al*., 2009), with higher levels (>6%) occurring in the outer crust due to excessive moisture loss resulting from air drying. Salt stress leads to loss of turgor pressure, which affects the cell physiology, enzyme activity, water activity and metabolism. Osmotolerant bacteria overcome this effect by maintaining low ΔOPi through increased production of osmolytes such as glycine betaine (Ibourahema *et al*., 2008). *Lb. plantarum* from the outer crust may have been similarly protected by this mechanism to be able to grow at 8% salt, albeit at a slower rate. There is scant information on quantitative differences in salt levels of the different sections (white core, blue veins and outer crust) of Stilton cheese and how these would change during ripening. Therefore, the data presented in this study may show some variation from the real growth kinetics of the *isolates in situ* in Stilton cheese, depending on the actual salt content of these cheese sites from which they were isolated.

5.5.4 Desiccation tolerance

Microorganisms survive prolonged drought in a desiccated state, where greater than 99% of the cell water has been lost (Chen and Alexander, 1973; Welsh, 2000; Alpert, 2005). Upon subsequent rehydration, the organisms rapidly swell and resume active metabolism, often within a few minutes. Whilst the underlying mechanisms of microbial desiccation tolerance are not fully understood, in yeast, the phenomenon has been shown to correlate with the accumulation of the non-reducing disaccharides, trehalose and to a lesser extent sucrose, and is independent of the growth phase (Welsh, 2000). The compatible solutes (CS) ectoine and hydroxyectoine as well as the induction of trehalose synthesis by osmotic shock promote increased desiccation tolerance in some strains of *E. coli* (Welsh, 2000; Vriezen *et al*., 2007).

Robert *et al*. (2000) reported that glycine betaine (an amino acid derivative) is the major effective osmoprotectant in *Lb. casei*, *Lc. lactis* and *Lb. plantarum*. However, these organisms do not synthesise the compound *de novo* but preferentially accumulate it from MRS broth during growth (Ibourahema *et al*., 2008) as the medium contains yeast and beef extracts which contain significant amounts of this CS as well as choline, its precursor. As glycine betaine is known to offer increased desiccation tolerance in some LAB (Santivarangkna *et al*., 2008), it could be possible that the *Lb. plantarum* isolates examined in this study could have accumulated the substance from MRS broth during growth prior to the experiment resulting in the varying degrees of desiccation tolerance observed. At the lowest tested RH (33%), drying in MRD provided further protection from desiccation stress (compared with SDW which led to cell death by 5 days of exposure), whereby isolates R2 and W30 from the outer crust and white core respectively, showed better survival than B14 from the blue veins. At a higher RH level (54%) however, survival was more variable whereby in MRD, all isolates lost viability by 7 days whereas in SDW, isolate R2 was more sensitive than B14 and W30. These results suggest that desiccation tolerance of the *Lb. plantarum* isolates could be an innate trait varying with the genotypic characteristics of the isolates obtained from different sites (outer crust, blue veins and white core), the medium in which the cells were suspended and dried, as well as the RH level applied. These results corresponded with the salt tolerance data (Section 5.4.3) showing, as expected, that the *Lactobacillus* isolate from the outer crust which had been shown to be the most halotolerant at 8% salt (Fig. 5.4) was also found to be the most desiccation tolerant at 33% RH in MRD, probably due to ecological selection.

The increased survival of cells suspended in MRD seen in some instances may have been due to the contribution of the medium to the cellular CS content which plays a vital role in protection of cells and intracellular macromolecules during desiccation (Santivarangkna *et al*., 2008). CS comprise low molecular weight organic solutes which do not interfere with cell functions but accumulate to high intracellular concentrations to balance the osmotic pressure of their surroundings and maintain cell turgor pressure enabling viability (Santivarangkna *et al*., 2008). CS also act as intracellular reserves of carbon, energy and nitrogen, and as more general stress metabolites involved in protection of cells against other environmental stresses including heat and freezing (Welsh, 2000). The high susceptibility of the *Lb. plantarum* isolates to desiccation at 33% in SDW may probably be due to increased plasmolysis following this treatment

(Iacobellist and Devay, 1986), whereas at 54% the effect was less pronounced which enhanced the survival (beyond 7 days) of isolate W30 from the white core.

It was evident from the current investigation that both MRD and drying at 33% RH were more protective to *Lb. plantarum* isolates than other treatments, whereby isolates R2 and W30 from the outer crust and white core respectively, had better survival than B14 from the blue veins. There is scant literature about the effect of MRD on microbial desiccation tolerance. However, Mary *et al*. (1985) reported some work on different strains of *Rhizobium* grown overnight at 30^{o}C. The cells were washed and suspended in a buffer (1 g of K_2HPO_4 and 0.2 g of $MgSO_4$ per litre of distilled water), and then subjected to desiccation at different RH following rapid (heated air), and slow drying as performed in this study. These researchers noted that after slow drying, viable numbers of all test strains decreased markedly throughout the first stages of storage (0-2 days) at 0, 22, and 43% RH. Thereafter, the number of cells declined at a slower rate up to the end of the experiment (14 days). They observed that under conditions of slow drying, most strains of the organism could withstand 22 and 43% RH better than 0 and 83% RH, which is comparable with the data from this study. The authors reported that harmful effects of rapid drying on survival of *Rhizobium* during storage at 31% RH were evident and increasing the RH from 30 to 92% adversely affected the organism. Further research on the behaviour of *Lb. plantarum* isolates rapidly dried and stored at RH greater than 54% is necessary to support the hypothesis that the organism would show increased susceptibility to desiccation.

Little is known about the possibility of rapid cell death following rehydration after desiccation. However, it was suggested that changes in membrane permeability, quantities of water retained at a known relative vapour pressure, dysfunction of intracellular enzymes, as well as rupture of the cell envelope when the internal pressure (due to water uptake) overcomes its weakened resistance may account for the low survivor counts obtained when desiccated cells are subsequently rehydrated (Mary *et al*., 1985; Vriezen *et al*., 2007).

Enhanced survival of some of the isolates dried in MRD may be partly attributed to intracellular accumulation of trehalose (Morgan *et al*., 2006; Fukuda *et al*., 2010). *E. coli* cells treated with exogenous trehalose or when osmotically induced to accumulate the

disaccharide, retained 2-6% of the initial culture viability following 50 days of storage in an evacuated desiccator, whereas for cells treated with glycine betaine or intracellularly induced to accumulate the substance, no viable cells were present after 7-20 days under the same conditions (Welsh, 2000; Santivarangkna *et al*., 2008). Similar observations were made in *Lactobacillus salivarius* subsp. *salivarius* frozen for 1 h at -85^{o}C and then subjected to freeze drying for 48 h. After these treatments, trehalose alone or in combination with sucrose enhanced survival of the organism from 13 to 85% and its subsequent stability during storage (50 days at -85^{o}C) compared with absence of these protective solutes (4% survival; Zayed and Roos, 2004).

Although trehalose is not protective to *Lb. plantarum* cells air dried at very low RH (such as 3%) compared with maltose and sorbitol which improve residual metabolic activity of the organism by 66 and 79% respectively, due to antioxidant properties (Linders *et al*., 1997), the substance is highly protective at higher RH (Welsh, 2000). The substance prevents inter-membrane fusion of the cell membrane, thereby maintaining cellular integrity upon subsequent rehydration (Morgan *et al*., 2006). Maltose protects cells from excessive loss of residual water (to ~2.8-5.6%) which is required for maintenance of metabolic activity (Zayed and Roos, 2004; Meng *et al*., 2008). Cells dried without sugars usually suffer fusion and complete loss of calcium transport activity upon rehydration. *Lb. plantarum* is among the 30 species of lactobacilli known to produce EPS including homopolymers, heteropolymers of neutral sugars (pentoses and hexoses) and anionic sugars (hexoses) (Badel *et al*., 2011). EPS has been shown to protect cells against physical stresses such as desiccation and osmotic stress (Fukuda *et al*., 2010; Badel *et al*., 2011).

The isolates used in the study were grown in MRS broth prior to the assay and caused acidification of the medium. Lowering pH during culturing of *Lactobacillus reuteri* induced greater protection to the cells during freeze drying, producing more than 90% cell recovery compared to 65% when the cells were grown at pH 6 (Morgan *et al*., 2006). Reducing pH of the growth medium produces changes in the bacterial membrane fatty acid composition and induces the production of acid shock proteins which also play a vital role in cellular protection during desiccation (Maus and Ingham, 2003). Microorganisms pre-exposed to low a_W due to salt in the medium (osmotic stress), usually show increased survival during desiccation (Chen and Alexander, 1973; Vriezen *et al*.,

2007). In Stilton cheese, the outer crust has lower a_W than the interior of the cheese, which may partly explain the higher desiccation tolerance of isolate obtained from this cheese section at 33% RH in MRD.

5.6 Conclusions

Lb. plantarum isolates with different genotypic characteristics have been found to have different heat tolerances and recovery patterns as well as tolerances to acid, salt and desiccation stresses. This shows that the isolates could have entered into the cheese through different routes or strains with distinct genotypic properties could have been selected to grow in the various microenvironments within the ripened product. The data have supported the hypothesis that lactobacilli in Stilton cheese are able to survive the standard milk heat treatment process, adapt to acid during the initial stages of fermentation with *Lc. lactis*, and to the physical-chemical changes which occur during the ripening process.

The different isolates showed optimal adaptation to the various stresses typical of Stilton cheese. For a strain to be used as a culture adjunct, as well as survival in the cheese, another important consideration would be its impact on the other microflora and in particular the *Lc. lactis* starter culture. In the next chapter, the antagonistic properties of the different isolates against the starter culture *Lc. lactis*, as well as some pathogenic and spoilage bacteria commonly associated with cheese are presented.

CHAPTER 6

ANTIMICROBIAL ACTIVITY OF *LACTOBACILLUS* ISOLATES

6.1 Introduction

Within the LAB group, the genus *Lactobacillus* displays the broadest antimicrobial spectrum due to a complex antagonistic system involving production of metabolites such as ethanol, organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetoin, acetaldehyde and bacteriocins in varying quantities (Amin *et al*., 2009; Essid *et al*., 2009). The ability of lactobacilli to produce these antimicrobial substances provides resistance against colonisation by spoilage and pathogenic microorganisms in food systems (Amin *et al*., 2009). The level and spectrum of activity of the antimicrobial agents (Table 6.1) is largely dependent on the pH, temperature, medium composition (Maldonado *et al*., 2003), as well as on LAB species, strain and growth conditions (Raccach *et al*., 1989; Ammor *et al*., 2006), some of which have been examined in this work. Antimicrobial activity of several species of the genus *Lactobacillus*, including *Lb. casei*, *Lb. rhamnosus*, *Lb. acidophilus*, *Lb. reuteri* and *Lb. fermentum*, has been previously studied and some of these organisms are used as commercial culture adjuncts in fermented milk (Amin *et al*., 2009). However, although *Lb. plantarum* was isolated as a major part of the Stilton cheese microflora, scant information is available on the role these play in influencing flora development or possible commercial applications of this species in Stilton and other blue cheeses.

Table 6.1 Mechanisms of antagonistic activity of some metabolites produced by LAB

Metabolic product	Mode of antagonistic activity
Carbon dioxide	Reduces cell membrane permeability though inhibition of decarboxylation enzymes
Diacetyl	Interacts with arginine binding proteins to inhibit the growth of Gram-negative bacteria
Hydrogen peroxide	Denatures enzymes and causes peroxidation of membrane lipids leading to loss of cell constituents due to high membrane permeability
Lactic acid	Un-dissociated lactic acid penetrates the membranes and lowers intracellular pH. Also interferes with cellular substrate oxidative phosphorylation
Bacteriocins	Damage cell membranes through pore formation in the phospholipid bilayer. Also inhibit DNA and protein synthesis

Source: Ammor *et al*. (2006)

Cheese, like other dairy products, has the potential to support the growth of unwanted microorganisms introduced from pasteurised milk (thermodurics), processing equipment, personnel or from the environment (Obadina *et al*., 2006). From the present work, the microflora of Stilton cheese appears to be dominated by *Lb. plantarum* (Section 4.2.3.4) which is acid and salt tolerant (Sections 5.3.2-5.3.3), and could therefore survive processing stages if it was applied as a starter culture adjunct. The ability of lactobacilli to inhibit growth of other bacteria through the production of bacteriocins is well known (Patil *et al*., 2007; Yi *et al*., 2010). These organisms are generally regarded as safe (GRAS) and so are their bacteriocins which do not affect humans and other eukaryotes. Therefore, the ability of Stilton cheese *Lb. plantarum* isolates to inhibit the growth of *L. monocytogenes* and other undesirable microflora through bacteriocin production could be an important criterion for developing the organism into a commercial culture adjunct for the bio-preservation of the cheese against undesirable microflora.

Bacteriocins are peptides categorised into different classes. Class I (also known as lantibiotics such as nisin) are elongated shaped (type A) or globular molecules (type B) produced as precursor peptides that usually undergo extensive post-translational modification (Eijsink *et al*., 2002). Nissen-Meyer *et al*. (2010) have summarised that class II members are non-modified heat stable peptides which are further divided into four subclasses, class IIa, IIb, IIc and IId. Class IIa contains the low molecular weight, anti-listerial one-peptide pediocin-like bacteriocins that have similar amino acid sequences; class IIb contains the two-peptide bacteriocins such as plantaricin EF whose genes are next to each other in the same operon; class IIc consists of the cyclic bacteriocins whose N- and C-termini are covalently linked; class IId contains the one-peptide non-cyclic bacteriocins that show no sequence similarity to the pediocin-like bacteriocins. Optimal antibacterial activity of the two-peptide batceriocins requires the presence of both peptides in about equal amounts. The two peptides of these bacteriocins form a membrane penetrating helix-helix structure involving helix-helix-interacting GxxxG motifs which induce the formation of pores in the sensitive bacteria due to conformational alteration in the membrane proteins of the latter which in turn causes membrane leakage. This mode of action is similar to the mode of action of the pediocin-like (class-IIa) bacteriocins and lactococcin A, a class IId bacteriocin.

A wide variety of bacteriocins has been previously isolated from LAB including nisin from *Lc. lactis* (Bromberg *et al*., 2004), helviticin J from *Lb. helveticus* (Bonade *et al*., 2001), bulgaricin from *Lb. delbrueckii* subsp. *lactis* (Boris *et al*., 2001) and various plantaricins from *Lb. plantarum* (van Reenen *et al*., 1998). A single strain of *Lb. plantarum* can produce multiple plantaricins encoded from the bacteriocin locus on the chromosome. The plantaricin gene cluster contains five plantaricin genes encoding for the two-peptide plantaricins EF, JK, NC8, J51 and a pheromone peptide plantaricin A with antimicrobial activity (Eijsink *et al*., 2002; Diep *et al*., 2009). van Reenen *et al*. (1998) determined that the organism also produces plantaricin 423, a heat resistant (80^{o}C) plasmid-encoded bacteriocin with strong inhibitory activity against *Bacillus cereus*, *Listeria*, *Clostridium* and *Staphylococcus* spp. Whereas plantaricin loci from different strains of *Lb. plantarum* resemble each other in the organization of the bacteriocin transport and regulatory genes, there is variation in the spectrum of antimicrobial activity of the specific plantaricins (Cho *et al*., 2010). This variation has been attributed to differences in length of the plantaricins, amino acid sequence and composition, secretion

and processing machinery, post-translational modifications and antimicrobial activity alone or in combination with other peptides (Eijsink *et al*., 2002). Moll *et al*. (1999) proposed that plantaricins EF and JK form pores in the membranes of target cells and dissipate the transmembrane electrical potential and pH gradient. The plantaricin EF pores conduct small monovalent cations, but conductivity for anions is low or absent. Plantaricin JK pores show high conductivity for specific anions but low conductivity for cations. These bacteriocins are translated as pre-peptides; these are then processed by cleavage of the double glycine leader sequences to form active peptides with sizes of 33 (PlnE), 34 (PlnF), 25 (PlnJ) and 32 amino acids (PlnK) (Diep *et al*., 2009). The individual peptides have low antimicrobial activity. However, potency increases by 1000-fold when combined with their cognate peptides indicating complementary ion selectivity which ensures efficient killing of target bacteria.

Plantaricins A, EF and JK display relatively narrow inhibitory spectra, mostly being active against some species of lactobacilli (*Lb. plantarum*, *Lb. casei*, *Lb. sakei* and *Lb. curvatus*) in addition to other Gram-positive bacteria closely related to the producer *Lb. plantarum* strains (Diep *et al*., 2009). Nissen-Meyer *et al*. (1993) pointed out that these bacteriocins have no antimicrobial activity towards Gram-negative bacteria or yeasts. It is, therefore, evident that plantaricins probably evolved to provide the producing organism with a selective advantage in a complex microbial ecosystem (Ammor *et al*., 2006). The aim of the current work was to evaluate the antimicrobial activity of *Lb. plantarum* isolates obtained from different sites in Stilton cheese and to examine the possible mechanisms of inhibition. This could explain why these isolates dominated the final cheese flora as observed in Chapter 4. From this, *Lactobacillus* isolates with the potential as food bio-preservatives may be identified.

6.2 Methods

Antimicrobial activity of MRS-grown broth cultures of 50 individual genotypes of *Lb. plantarum* against the various indicator bacteria (Table 3.5) was evaluated using the agar overlay and paper disc diffusion assays as outlined in Section 3.5. This range of Gram-positive and Gram-negative indicator strains with different cell wall properties was chosen to establish whether antimicrobial activity was due to production of bacteriocins (plantaricins), organic acids, hydrogen peroxide or a combination of these compounds. *Lb. plantarum* is a facultative homofermentative organism and produces lactic as the dominant metabolite. However, depending on the environmental conditions, the organism can also produce acetic acid and other metabolites (in smaller quantities) with synergistic activity against yeasts, moulds and most Gram-negative bacteria. *Lb. plantarum* produces smaller quantities of hydrogen peroxide compared with other LAB such as *Leuconostoc* (Adesokan *et al*., 2010). The compound is inhibitory to some Gram-negative bacteria such as *Pseudomonas* spp. (Salminen *et al*., 2004) but is mostly active against LAB as well as other Gram-positive bacteria including *Cl. butyricum* and *Cl. tyrobutyricum* (Tuma *et al*., 2008). *Staph. aureus* produces catalase which breakdown hydrogen peroxide to enhance its survival from deleterious effects. This range of indicator bacteria was also used to demonstrate why lactobacilli prevail throughout milk fermentation to dominate the cheese microflora. *Ped. acidilactici* was included as the bacteriocin-producing control. The latter had been shown to exert antimicrobial activity against a broad range of organisms including *L. monocytogenes* and *Salmonella* Typhimurium, Enterobacteriaceae, *Staphylococcus*, yeasts and moulds (Olaoye and Dodd, 2010).

In order to establish the nature of any antimicrobial activity, the MRS broth culture was centrifuged (3400 *g*) to obtain a cell-free supernatant (CFS). Paper discs were soaked in CFS and in CFS treated with sodium hydroxide, catalase and a combination of sodium hydroxide & catalase in order to neutralise acids and hydrogen peroxide, respectively. Subsequently, the discs were applied onto agar overlays of the indicator strains as described in Section 3.5.2. CFS treated with proteinase K was used to determine if antimicrobial activity was due to plantaricin production as all these compounds are either proteins or peptides (Ammor *et al*., 2006). Paper discs treated with sterile MRS broth and 100 μg/ml chloramphenicol in MRS broth were used as the negative and positive controls, respectively. As *Lb. plantarum* strains usually harbour the genetic determinants for at least three bacteriocin systems i.e., plantaricin N, as well as the two-peptide bacteriocins

plantaricins EF and JK (Diep *et al*., 1996; Kleerebezem *et al*., 2003), a PCR method (Section 3.3.4.3) adapted from Cho *et al.* (2010) and Yi *et al*. (2010) was used to ascertain the presence of plantaricin N, EF and JK operons encoding the respective plantaricin bacteriocins among the *Lactobacillus* isolates. However, production of other bacteriocins such as plantaricin W and NC8 has also been reported (Eijsink *et al*., 2002; Diep *et al*., 2009). Sequencing and identification of the plantaricin genes determined by the operon was performed as in §3.3.4.3.

6.3 Results

6.3.1 Antimicrobial activity of lactobacilli using the agar overlay method

In this assay, *Lb. plantarum* (50 isolates; 7 from outer crust, 19 from blue veins, 24 from white core) were evaluated for antimicrobial activity against examples of spoilage, pathogenic and closely related LAB strains (Table 3.4). Each of the *Lb. plantarum* genotypes showed antimicrobial activity against more than one bacterial species but the number of sensitive species varied with the *Lactobacillus* isolate and its site of isolation (Appendix 6.1). The majority (84% of 50) of *Lb. plantarum* isolates gave a positive reaction and formed a halo around their colonies on the plates due to lysis of the sensitive strains (Figure 6.1). In order to determine the antimicrobial effect of the different strains of the organism against the primary starter culture for Stilton cheese, *Lactobacillus* isolates from the different sites were tested against *Lc. lactis*. All the isolates obtained from the outer crust and blue veins were able to inhibit *Lactococcus*, whereas only 24% of the white core strains could inhibit the growth of this organism (Table 6.2).

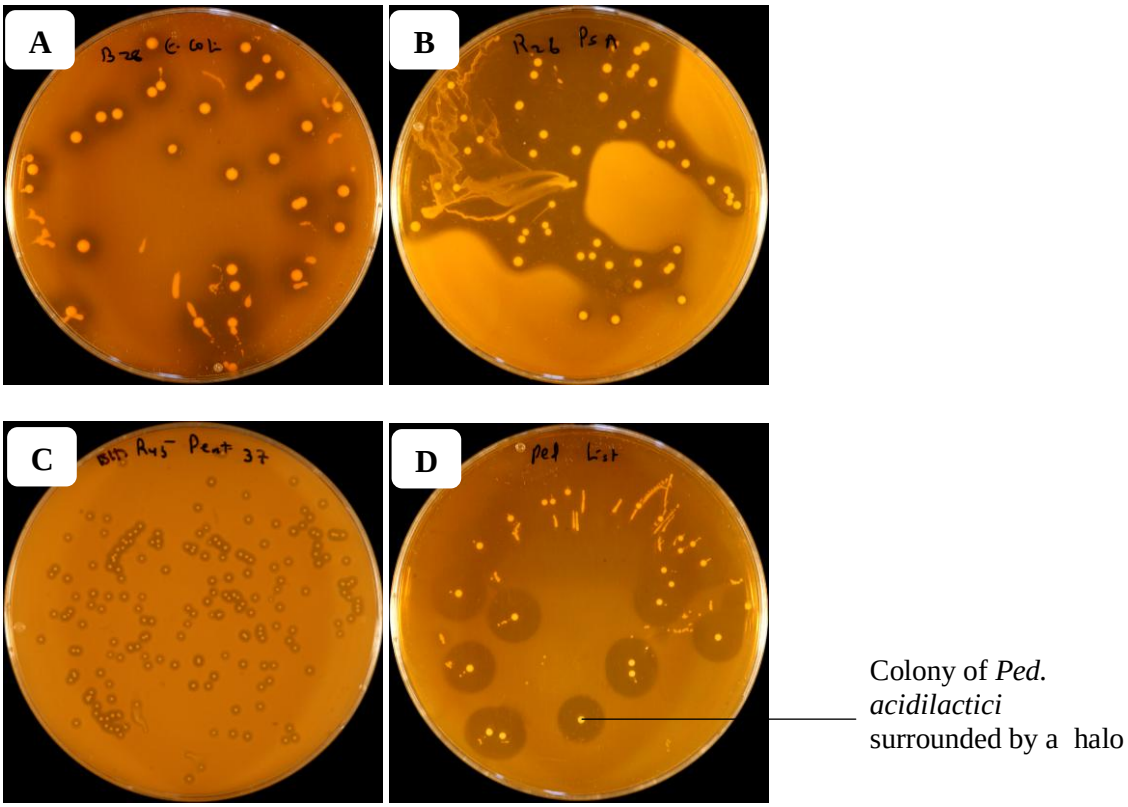


Figure 6.1 Antimicrobial activity of different genotypes of *Lb. plantarum* obtained from Stilton cheese. Isolates: (**A**) B28 (blue veins) against *E. coli*; (**B**) R26 (blue veins) against *Ps. aeruginosa*; and (**C**) R45 (white core) against *Lactobacillus pentosus*. (**D**) Antimicrobial activity of the pediocin-producing control strain *Ped. acidilactici* NCIMB 700993 against *L. monocytogenes* NCTC 11944. Antagonism is demonstrated by the presence of a halo around the colony of the producing organism.

Table 6.2 Proportion of *Lb. plantarum* isolates that demonstrated inhibitory activity against the various indicator bacteria based on agar plate overlay assay

Indicator strain	*% *Lb. plantarum* strains inhibitory against the indicator strains			
	Crust (n=7)	Veins (n=19)	Core (n=24)	(N=50)
L. monocytogenes NCTC 11944	100	100	100	100
Staph. aureus (Stilton isolate)	86	89	96	92
Staph. aureus NCTC 12100	100	95	96	96
E. coli 0157: H7-stx	100	100	100	100
Salm. Typhimurium	100	95	96	96
Ps. aeruginosa glaxo-3	100	100	100	100
Cl. sporogenes	000	000	000	000
Lc. lactis NCIMB 9918	100	100	24	100
Lb. pentosus NCIMB 8026	100	100	100	100

*Based on the presence (+) or absence (-) of a halo around the colonies of LAB strains

There was no apparent pattern in antimicrobial activity, even amongst *Lactobacillus* isolates obtained from the same site. However, *Lb. pentosus*, *Ps. aeruginosa*, *E. coli* and *L. monocytogenes* were the most inhibited organisms showing 100% susceptibility, whereas the *Staph. aureus* isolate obtained from Stilton cheese (92%) and *Salm.* Typhimurium (96%) were inhibited by all but a few isolates (Table 6.2). Of the seven tested *Lactobacillus* isolates obtained from the outer crust, R5 and R6 were found to have a weaker antimicrobial activity against *E. coli* and *Lc. lactis* than other genotypes whereas strain R1 had no inhibitory activity against the Stilton cheese *Staph. aureus* isolate (Appendix 6.1). Isolates R22 and R23 obtained from the blue veins as well as R37 from the white core also exerted no antimicrobial activity towards the Stilton cheese *Staph. aureus* isolate. Similar results were obtained for *Lb. plantarum* isolates B30 and W13 from the blue veins and white core respectively, on *Salm.* Typhimurium; as was the case for W30 (white core) on *Staph. aureus* NCTC12100. Compared with the isolates from the blue veins and white core, *Lb. plantarum* isolates from the outer crust had the highest level of inhibitory activity with only *Cl. sporogenes* and the Stilton cheese *Staph. aureus* isolate showing some resistance to these *Lactobacillus* isolates. Hence the study demonstrated that both Gram-positive and Gram-negative species were inhibited. However, none of the *Lactobacillus* isolates was inhibitory against *Cl. sporogenes*

suggesting spore-forming bacteria such as *Clostridium* could be resistant because their spores are not affected by the antimicrobials produced.

The results indicated that *Lb. plantarum* genotypes could produce different antimicrobial compounds active against a broad range of bacterial species including LAB as well as Gram-negative and other Gram-positive organisms. As *Lb. plantarum* produces plantaricins with antimicrobial activity only against closely related LAB spp. (Diep *et al*., 2009), the ability of the isolates to inhibit growth of other bacterial species could be attributed to other mechanisms. Hence, the isolates were further examined for inhibitory activity due to production of the diffusible substances including bacteriocins, acids, hydrogen peroxide or a combination of these compounds usually produced by the *Lactobacillus* spp. (Essid *et al*., 2009). The phenomenon was assessed on the most sensitive strains utilising paper discs soaked in cell-free supernatants (CFS) and CFS treated to eliminate the effect of acids and hydrogen peroxide in order to understand which antimicrobials could be acting against specific groups of the sensitive bacteria (Section 6.3.2).

6.3.2 Antimicrobial activity of *Lb. plantarum* isolates using paper disc diffusion assay

Cell-free supernatants from 50 *Lb. plantarum* isolates obtained from different sites within a Stilton cheese were examined for cross antagonistic activity against *L. monocytogenes*, *E. coli* and *Ps. aeruginosa* using the paper disc diffusion assay. This assay allowed for comparison of results obtained using the overlay method and also eliminated the possibility of *Lb. plantarum* growth being an inhibitory factor to the sensitive strains. In all cases, the cell-free supernatant (CFS) had a narrow spectrum of activity and only showed inhibitory reactions against *E. coli* and *Ps. aeruginosa*. The CFS could not inhibit the growth of *L. monocytogenes* and only formed a small faint halo regarded as negative for this indicator bacterium. Further examination of the effects of the CFS on the closely related LAB strains demonstrated that seven (of the 50) isolates had inhibitory activity against *Lb. pentosus*, but none was inhibitory against other LAB strains including *Leu. mesenteroides*, *Lb. plantarum* NCIMB 138914, *Lc. lactis*, *Lb. fermentum* and *Strep. thermophilus*. These results suggested that under the experimental conditions examined in this study, the CFS of *Lb. plantarum* isolates could only show antagonism against closely related members of the LAB group such as *Lb. pentosus*. *Lb. pentosus* is a pentose degrading organism commonly isolated from sewage and exhibits considerable

phenotypic and genomic homology with *Lb. plantarum*, which is why strains of this species are generally treated as synonymous with the latter and sometimes occupy the same niche (Zanon *et al*., 1987).

CFS with inhibitory activity against *Lb. pentosus* was only obtained from the *Lb. plantarum* isolates obtained from the blue veins (1 of 7 strains) and white core (6 of 7 strains) (Table 6.3). The growth conditions of *Lb. plantarum* isolates which produced antimicrobial activity against *Lb. pentosus* were examined at 30 and 37°C for 14, 24 and 48 h, aerobically & anaerobically, with & without agitation in MRS broth supplemented with 0.6, 10 and 20% (w/v) glucose in an effort to establish the optimum conditions for production of antimicrobial substance(s). Maximal inhibition of *Lb. pentosus* was obtained when *Lb. plantarum* isolates were grown for 24 h at 30^{o}C at 100 rpm in MRS broth supplemented with at least 0.6% glucose; the rest of the conditions had minimal influence. Consequently, these conditions were employed to obtain the CFS which were treated to neutralise acids and hydrogen peroxide in order to establish the mechanism of inhibitory activity.

Table 6.3 Inhibitory activity of treated and untreated cell-free supernatants of *Lb. plantarum* isolates against *Lb. pentosus* NCIMB 8026, and *Ped. acidilactici* NCIMB 700993 against *L. monocytogenes* NCTC 11944

Extract	Control strain	*Lb. plantarum* strains isolated from the						
		Blue veins	White core					
	Ped. acidilactici[*]	R23[**]	R36[**]	R37[**]	R38[**]	R39[**]	R42[**]	R45[**]
CFS	+++	+++	+++	++	+++	+++	+++	+++
CFS-N	+++	+++	++	++	++	++	+++	++
CFS-C	+++	+++	++	++	++	++	+++	+++
CFS-N-C	+++	++	++	++	++	+++	++	++
CFS-P	-	-	-	-	-	-	-	-

(CFS) cell-free supernatant; (CFS-N) neutralised cell-free supernatant; (CFS-C) cell-free supernatant treated with catalase; (CFS-N-C) neutralised cell-free supernatant treated with catalase; (CFS-P) cell-free supernatant treated with proteinase K.

(-) indicates that no zone of inhibition was observed or a zone less than 0.5 mm in diameter.

(+) a zone of inhibition greater than or equal to 0.5mm but less than or equal to 1 mm in diameter.

(++) a zone of inhibition greater than 1mm and less than or equal to 2 mm in diameter.

(+++) a zone of inhibition greater than 2 mm in diameter

* tested against *L. monocytogenes*

** tested against *Lb. pentosus*

Untreated cell-free supernatants showed zones of inhibition greater than 2 mm (Table 6.3). Varying inhibitory reactions against *Lb. pentosus* were observed when treated cell-free supernatants were applied. Neutralised cell-free supernatants adjusted to pH 6.5-7 (CFS-N) and catalase-treated cell-free supernatants (CFS-C) from *Lb. plantarum* isolates R36, R37 and R38 (white core) formed smaller halos (1-2 mm), whereas the corresponding cell-free supernatants from isolates R23 (blue veins), R42 and R45 (white core) showed halos greater than 2 mm. With the exception of *Lb. plantarum* R39 from the white core, catalase-treated cell-free supernatants adjusted to pH 6.5-7 (CFS-N-C) from all other *Lb. plantarum* strains formed halos of 1-2 mm against *Lb. pentosus*. For each of the isolates R23 (blue veins) and R42 (white core), a decrease in antimicrobial activity was only noted when the neutralised CFS treated with catalase (CFS-N-C) was applied

indicating the acid and hydrogen peroxide had similar levels of activity which was subsequently reduced after removal of both substances. The observed reduction in antimicrobial activity when neutralised (CFS-N) and catalase-treated CFS (CFS-C) were applied suggests that activity could, in part, be attributed to production of acid and hydrogen peroxide. These results were compatible with the data obtained when the growth medium was supplemented with glucose; this led to a greater pH reduction and corresponded with greater antimicrobial activity (data not shown). In contrast, all treated and untreated CFS of the pediocin producing control strain, *Ped. acidilactici* showed strong inhibition forming halos greater than 2 mm against *L. monocytogenes*. Activity of the untreated CFS was not different from that of the neutralised and catalase-treated CFS (Table 6.2) indicating further mechanisms other than acid and hydrogen peroxide production could account for inhibitory activity of the latter against *L. monocytogenes*.

For each of the *Lb. plantarum* isolates tested, treated and untreated cell-free supernatants retained some inhibitory activity against *Lb. pentosus* (Fig. 6.2, A-E) implying that bio-active substances additional to acids and hydrogen peroxide were produced. Cell-free supernatants treated with the proteolytic enzyme proteinase K lost inhibitory activity suggesting that a key part of the antagonism was due to a proteinaceous bacteriocin. Similar results were obtained with the pediocin producing control strain *Ped. acidilactici* against *L. monocytogenes* (Fig. 6.2, F-J), highlighting the proteinaceous nature of the bio-active compound(s) involved. In cases where positive results were obtained, all catalase-treated cell-free supernatants were heat stable and retained activity when boiled for ~1 min at 90^{o}C. The combined analysis of data from this assay and results from Section 6.3.1 suggested that the antimicrobial activity of *Lb. plantarum* isolates obtained from Stilton cheese could be attributed to acid, hydrogen peroxide and bacteriocin production and provided the basis for further screening of the strains for presence of genes encoding the class IIb plantaricin bacteriocins as outlined in Section 6.3.3.

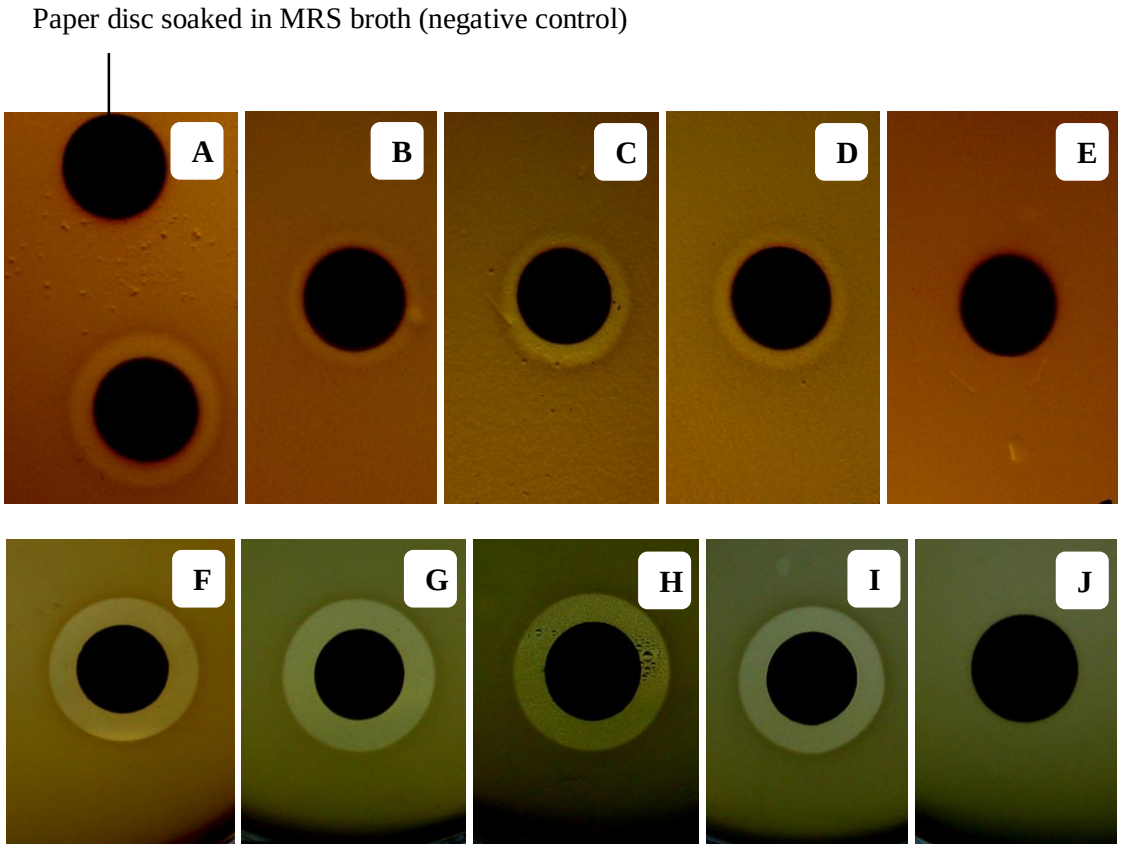


Figure 6.2 (**A-E**) Antimicrobial activity of the *Lb. plantarum* isolate R45 obtained from the white core of Stilton cheese; and (**F-J**) *Ped. acidilactici* NCIMB 700993. Positions: (**A & F**) cell-free supernatant; (**B & G**) neutralised cell-free supernatant; (C **& H**) cell-free supernatant treated with catalase (500U/ml); (**D & I**) neutralised cell-free supernatant treated with catalase; (**E & J**) cell-free supernatant treated with proteinase K (1mg/ml). (**A-E**) *Lb. pentosus* NCIMB 8026 (~10^6 CFU/ml); (**F-J**) *L. monocytogenes* NCTC 11944 (~10^6 CFU/ml,) were used as the sensitive strains. Inhibitory activity is demonstrated by formation of a halo around paper discs soaked in the supernatants for 30-60 min at room temperature.

6.3.3 Prevalence of plantaricin encoding genes

Fifty four *Lb. plantarum* isolates were screened for presence of plantaricin N, EF and JK operons using PCR. The technique is highly sensitive (Yi *et al*., 2010), and therefore was used to ascertain isolates harbouring the operons in order to complement the results of qualitative assays obtained (Sections 6.3.1-6.3.2). Genomic DNA extracts (Section 3.3.1) used in PCR have been found to be appropriate for this work (Maldonado-Barragán *et al*., 2009; Yi *et al*., 2010). The representative agarose gel of PCR amplification of the plantaricin EF operons is shown in Fig. 6.3. The majority (64.8% of the 54) of the isolates were successfully amplified with the primers to produce an amplicon of 428 bp. Confirmation that the amplimers were the plantaricin EF operons was ascertained by sequencing the PCR product followed by NCBI database search using the BLAST programme. PCR did not yield successful results when primers targeting amplification of other plantaricin operons including plantaricins JK and N were applied, and so the genes were regarded as absent from the genome.

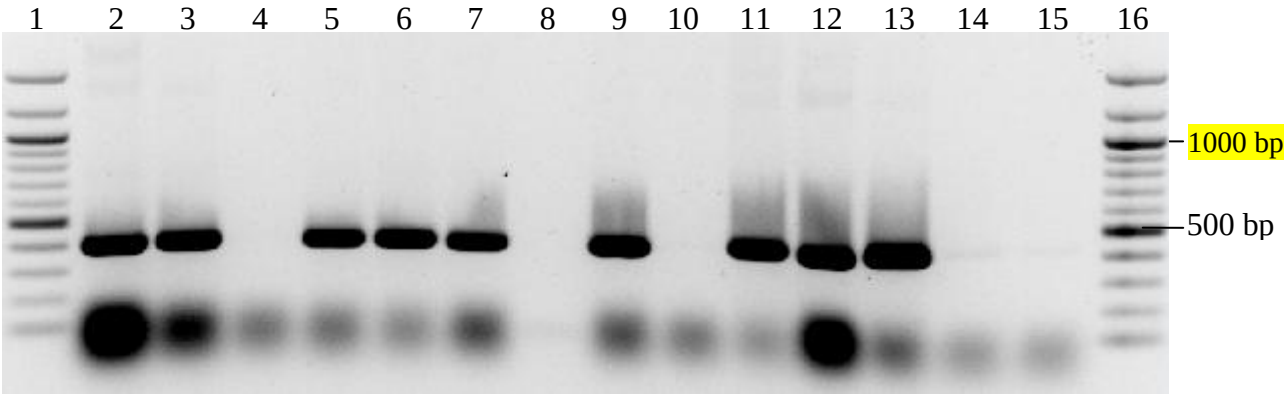


Figure 6.3 Agarose gel electrophoresis of PCR products for the detection of plantaricin EF operons in *Lb. plantarum* isolates obtained from Stilton cheese. Lanes: (1&16) 100 bp marker; (2-5) isolates from the outer crust R1, R2, R3 & R6; (6-10) isolates from the blue veins R25, B11, B13, B25 & B29; (11-14) isolates from the white core R37, R40, R45 & W24; (15) *Lb. brevis* (negative control). The samples were run on 1% (w/v) agarose gel in 1X TAE buffer for 2 h at 75 V.

The amplicon was mostly (6 out of 7) obtained from isolates from the outer crust and was least (15 out of 32) observed among the isolates obtained from the white core (Table 6.4). BLAST matches of the sequences obtained from amplimers of the isolates obtained from the blue veins and white core were identified as putative plantaricin (pln) EF bacteriocin-encoding gene from closest relatives of *Lb. plantarum* JDM1. Conversely, sequences of PCR product from the isolates obtained from the outer crust were identified as putative pln EF bacteriocin-encoding gene from closest relatives of *Lb. plantarum* WCFS1. For all samples analysed, the identification level ranged from 97 to 99% ($E=2^{-132}$ to 9^{-167}) homology and the gene translation gave the predicted protein sequence MKKFLVLSDR ELNAISGGVFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG (52 amino acids).

Table 6.4 Prevalence of plantaricin EF encoding operons among *Lb. plantarum* isolates obtained from different sites in Stilton cheese

Site of isolation	Sample size	Number of positive samples	%
Outer crust	07	06	85.7
Blue veins	22	14	63.6
White core	32	15	46.9
Total	54	35	64.8

These findings correlated with the results of the agar overlay assay (§6.3.1) and indicated that *Lb. plantarum* isolates obtained from the outer crust were more inhibitory against the various indicator bacterial spp. tested. In fact, all isolates which were found not to contain the plantaricin EF operons could still exert antimicrobial activity albeit at a lower level (as demonstrated by smaller halos), with *Salm.* Typhimurium and *Staph. aureus* NCTC 121000 showing some resistance to these isolates (Appendix 6.1). This was the case for isolate B30 from the blue veins as well as W13 and W30 obtained from the white core (Appendix 6.1). This therefore suggests that antagonism by these isolates could mainly be attributed to acid and hydrogen peroxide production as shown in Section 6.3.2.

Presence of the plantaricin EF operons amongst the isolates showed some correspondence with the results from the agar overlay and paper disc assays (§6.3.2). For instance, all the isolates which gave positive results with *Lb. pentosus* after neutralisation and catalase treatment of their CFS (Section 6.3.2) were also found harbour the plantaricin EF operons suggesting expression. However, despite the high prevalence of the operons among *Lb.*

plantarum isolates obtained from the outer crust, none of these isolates could show CFS activity against *Lb. pentosus* implying failure to express the genes as will be discussed later in Section 6.4.2. In general, the study suggested that antimicrobial activity of *Lb. plantarum* isolates against non-LAB spp. could only be attributed to production of acid, hydrogen peroxide and/or other metabolites whereas in case of *Lb. pentosus*, antagonism from some of the *Lactobacillus* isolates was due to synergistic action of acid, hydrogen peroxide and plantaricin EF bacteriocins. As already discussed in the introduction, the overall results were in agreement with the reported biological activity of plantaricins. Nissen-Meyer *et al*. (1993) and Diep *et al*. (2009) pointed out that these bacteriocins have a relatively narrow inhibitory spectrum, mostly being active against bacterial species closely related to the producer *Lb. plantarum* strains.

6.4 Discussion

6.4.1 Antimicrobial activity of the *Lactobacillus* isolates

Results from the agar plate overlay indicated that *Lb. plantarum* isolates had a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria. These results are supported by some of the available literature whereby *Lb. plantarum* from natural fermented foods was reported to show antimicrobial activity against a wide variety of bacteria including *L. monocytogenes*, *B. cereus*, *Cl. perfringens*, *Staph. aureus* and *E. coli* (Wilson *et al*., 2005; Sawitzki *et al*., 2009; Yi *et al*., 2010). In the present assay, there were some variations in levels of activity against the various indicator bacterial species tested. However, the majority of *Lb. plantarum* isolates obtained from different sites (outer crust, blue veins and white core) of Stilton cheese exhibited antimicrobial activity against 8 of the 9 tested indicator bacteria, highlighting their prospects as potential bio-preservatives for Stilton and other dairy products.

The antagonistic effect of treated and untreated cell-free supernatants of the MRS broth cultures on various bacteria was also tested. Cell-free supernatants had a narrow spectrum of activity only showing inhibitory reactions against *E. coli*, *Ps. aeruginosa* and *Lb. pentosus*, which together with leuconostocs, pediococci and streptococci, is regarded as a natural competitor of *Lb. plantarum* (Gonzalez *et al*., 1994). These results are similar to those from other studies (Ennahar *et al*., 1996; Wilson *et al*., 2005; Obodai, 2006). The current study demonstrated that inhibition of *Lb. pentosus* was partly due to plantaricin production in synergy with acid and hydrogen peroxide production. Maximal activity was

recorded at early stationary phase (24 h, 8-9 $\log_{10}$ CFU/ml) at 30^{o}C, which is similar to the data reported by other researchers (Nissen-Meyer *et al*., 1993; Messi *et al*., 2001; Todorov and Dicks, 2005). Todorov and Dicks (2005) reported that optimal bacteriocin production in *Lb. plantarum* may also occur at pH above 4.5 in presence of the surfactant Tween 80 which facilitates discharge of the bacteriocin from the cell surface of the producer strain. Bacteriocin production in the organism is also enhanced by tryptone (20 g/L) and mannose (30 g/L) but supplementing MRS with more than 1 g/L glycerol inhibits production due to increased osmotic stress (Todorov and Dicks, 2005). This study recorded loss of bacteriocin activity after 24 h suggesting degradation; however, the isolates examined could not hydrolyse casein on agar plates (Section 7.3.3) showing the strains examined here could not produce extracellular proteases.

Production of some other intracellular peptidases active against the bacteriocin may account for loss of activity due to degradation (Todorov and Dicks, 2005). Moll *et al*. (1999) reported that plantaricins EF (and JK) are unstable at low pH and suggested that activity of these bacteriocins occurs at a pH optimum of 6-6.5. In milk, *Lb. plantarum* usually employs a complex cell wall bound proteolytic system which is optimally active at 30^{o}C and high pH; and consists of proteinases, peptidases, amino and peptide carriers used for degradation of casein to provide essential amino acids for growth (Marathe and Ghosh, 2009). However, this phenomenon cannot be extrapolated to the results obtained in the current study as the pH was found to be low (less than 4) and glucose was added to MRS broth in which the isolates were grown; this would enhance more acid production.

None of the other Gram-positive and Gram-negative bacteria was inhibited by the cell-free supernatants. Using thin layer chromatography, a previous study investigating the mechanism of anti-listerial activity of *Lb. plantarum* in MRS broth cell-free supernatant showed that inhibitory activity against *L. monocytogenes* occurs during late log or early stationary phase of *Lb. plantarum* growth and was attributed to lactic acid production alone (Wilson *et al*., 2005). Other studies have reported that *Lb. plantarum* produces bacteriocins with inhibitory activity against *L. monocytogenes* (Messi *et al*., 2001) but with no potency towards Gram-negative bacteria (Messi *et al*., 2001; Todorov and Dicks, 2005). From the stand point of this study, antimicrobial activity of cell-free supernatants from *Lb. plantarum* was greatest in the presence of glucose which implies high acid conditions. There was weak evidence to assert with confidence that some of the isolates

could produce plantaricins as these only gave small halos (1-2 mm) against *Lb. pentosus*. However, the inhibitory spectrum of plantaricins from the producer *Lb. plantarum* isolates was consistent and appeared to be similar to that of the bacteriocins from *Lactobacillus* spp. such as lactacin B and helveticin J (Boris *et al*., 2001) whose activity spectra are rather narrow and include only strains belonging to the same genus (Gonzalez *et al*., 1994). Further research is required to establish why cell-free supernatants had weaker activity even though there was clear evidence of antagonism when the organism was tested using the plate agar overlay assay. None-the-less, antimicrobial activity may partly account for the presence and dominance of *Lb. plantarum* in Stilton cheese as evidenced in Chapter 4, with the different genotypes showing varying levels of activity towards the sensitive microbial strains. However, on the basis of the results from this study, the precise number of *Lactobacillus* clones isolated from Stilton cheese could not be effectively established and this an issue which should be further examined.

Bacteriocins may be produced directly in foods as a result of starter culture or co-culture activity. Indeed, several studies have indicated that lactobacilli as starter cultures or co-cultures are capable of producing the compounds in food matrices and consequently display antimicrobial activity against sensitive spoilage or pathogenic bacteria. This effect has been documented for meat products (Bromberg *et al*., 2004; Albano *et al*., 2007; Essid *et al*., 2009), sauerkraut (Wilson *et al*., 2005), sourdough (De Vuyst and Leroy, 2007), and fermented dairy products such as Kefir (Powell *et al*., 2007) & soft cheese (Ennahar *et al*., 1996), and therefore has potential application in Stilton cheese. Stability of plantaricin EF produced by the *Lb. plantarum* isolates in the cheese matrix needs further investigation as this may offer potential application in Stilton cheese and other fermented foods. In order to select a microorganism as starter culture or starter culture adjunct, it is often recommended to consider evaluating the strain against a broad spectrum of microorganisms including pathogenic moulds and fungi (Sawitzki *et al*. 2009) which is an aspect that needs further investigation for the isolates assessed in this study.

Gálvez *et al*. (2007) compared the data from culture media with those from food systems and revealed that the efficacy of bacteriocins is about 10-fold lower in the latter and greatly depends on a number of food related factors including interaction with food components, precipitation, inactivation and poor solubility at pH greater than 6. Further

research should focus on predicting the kinetics of bacteriocin production by *Lb. plantarum* isolates *in situ* in Stilton cheese through mathematical modelling in conjunction with positive predictive microbiology (Khalid, 2011). The relationship between food, environment and kinetics of the starter cultures has been previously established (De Vuyst and Leroy, 2007; Varghese and Mishra, 2008; Zhang *et al*., 2012) and provided important insights about the potential of *in situ* production of bacteriocins and their effect on the sensitive target strains. Further research in this area for bacteriocin producing *Lb. plantarum* isolates is important given the increasing commercial application of bacteriocins and bacteriocin-producing LAB in various food systems (Gálvez *et al*., 2007).

6.4.2 Prevalence of plantaricin EF genes

In *Lb. plantarum* and most other LAB, two-peptide bacteriocins are the most potent antimicrobial peptides. They are characterised by the occurrence of the conserved amino acid sequence motif 'YGNGVXCXXXXCXV' in the N-terminus of the molecules (Yi *et al*., 2010). Currently, the ability to establish the presence of the gene encoding for this sequence motif is the basis for rapid screening of LAB which produce these bacteriocins (Eijsink *et al*., 2002; Yi *et al*., 2010).

The prevalence of plantaricin EF operons among *Lb. plantarum* isolates was in agreement with other studies (Rojo-Bezares *et al*., 2007; Yi *et al*., 2010). These researchers proposed that when the gene encoding plantaricin F is expressed in *Lb. plantarum*, a heat stable bacteriocin (plantaricin F) normally active in a narrow pH range is produced, which may partly explain low activity of the CFS as observed in Section 6.3.2. This study indicated that the operons encoding plantaricin EF occur in *Lb. plantarum* isolates obtained from Stilton cheese (pH, 4.5-6). If the gene encoding for plantaricin F was induced to produce the bacteriocin, it is possible the peptide would have lower antimicrobial effect, given the low pH of the product. The narrow spectrum of activity (i.e. small range of species acted against) may be partly attributed to an imbalance in the proportion of the two peptides (E and F). Nissen-Meyer *et al*. (2010) suggested that two peptide bacteriocins such as plantaricin EF display optimal activity when both peptides are present in about equal amounts. As these bacteriocins act by inducing formation of pores that display specificity with respect to the transport of molecules in the target cells (Nissen-Meyer *et al*., 2010),

the current study could also have been limited by the number of possible sensitive microbial groups examined.

This study has shown that the genes encoding plantaricin EF were highly prevalent among the *Lb. plantarum* isolates with the highest level (86%) detected in the isolates obtained from the outer crust and the lowest (47%) from lactobacilli from the white core. These prevalence levels correlated with the data from the agar overlay assay (Section 6.3.1) when the organism was cultured on solid media but could not be related with the CFS assays (Section 6.3.2) as there was no evidence for bacteriocin expression among the outer crust isolates in the latter assay. Also, as already mentioned in Section 6.4.1, the percent prevalence of the genes ending plantaricin EF obtained from this work (Table 6.4) could have been biased by failure to establish the precise number of *Lactobacillus* clones isolated from the cheese. Molecular techniques presented in Chapter 2 (Section 2.1.2) would be would provide future prospects to overcome this limitation. Messi *et al*. (2001) and Maldonado-Barragán *et al*. (2009) have reported that on solid media, well isolated colonies of *Lb. plantarum* normally produce acid, hydrogen peroxide and plantaricins B, 423 and F which have broad spectrum of activity implying the phenomenon is a constitutive trait. However, the organism rarely produces the bacteriocins in liquid broth.

In most organisms, bacteriocin production has been shown to be an unstable phenotype and environmental factors play an important role in regulation of the process. For example, sakacin A and curvaticin production by *Lb. sakei* and *Lb. curvatus*, respectively, is a temperature-sensitive process (Maldonado *et al*., 2004). Nevertheless, the mechanism by which the environment interacts with regulation of bacteriocin production is still poorly understood. Studies have proposed that most bacteriocins can be produced in liquid media when appropriate growth conditions have been fulfilled. Maldonado-Barragán *et al*. (2009) have summarised that *Lb. plantarum* and some lactococci lose the ability to produce bacteriocins when inoculated in liquid media below a specific inoculum size (~10^5 CFU/ml). In this case, the bacteriocin producing (Bac+) phenotype can be restored when the culture is either streaked onto solid media or by addition of the cell-free supernatant from a previous Bac+ culture.

Previous studies have shown that sometimes *Lb. plantarum* does not produce bacteriocins (including plantaricin EF) when inoculated as a pure culture in liquid medium regardless of inoculum size and growth conditions (Maldonado *et al*., 2004). However, co-culture of the organism with other specific Gram-positive bacteria or addition of its own specific auto-inducer peptide to broth culture results in bacteriocin production (Maldonado *et al*., 2004). From this perspective, the isolates examined in this study were subsequently separately co-cultured with live cells of *Lc. lactis*, *Ped. pentosaceus* and *Lb. acidophilus*; these had been previously shown to induce plantaricin production in *Lb. plantarum* (Rojo-Bezares *et al*., 2007). However, no induction of antimicrobial activity was observed in any case.

Bacteriocin production in such LAB strains is regulated by a three component regulatory system composed by an auto-inducer peptide (AIP), histidine-kinase protein (HK) and response regulator (RR). AIP acts as an indicator of the cell density which is sensed by the corresponding HK, resulting in activation of RR which finally activates expression of all operons necessary for bacteriocin synthesis, transport and regulation. This quorum sensing (QS) or auto-induction mechanism mediated by AIP was found in various lactobacilli including *Lb. salivarius*, *Lb. sake* (Diep *et al*., 2009) and *Lb. plantarum* (Di Cagno *et al*., 2010). Maldonado-Barragán *et al*. (2009) suggested that differences in bacteriocin production between solid and liquid media may be attributed to differences in the rate of diffusion of the corresponding AIP. These authors reported that AIP does not diffuse in solidified agar as readily as in solution, allowing cells on solid media surface to come in closer contact with secreted AIP than in liquid medium. The possible functionality of AIP mechanism on solid and in liquid media needs to be addressed for the *Lb. plantarum* isolates examined in this study. However, this phenomenon would be important for *Lb. plantarum* growing in cheese where diffusion is likely to be limited due to its solid nature.

The results of this work suggest that the substance(s) responsible for inducing production of plantaricin EF in *Lb. plantarum* may be either synthesised or activated by close cellular contact with the competing/sensitive microorganisms (Rojo-Bezares *et al*., 2007). This may explain why the isolates that were examined in this study maintained antimicrobial activity against other Gram-positive bacteria when both were co-cultured together on agar overlays but after removal of target cells (application of the CFS), the effect was only

expressed by fewer *Lb. plantarum* strains and antagonism was only limited to *Lb. pentosus*.

In food fermentations, the narrow spectrum of antimicrobial activity of the CFS from *Lb. plantarum* isolates may be exploited in control of specific sensitive undesirable organisms without compromising the beneficial microflora of the product. There is the need to determine the genetic function and organisation of the regulon implicated in bacteriocin production among the *Lb. plantarum* isolates. This would give better understanding of how environmental conditions and other factors such as presence of target cells induce production and activity of plantaricin EF bacteriocins in the organism. In general, *Lb. plantarum* isolates presented interesting prospects as bio-preservatives due to their anti-pathogen activity demonstrating that the isolates have potential to compete with other bacteria in solid systems such as cheese. However, prospects for application of the isolates as adjunct cultures for cheese production could be undermined by the observed ability of some isolates to inhibit *Lc. lactis*. Investigations on the viability of the organism as a probiotic *in vivo* in the human gut are worthwhile. Purification of the bacteriocins produced by the *Lb. plantarum* isolates would allow a more targeted approach for further evaluation *in situ* in Stilton cheese in order to control the undesirable bacteria such as *Listeria* in the final product.

6.5 Conclusions

In light of the results obtained using qualitative methods and PCR, it could be concluded that a cell contact inducible activity of antimicrobial activity against a wide range of other bacteria, including pathogens and spoilage organisms, was detected in *Lb. plantarum* isolates obtained from Stilton cheese. Whereas different antimicrobials were produced by different *Lactobacillus* isolates, activity against non-LAB bacterial species was mainly due to acid, and to a less extent, hydrogen peroxide production.

Although the isolates had a high prevalence of plantaricin EF genes, there was weak evidence for plantaricin production in liquid medium assays. The plantaricin EF had a narrow spectrum of activity; this was only demonstrable among *Lb. plantarum* isolates obtained from the blue veins and white core against *Lb. pentosus*, implying the phenomenon was largely dependent on the genotype/isolate of *Lb. plantarum* and was only active against closely related lactic acid bacteria.

These results, together with the data presented in Chapters 4 and 5, showed that there were different genotypes of *Lb. plantarum* in the blue veins and white core compared with the isolates from the outer crust. The latter group could only show antimicrobial properties on solid medium (agar plates) whereas the antimicrobial compounds from the former group were active both in liquid (CFS) as well as on solid media.

At present *Lb. plantarum* is fortuitous in Stilton cheese, thus production of antimicrobials could influence the cheese microflora giving rise to batch-to-batch variation in the flora of the final product which may influence sensory characteristics. In the next chapter, different genotypes of the organism were examined for growth interaction(s) with the starter culture *Lc. lactis* and how the possible interactions could affect the aroma profile of milk under simulated cheese ripening conditions.

CHAPTER 7

ACID PRODUCTION, POPULATION DYNAMICS AND VOLATILE PROFILES OF *LACTOBACILLUS PLANTARUM* ISOLATES

7.1 Introduction

It has previously been demonstrated that the non-starter bacterial flora from different sites (outer crust, blue veins and white core) of Stilton cheese is dominated by *Lb. plantarum* (Section 4.2.2) and that genotypically different isolates of the organism tolerate stress conditions typical of the microenvironments from which they were isolated (Sections 5.3.2-5.3.4). A previous study by Gkatzionis *et al*. (2009) has also established that the key aroma compounds (alcohols and ketones) are differentially distributed in the above cheese sites. *Lb. plantarum* is known to contribute to flavour development in cheese through acid production and aroma formation (Whitley, 2002; Ghotbi, 2011). Moreover, the process involves complex metabolic pathways which can follow different routes in pure or mixed cultures (Gkatzionis, 2010). As the volatile aroma compounds in cheese originate from microbial, enzymatic and chemical transformation of substrates present in milk (Vítová *et al*., 2006), the current study aimed to examine how the different genotypes of the organism may influence the aroma profile of cheese and how this can be influenced by co-culture with different strains of *Lc. lactis* and salt addition. Isolates of the organism have been found to possess a broad antimicrobial spectrum on solid medium (Section 6.3.1) and some isolates could produce bacteriocins (Sections 6.3.2). Therefore, it was also deemed interesting to establish the effect of the different genotypes of the organism on the growth and survival of the primary starter culture *Lc. lactis*. Indeed, Stilton cheese producers often have to deal with poor quality cheeses having low aroma scores (Whitley, 2002) which could be due to low starter culture activity and it is important to establish if NSLAB could be a cause of such effects.

7.2 Methods

7.2.1 Acid production

In order to quantify acid production, *Lb. plantarum* isolates representing each sampling site (n=6) were selected from the six clusters of the PFGE dendrogram and evaluated for growth, and lactic and acetic acid production (dominant organic acids produced by LAB) under optimal growth conditions in MRS broth at 30^{o}C as described in Section 3.8. *Ped. acidilactici* was included as an acid-producing control strain. The pH was measured to monitor the progress acid production in the medium. Organic acids were extracted from the cell-free supernatants at different growth time intervals and analysed by HPLC; samples were eluting through the solid phase extraction (SPE) cartridges which had been pre-conditioned with 100% methanol and SDW as outlined in Section 3.8.2. Un-inoculated MRS broth was used to set the baseline level for quantification of lactic and acetic acid.

7.2.2 Dynamics of viable populations and pH changes and volatile profiles analysis

The dynamics of growth and survival, and the concomitant volatile production of pure and mixed cultures of the different *Lb. plantarum* geno-groups (R2, outer crust, B30, blue veins, W8, white core), and different strains of *Lc. lactis*: *Lc. lactis* subsp. *lactis* (acid producer) and *Lc. lactis* NCIMB 9918 (non acid-producer) was examined by culturing the isolates as pure or mixed cultures in UHT cows' milk (3% fat) for 48 h at 30^{o}C as in Section 3.6.1. Subsequently, each sample was subdivided into two portions; 3.5% NaCl was added to one portion whilst the other portion (control) was unsalted. All samples were further incubated for 12 weeks at 18^{o}C and viable counts enumerated as detailed in §3.6.1. Simultaneously, 5 ml aliquots were also taken at 24 and 48 h (fermentation), and then at 4 and 7 weeks (ripening) for headspace analysis using SPME GC-MS as in Section 3.9.2. Un-inoculated milk was included as a control.

These conditions were chosen to simulate, as much as possible, the initial stages of milk fermentation at 28-30^{o}C and subsequent cheese ripening at 15-20^{o}C. UHT milk was chosen as the model medium as it is a microbially controlled medium and has been used previously for studying microbial interactions in dairy matrices (Gkatzionis *et al*., 2009). *Lc .lactis* subsp. *lactis* was selected to represent, as much as possible, the commonly used lactic acid-producing primary starter culture (acid producer) during cheese production. *Lc. lactis* NCIMB 9918 was included in order to examine the effect on the aroma profile,

of the possible growth interactions between the non-starter *Lb. plantarum* isolates and the non acid-producing *Lc. lactis* strains which may occur naturally.

Lb. plantarum from pure and mixed cultures was enumerated on Rogosa agar, a selective medium which does not support the growth of *Lc. lactis*. *Lc. lactis* was enumerated indirectly from MRS agar counts (Fig. 7.1) as detailed in Section 3.6.3. The difference between the total LAB viable counts obtained on MRS agar and *Lactobacillus* counts on Rogosa agar plates was used to ascertain the *Lactococcus* counts. The changes in pH were monitored using a pH meter as described in Section 3.6.2. Aroma compounds in the headspace above the sample in a 20 ml sealed vial were sampled using a stableflex SPME fibre, detected using GC-MS and identified and quantified based on their linear retention indices and mass spectra as in Section 3.9.2.

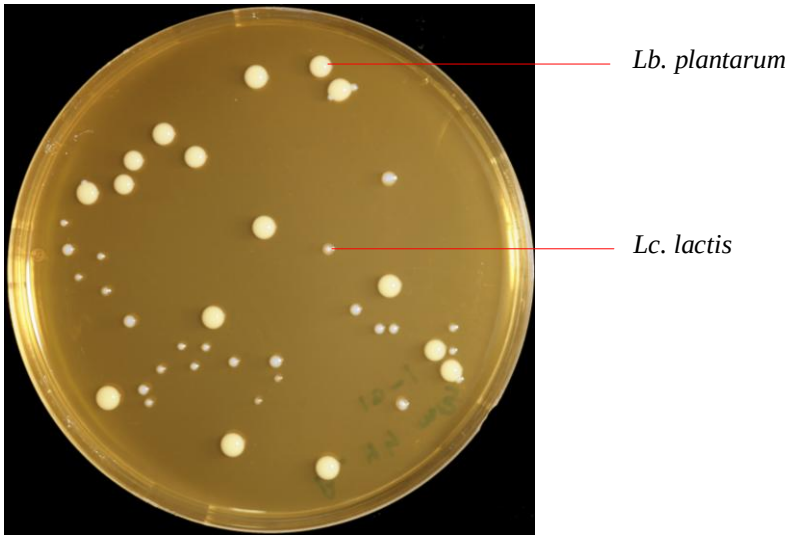


Figure 7.1 Colony morphology of *Lb. plantarum* B30 obtained from the blue veins and *Lc. lactis* subsp. *lactis* (UNFCC) co-cultured in cows' UHT milk for 24 h at 30^{o}C. The sample (0.1 ml) was surface spread on MRS agar and incubated anaerobically for 48 h at 30^{o}C.

7.3 Results

7.3.1 Acid production

The six isolates chosen for this study were selected to represent each of the cheese sites as well as the major clusters of the PFGE genotyping dendrogram. The data obtained from growth characteristics, pH changes and acid production of the *Lb. plantarum* isolates and *Ped. acidilactici* (control) are presented in Table 7.1. Fig. 7.2(A) represents the typical HPLC chromatogram obtained from standard solutions of lactic (1.25 g/L) and acetic acid (0.62 g/L). Fig. 7.2(B) shows the HPLC chromatogram of MRS broth inoculated with *Lb. plantarum* W30 obtained from the white core and incubated for 48 h at 30^{o}C. The plot of the data in Table 7.1 is shown in Figure 7.3. From Fig 7.2A, there was good separation of lactic and acetic acid eluting at 16 and 19 min, respectively. Two of the peaks in the sample extracts could be identified as lactic and acetic acid because they gave similar elution times to those of the standard solutions. It was anticipated that the large peak at 11.8 min could be attributed to glucose; this peak gave a decreasing trend as fermentation progressed. The method was fast and provided high throughput for sample analysis.

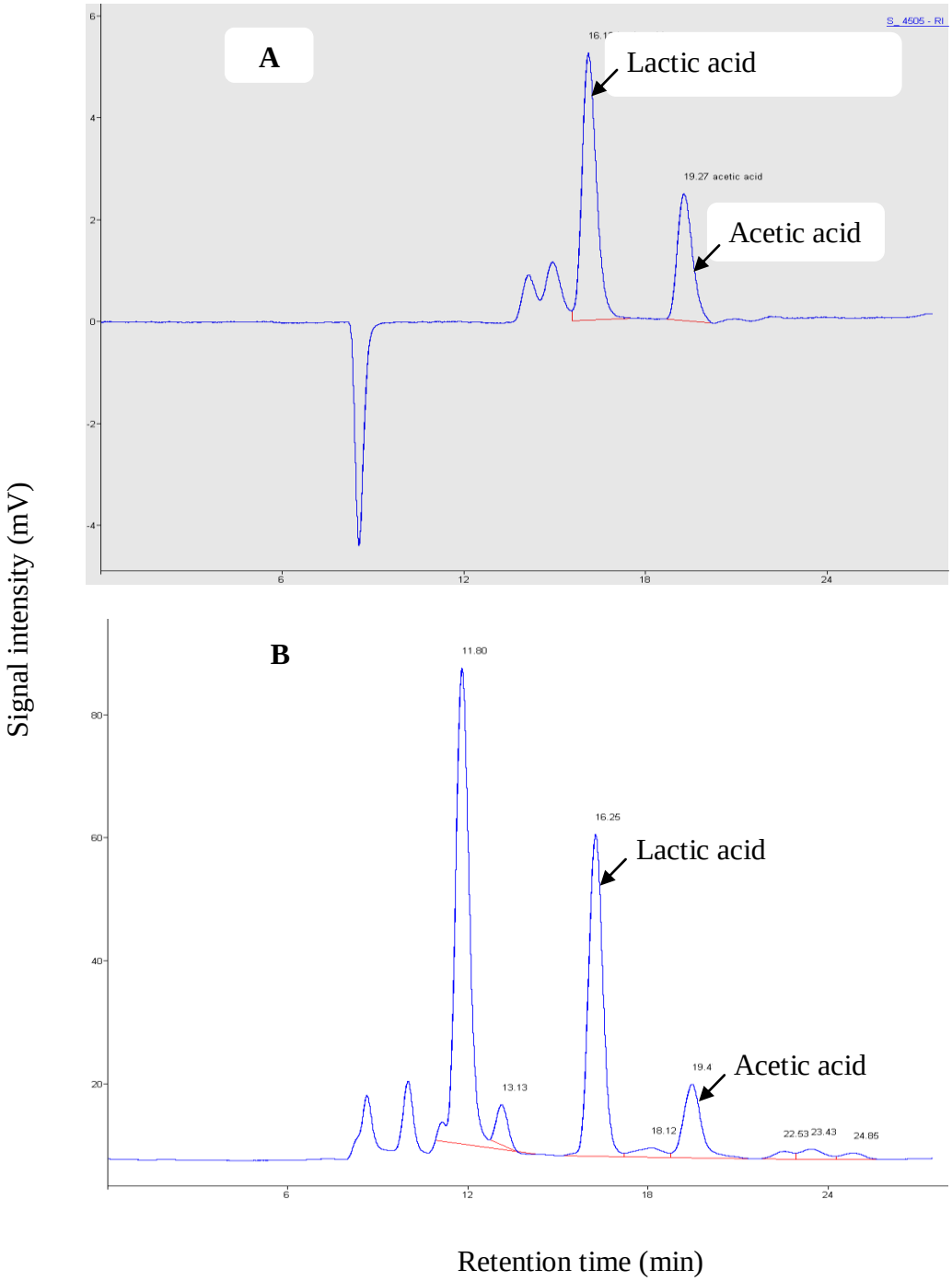


Figure 7.2 Chromatograms showing HPLC detection of lactic (1.25g/L) and acetic acid (0.62g/L) in (**A**) the standard solution; and (**B**) cell-free supernatant of MRS broth culture of *Lb. plantarum* W30 (white core) grown for 48 h at 30^{o}C

Table 7.1 Viable counts, lactic and acetic acid production and pH changes of MRS broth inoculated with *Ped. acidilactici* NCIMB 700993 and different genotypes of *Lb. plantarum* obtained from Stilton cheese. Values are means of three independent determinations ± standard errors of the means, $p = 0.05$.

Time (h)	R2 (crust)	R6 (crust)	B30 (veins)	R25 (veins)	R40 (core)	W30 (core)	*Ped. acidilactici*
				Log_{10} CFU/ml			
0	$3.69\pm0.1^{f1,2}$	$3.69\pm0.5^{d1,2}$	$3.58\pm0.1^{e1,2}$	3.18 ± 0.2^{f2}	3.31 ± 0.2^{d1}	$3.89\pm0.1^{e1,2}$	$3.79\pm0.1^{e1,2}$
3	$4.17\pm0.2^{e1,2}$	3.77 ± 0.3^{d2}	$4.08\pm0.1^{d1,2}$	3.74 ± 0.1^{e2}	3.74 ± 0.2^{d1}	4.46 ± 0.1^{d2}	$4.11\pm0.2^{e1,2}$
6	4.71 ± 0.1^{d2}	$4.36\pm0.2^{c,d2}$	4.71 ± 0.2^{c2}	4.56 ± 0.1^{d2}	4.56 ± 0.2^{c1}	5.32 ± 0.1^{c2}	5.58 ± 0.2^{d1}
9	5.24 ± 0.2^{c3}	4.97 ± 0.3^{c3}	$5.43\pm0.2^{b2,3}$	4.97 ± 0.1^{c3}	5.00 ± 0.1^{c2}	5.85 ± 0.1^{b3}	6.76 ± 0.1^{c1}
24	8.32 ± 0.2^{b3}	7.63 ± 0.5^{b4}	$8.43\pm0.1^{a2,3}$	$8.10\pm0.1^{b3,4}$	$7.90\pm0.2^{b1,2}$	$9.03\pm0.2^{a3,4}$	9.28 ± 0.1^{a1}
48	$8.81\pm0.1^{a2,3}$	$8.96\pm0.2^{a1,2}$	$8.52\pm0.1^{a3,4}$	$9.01\pm0.1^{a1,2}$	8.77 ± 0.1^{a1}	$9.13\pm0.1^{a2,3}$	8.45 ± 0.1^{b4}
				pH of the samples			
0	5.79 ± 0.0^{a1}	5.79 ± 0.0^{a1}	5.79 ± 0.0^{a1}	5.79 ± 0.0^{a1}	5.79 ± 0.0^{a1}	5.79 ± 0.0^{a1}	5.79 ± 0.0^{a1}
3	5.69 ± 0.1^{b1}	5.53 ± 0.1^{b2}	5.46 ± 0.1^{c3}	5.43 ± 0.1^{c3}	5.46 ± 0.1^{c3}	5.41 ± 0.1^{c3}	5.39 ± 0.2^{b3}
6	$5.57\pm0.1^{c1,2}$	$5.59\pm0.1^{b1,2}$	$5.58\pm0.1^{b1,2}$	5.65 ± 0.1^{b1}	$5.57\pm0.1^{b1,2}$	5.56 ± 0.1^{b2}	5.36 ± 0.2^{b3}
9	5.56 ± 0.1^{c2}	5.72 ± 0.1^{a1}	5.60 ± 0.2^{b2}	5.79 ± 0.1^{a1}	5.57 ± 0.2^{b2}	5.61 ± 0.1^{b2}	5.34 ± 0.2^{b3}
24	5.01 ± 0.1^{d3}	5.44 ± 0.1^{c1}	4.64 ± 0.1^{d4}	5.04 ± 0.1^{d3}	5.25 ± 0.1^{d2}	4.26 ± 0.1^{d5}	3.79 ± 0.1^{d6}
48	$4.07\pm0.1^{e1,2}$	4.09 ± 0.1^{d1}	3.92 ± 0.1^{e3}	$3.93\pm0.1^{e2,3}$	$3.98\pm0.1^{e1,2,3}$	3.76 ± 0.1^{e4}	$3.98\pm0.1^{c2,3}$

Values in columns with same superscript letters are not significantly different ($p>0.05$)

Values in rows with same superscript numbers are not significantly different ($p>0.05$)

Time (h)	R2 (crust)	R6 (crust)	B30 (veins)	R25 (veins)	R40 (core)	W30 (core)	*Ped. acidilactici*
			Lactic acid (g/L)				
0	0.00±0.0^{c1}	0.00±0.0^{c1}	0.00±0.0^{c1}	0.00±0.0^{c1}	0.00±0.0^{c1}	0.00±0.0^{c1}	0.00±0.0^{c1}
3	0.02±0.1^{c1}	0.07±0.1^{c1}	0.09±0.03^{c1}	0.02±0.1^{c1}	0.07±0.03^{c1}	0.07±0.03^{c1}	0.05±0.01^{c1}
6	0.03±0.1^{c1}	0.09±0.02^{c1}	0.01±0.1^{c1}	0.05±0.01^{c1}	0.04±0.03^{c1}	0.04±0.04^{c1}	0.01±0.1^{c1}
9	0.02±0.03^{c1,2}	0.05±0.03^{c1,2}	0.08±0.02^{c1}	0.04±0.03^{c1,2}	0.06±0.03^{c1,2}	0.02±0.05^{c2}	0.05±0.02^{c1,2}
24	2.14±0.1^{b4}	0.75±0.3^{b4}	4.36±0.4^{b3}	1.84±0.5^{b4}	1.14±0.2^{b4}	7.99±0.9^{b2}	10.30±0.9^{b1}
48	13.63±0.8^{a2,3}	13.91±0.3^{a2,3}	14.06±0.8^{a2,3}	15.19±0.9^{a2}	13.64±0.4^{a2,3}	21.40±1.5^{a1}	12.21±0.8^{a3}
			Acetic acid (g/L)				
0	0.00±0.0^{b1}	0.00±0.0^{b1}	0.00±0.0^{b1}	0.00±0.0^{b1}	0.00±0.0^{b1}	0.00±0.0^{b1}	0.00±0.0^{c1}
3	0.15±0.5^{b1}	0.30±0.22^{b1}	0.26±0.2^{b1}	0.35±0.3^{b1}	0.33±0.3^{a,b1}	0.27±0.3^{b1}	0.60±0.2^{b1}
6	0.15±0.4^{b1}	0.47±0.4^{b1}	0.31±0.3^{a,b1}	0.24±0.3^{b1}	0.15±0.2^{a,b1}	0.16±0.1^{b1}	0.55±0.3^{b,c1}
9	0.15±0.3^{b1}	0.24±0.3^{b1}	0.37±0.3^{a,b1}	0.23±0.2^{b1}	0.26±0.3^{a,b1}	0.07±0.2^{b1}	0.66±0.1^{b1}
24	0.29±0.2^{b2}	0.18±0.2^{b2}	0.31±0.2^{a,b2}	0.55±0.2^{a,b1,2}	0.28±0.2^{a,b2}	0.39±0.3^{b1,2}	1.02±0.3^{a,b1}
48	1.75±0.5^{a1}	1.37±0.5^{a1,2}	0.99±0.3^{a1,2}	1.06±0.1^{a1,2}	0.71±0.2^{a2}	1.34±0.1^{a1,2}	1.43±0.3^{a1,2}

As evident from the plot of the data (Fig. 7.3), lactic acid production was strongly associated with the growth phase, whereas the relationship was weaker for acetic acid production. At 0-9 h, there was minimal lactic acid production for all the tested isolates probably due to lag phase (Passos *et al*., 1994) or due to inability of the isolates to produce it during this growth phase. Exponential growth phase was observed at 9-24 h, during which noticeable pH decrease was detected due to concomitant lactic acid production. Subsequently (24-48 h), further pH decrease was recorded to the lowest value of 3.76±0.01 from *Lb. plantarum* W30 obtained from the white core. The low pH range (3.76±0.01-4.09±0.1) correlated with inhibition of cell growth. Between 24-48 h however, maximal lactic acid production was obtained for all the isolates (Fig. 7.3) which is why this phase was associated with the greatest pH decrease. At 48 h, *Lb. plantarum* W30 obtained from the white core produced the highest level (21.40±1.5 g/L) of lactic acid, whereas *Ped. acidilactici* had the lowest (12.21±0.8 g/L) compared with other isolates examined (Table 7.1, $p<0.05$).

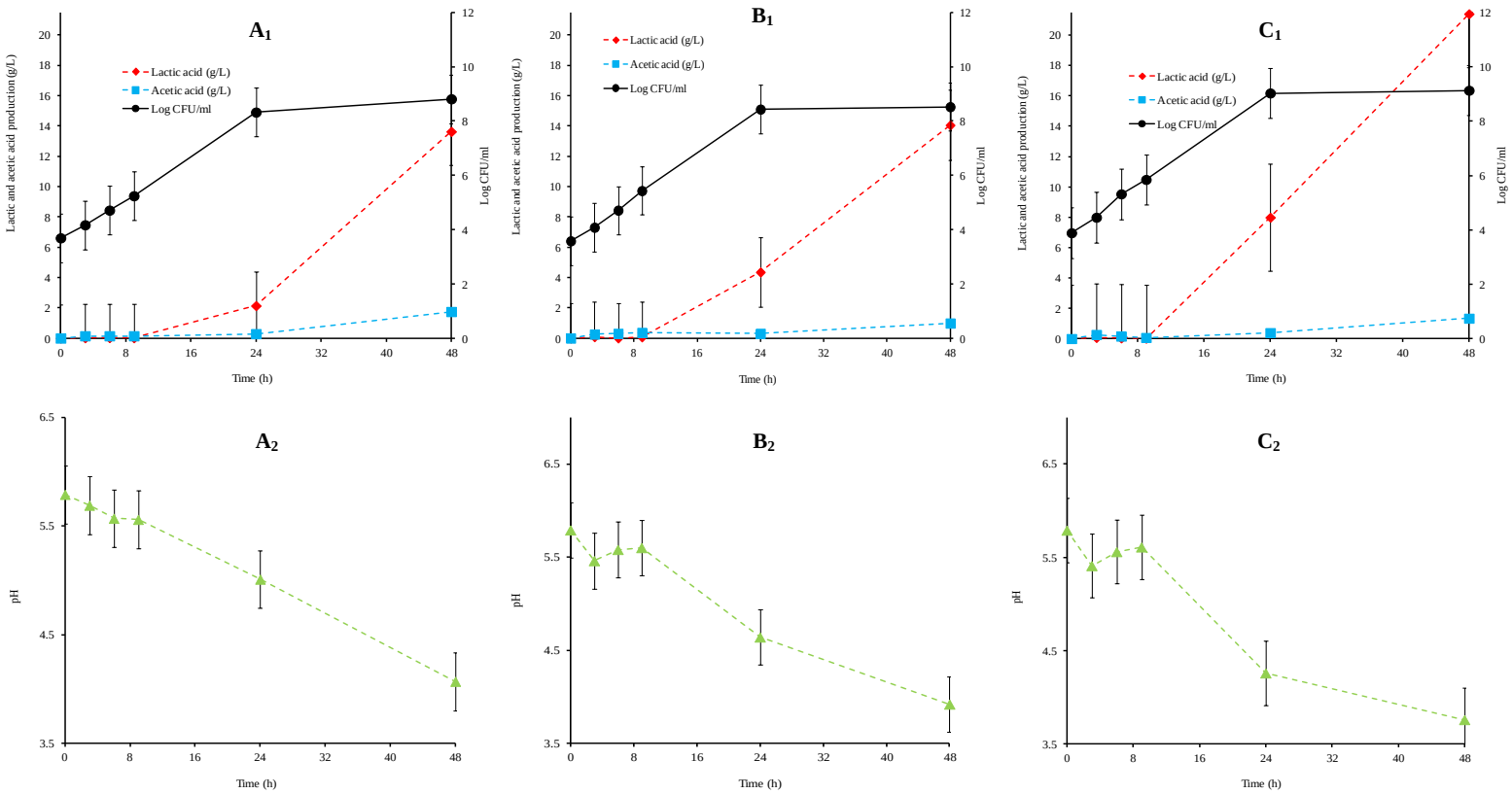


Figure 7.3 Data from Table 7.1 showing plots of lactic and acetic production (g/L), growth (log CFU/ml) (A_1-C_1) and pH changes (A_2-C_2) of representative *Lb. plantarum* isolates obtained from Stilton cheese. Isolates: (panel A) R2 from the outer crust, (panel B) B30 from the blue veins, (panel C) W30 from the white core. Solid line (black circle) log CFU/ml; broken line: (red diamond) lactic acid production, (blue square) acetic acid production; (green triangle) pH changes. The isolates were cultured in MRS broth for 48 h at 30^{o}C. Points are means of nine technical replicates obtained from three independent experiments and error bars are ± standard errors of the means, p = 0.05.

All isolates produced low levels (0.15±0.5-1.75 g/L) of acetic acid throughout the growth period. At 48 h, the lowest proportion of acetic acid (0.71±0.2 g/L) was obtained from *Lb. plantarum* R40 (white core), whereas the highest (1.75±0.5 g/L) was produced from R2 (outer crust) (Table 7.1, $p<0.05$). On the basis of these results, it was possible to suggest that during short term fermentation in MRS broth (24-48 h), the different geno-groups of *Lb. plantarum* caused acidification of the medium mainly due to lactic acid production, which is consistent with the view that the organism is homofermentative. *Lb. plantarum* isolates obtained from the white core produced the highest level of lactic acid whereas the lowest was recorded from isolates obtained from the outer crust. A vice versa relationship was noted for acetic acid production. Production of lactic acid is of significance in fermented foods where low pH due to acid formation is a desired flavour attribute as well as important for exerting antimicrobial effects in the final product (Ammor *et al*., 2006). Acetic acid has stronger antimicrobial properties than lactic acid and is often associated with a sour/vinegar note in ripened cheese. Therefore, the isolates of *Lactobacillus* were subsequently examined for possible growth interactions with *Lc. lactis* (Section 7.3.2) and how these could influence the aroma profile of fermented milk (Section 7.3.3).

7.3.2 Dynamics of population changes

NSLAB have been previously reported to influence flavour properties of ripened cheese positively (Grappin and Beuvier, 1997). Given that microbial aroma formation in milk follows a series of complex metabolic pathways driven by interactions between species (Gkatzionis *et al*., 2009), the current work aimed to establish how the possible population interactions (synergistic, competitive or antagonistic) between the different genotypes of *Lb. plantarum* (the dominant non-starter bacterial flora in Stilton cheese) and different strains of *Lc. lactis* could influence the profile of aroma compounds in milk. As there was little difference in acidification capabilities of *Lactobacillus* isolates obtained from a given cheese site (Table 7.1), one isolate from each site was considered for this part of the study. From the outer crust, isolate R2 was selected whereas B30 was chosen to represent the blue veins. None of the *Lactobacillus* isolates R40 and W30 (white core) was chosen for this part of the study. This is because preliminary work looking at flavour volatiles suggested that another isolate (W8) from this cheese section gave distinctive volatile spread. As these samples were to be used for growth and aroma analysis, it was regarded that W8 more appropriate (than R40 or W30) for examining the possible

differences in aroma properties of *Lactobacillus* isolates obtained from the different sites. In addition, only *Lactobacillus* isolates that were carriers of plantaricin EF genes were considered for the possible growth effects on the starter culture *Lc. lactis*. Isolate W8, like R2 and B30, carried these genes whereas W30 did not.

7.3.2.1 Early fermentation

During early fermentation (0-48 h) at 30^{o}C, the three *Lb. plantarum* isolates in pure culture had similar growth characteristics (Fig. 7.4A_1, p>0.05). Also, *Lc. lactis* subsp. *lactis* and *Lc. lactis* NCIMB 6681 (acid producers) in pure (Fig. 7.4B_1) or in mixed culture (Fig. 7.5A_2 and B_2) had indistinguishable growth patterns irrespective of the isolate of *Lb. plantarum* inoculated in the co-culture (p>0.05). At 48 h, the final cell concentration of acid-producing *Lc. lactis* strains in pure (Fig. 7.4B_1) or in mixed cultures (Fig. 7.5A_2 and B_2) was higher than that of *Lc. lactis* NCIMB 9918 (non acid-producer) in pure (Fig. 7.4B_1) or in mixed cultures (Fig. 7.5C_2) and *Lb. plantarum* isolates in pure (Fig. 7.4A_1) or in mixed cultures (Fig. 7.5A_1-C_1, p<0.05).

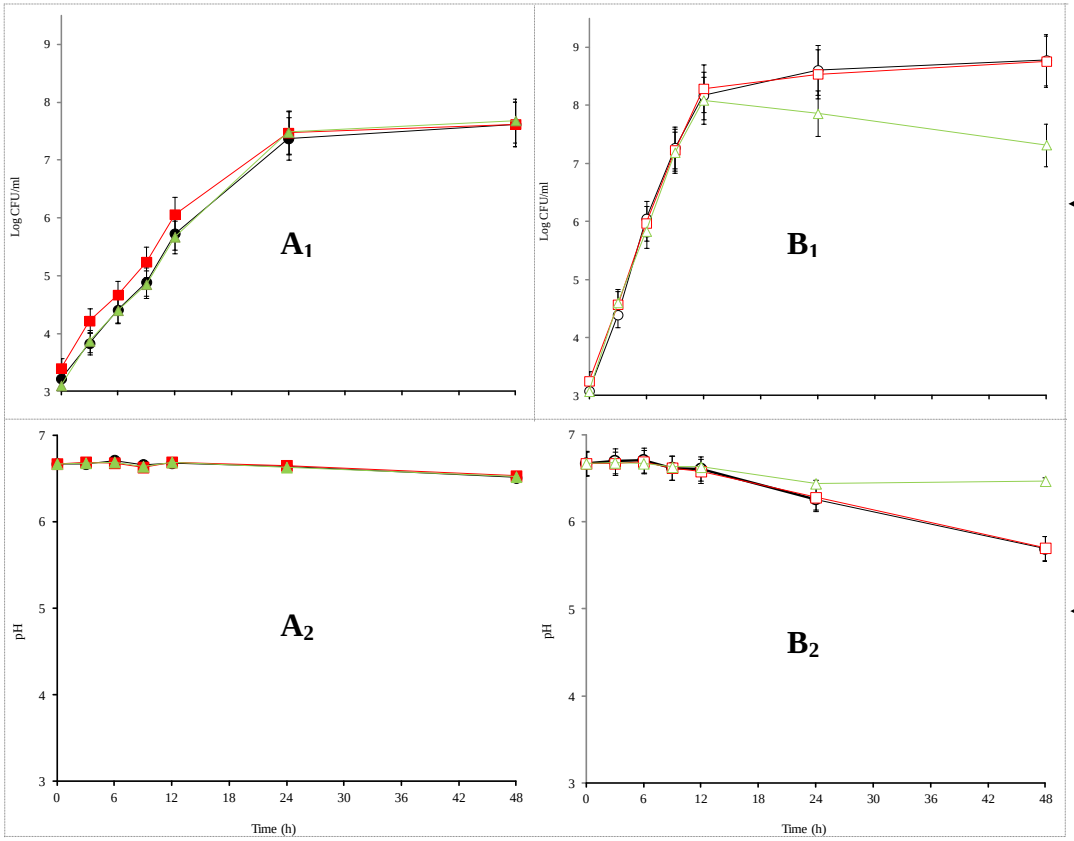


Fig. 7.4 Viable populations (A_1 & B_1) and pH changes (A_2 & B_2) of pure cultures of: (panel A) *Lb. plantarum* isolates obtained from: outer crust (strain R2, black circle), blue veins (B30, red square) and white core (W8, green triangle) of Stilton cheese; (panel B) *Lc. lactis* NCIMB 6681 (open black circle), *Lc. lactis* subsp. *lactis* (UNFCC, open red square) and *Lc. lactis* NCIMB 9918 (open green triangle). Strains were cultured in cows' milk for 48 h at 30°C. Enumeration was performed on Rogosa (for lactobacilli) and difference in counts between Rogosa and MRS agar (for lactococci). Points are means of nine technical replicates obtained from three independent experiments and error bars are ± standard errors of the means, $p = 0.05$.

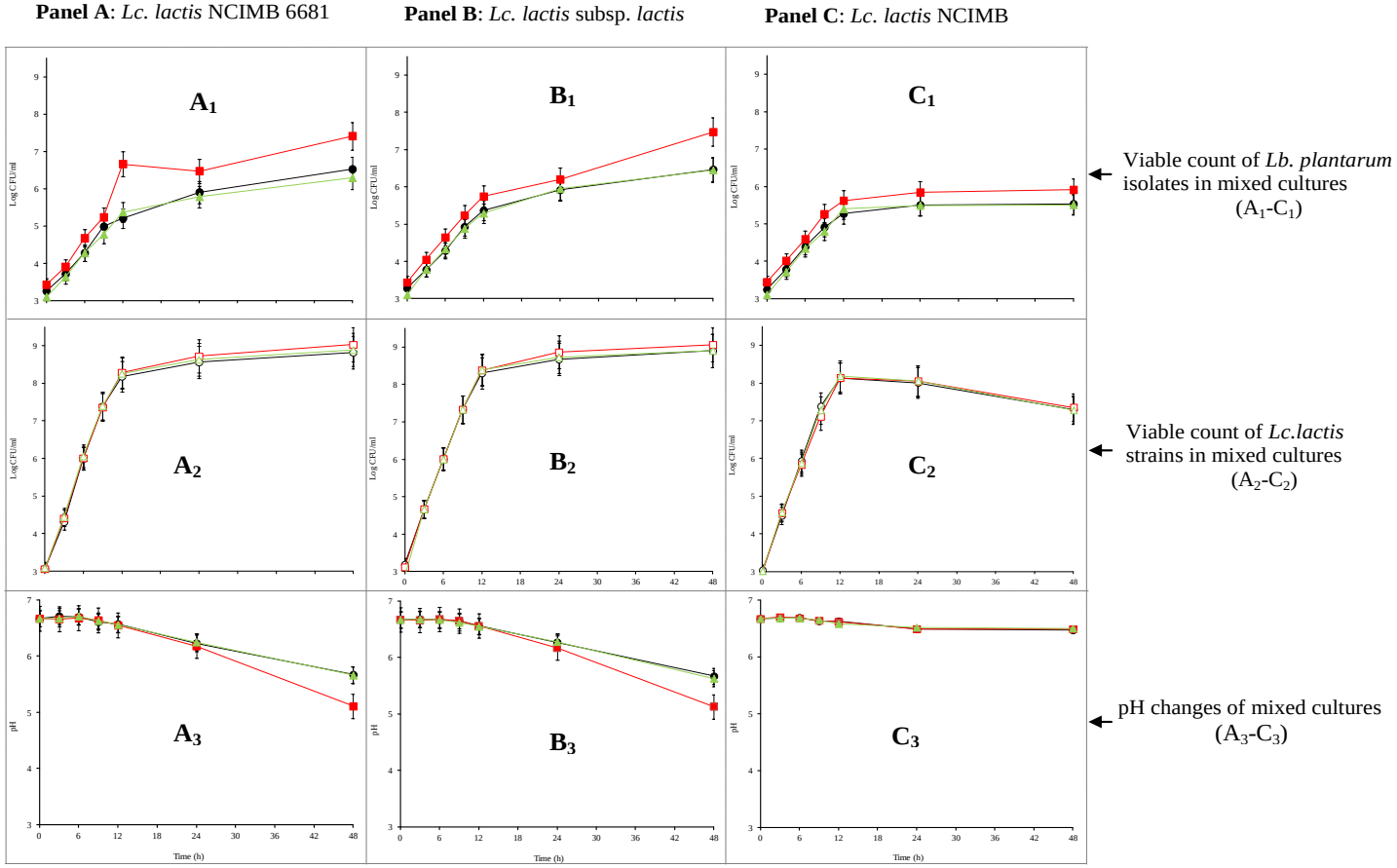


Fig. 7.5 Viable populations of (A_1-C_1) *Lb. plantarum* isolates obtained from outer crust (strain R2, black circle), blue veins (B30, red square) and white core (W8, green triangle) of Stilton cheese in co-culture with: (panel A) *Lc. lactis* NCIMB 6681; (panel B) *Lc. lactis* subsp. *lactis*; (panel C) *Lc. lactis* NCIMB 9918. (A_2-C_2) viable populations of lactococci strains, (A_3-C_3) pH changes of the mixed cultures. Enumeration was performed on Rogosa (for lactobacilli) and difference in counts between Rogosa and MRS agar (for lactococci). Points are means of three independent experiments and error bars are ± standard errors of the means, $p = 0.05$.

Different genotypes of *Lb. plantarum* had no significant effect on the growth characteristics of the individual lactococci strains in mixed culture (p>0.05). In general, *Lc. lactis* grew rapidly in milk to reach 8-9 $\log_{10}$ CFU/ml at 12-48 h (Fig. 7.4B_1 & 7.5A_2-C_2). The viable counts of acid-producing lactococci remained stable at this level for both pure (Fig. 7.4B_1) and mixed culture (Fig. 7.5A_2-B_2) treatments, whereas that of *Lc. lactis* NCIMB 9918 (non acid-producer) gradually decreased to 6-7 $\log_{10}$ CFU/ml (Fig. 7.4B_1 & 7.5C_2). For each of the acid producers and non acid-producing lactococci, there was no significant difference in overall growth patterns of the strains in pure or in mixed cultures (p>0.05) although the acid producers attained a higher final count at 48 h than the non acid producing *Lc. lactis*.

At 48 h, *Lb. plantarum* isolates had better overall growth in pure culture than in mixed culture (Fig. 7.6A-C). However, the final cell density of the isolates was higher when they were co-cultured with the acid-producing *Lc. lactis* subsp. *lactis* and *Lc. lactis* NCIMB 6681 than with *Lc. lactis* NCIMB 9918 (non acid-producer) (Fig. 7.6A-C, p<0.05). At entry into stationary phase, the viable count of *Lactobacillus* isolates was higher when they were co-cultured with acid-producing lactococci compared with that obtained when co-inoculated with the non acid-producing *Lc. lactis* (p<0.05).

During this growth phase (0-48 h), the viable count of *Lb. plantarum* B30 from the blue veins (Fig. 7.6B) was higher than that of the isolates obtained from the outer crust (Fig. 7.6A, p<0.05) and the white core (Fig. 7.6C). Although *Lb. plantarum* B30 grew better when co-cultured with acid-producing lactococci (Fig. 7.6B), the isolate also exhibited better growth characteristics than other *Lb. plantarum* isolates in mixed culture (Fig. 7.6A-C). Therefore this difference may be due to a difference in competitiveness of this isolate rather than it having an inherently greater growth rate than the other *Lactobacillus* isolates. Pure culture data showed no significant difference in the growth patterns of the *Lactobacillus* isolates over the 48 h period as evident in Fig. 7.6 (p>0.05).

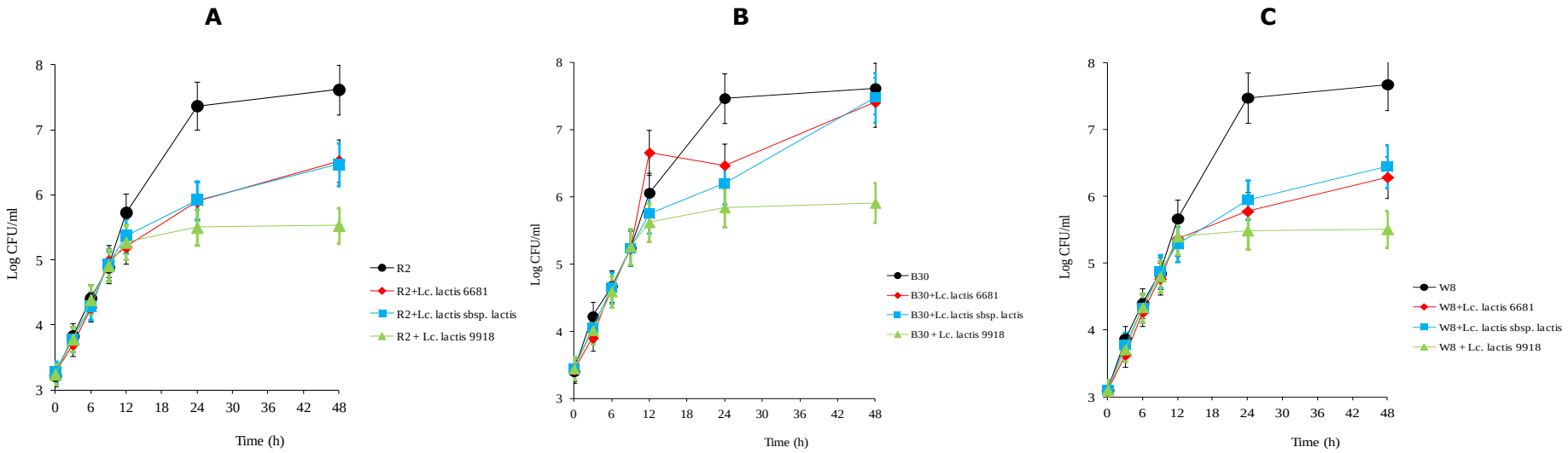


Figure 7.6 Replotting of data from Figs. 7.4A$_1$ and 7.5A$_1$-C$_1$ showing comparative plots of viable populations of *Lb. plantarum* isolates alone and in co-culture. (A) isolate R2 from the outer crust, (B) isolate B30 from the blue veins, and (C) isolate W8 from the white core. The isolates were grown in cows' milk for 48 h at 30^{o}C as pure cultures (black circle), or as co-cultures with: (red diamond) *Lc. lactis* NCIMB 6681, (blue square) *Lc. lactis* subsp. *lactis*, (green triangle) *Lc. lactis* NCIMB 9918. Enumeration was performed on Rogosa (for lactobacilli) and difference in counts between Rogosa and MRS agar (for lactococci). Points are means of nine technical replicates obtained from three independent experiments and error bars are ± standard errors of the means, $p = 0.05$.

Lb. plantarum isolates in pure culture (Fig. 7.4A_2) or in co-culture with *Lc. lactis* NCIMB 9918 (Fig. 7.5C_3) showed no significant pH drop (p>0.05) probably because of the high buffering capacity of the milk medium (Robinson, 1990). Similarly, there was no pH drop in the pure culture of *Lc. lactis* NCIMB 9918 (Fig. 7.4B_2). Pure culture of *Lc. lactis* subsp. *lactis* and *Lc. lactis* NCIMB 6681 reduced the pH from 6.5 to 5.5 (Fig. 7.4B_2, p<0.05). *Lb. plantarum* R2 obtained from the outer crust and W8 from the white core in co-culture with the acid-producing *Lc. lactis* strains (Fig. 7.5A_3-B_3) had a similar pH drop to that of these lactococci strains alone (Fig. 7.4B_2). However, the acid-producing *Lc. lactis* strains in co-culture with *Lb. plantarum* B30 from the blue veins showed a significantly greater pH drop (p<0.05) (Fig. 7.5A_3-B_3) than all other treatments. The results are compatible with the data obtained from Section 7.3.1 (Table 7.1) and further highlight that *Lactobacillus* isolate B30 from the blue veins had better acid producing ability than isolate R2 from the outer crust. These results suggested that during early fermentation in milk, there was some suppression in overall growth characteristics of the different genotypes of *Lb. plantarum* due to low pH arising from acidification by *Lc. lactis*. However, isolate B30 tolerated this effect more than R2 and W8, adapting to attain a final viable count similar to that obtained in pure culture (Fig. 7.6B). The increased suppression of *Lactobacillus* isolates in co-culture with *Lc. lactis* NCIMB 9918 may be attributed to factors other than pH drop as this lactococci strain was a non acid-producer. It was also noticeable that pH changes at 48 h were mainly caused by growth of acid-producing lactococci rather than the non acid-producing lactococci strain and *Lb. plantarum* isolates. It is possible that the *Lactobacillus* isolates and *Lc. lactis* NCIMB 9918 had produced some acid but this was buffered by the milk medium due to its high protein content (Robinson, 1990).

7.3.2.2 Long term ripening

The effect of low temperature (18^{o}C) and salt addition (3.5%, w/v) on the growth characteristics of *Lb. plantarum* isolates in pure or in co-culture with different strains of *Lc. lactis* is shown in Figs. 7.7-7.9. The viable counts of the *Lb. plantarum* isolates in pure (Fig. 7.7A_1) or in mixed cultures (Fig. 7.8A_1-C_1) remained stable at 7-8 $\log_{10}$ CFU/ml in both salted and unsalted milk. The viable counts of *Lc. lactis* NCIMB 9918 (non acid-producer) in pure (Fig. 7.7B_1) or in mixed cultures (Fig. 7.8C_2) also remained stable at 6-7 $\log_{10}$ CFU/ml. However, the viable count of the pure cultures of *Lc. lactis*

subsp. *lactis* and *Lc. lactis* NCIMB 6681 (acid producers) in salted and unsalted milk decreased from 9 to 2-3 $\log_{10}$ CFU/ml (Fig. 7.7B_1, $p<0.05$) by 12 weeks.

After one week in salted milk, the viable count of acid-producing lactococci (APL) in co-culture with *Lb. plantarum* B30 rapidly declined from 9 to 6.5 $\log_{10}$ CFU/ml followed by gradual decrease, whereas the population of these lactococci remained relatively stable at 8-9 log10 CFU/ml in unsalted milk or when they were co-cultured with the other *Lb. plantarum* isolates (Fig. 7.8A_2 and B_2). Further incubation (2-7 weeks) resulted in similar overall death patterns for APL lactococci strains irrespective of salt addition or the isolate of *Lb. plantarum* with which they were co-cultured. At 12 weeks, APL in mixed culture (Fig. 7.8A_2-B_2) were undetectable (LOD, 3 $\log_{10}$ CFU/ml). Although results of early fermentation (48 h at 30^{o}C) showed that the final cell density of *Lb. plantarum* isolates in mixed culture was lower when they were co-cultured with *Lc. lactis* NCIMB 9918 (non acid-producer; Fig. 7.6A-C), further incubation (4 weeks) at 18^{o}C produced an increase in growth of lactobacilli isolates in co-culture with this lactococcal strain (Fig. 7.9A-C, $p<0.05$), attaining a similar final level to growth with the other lactococci.

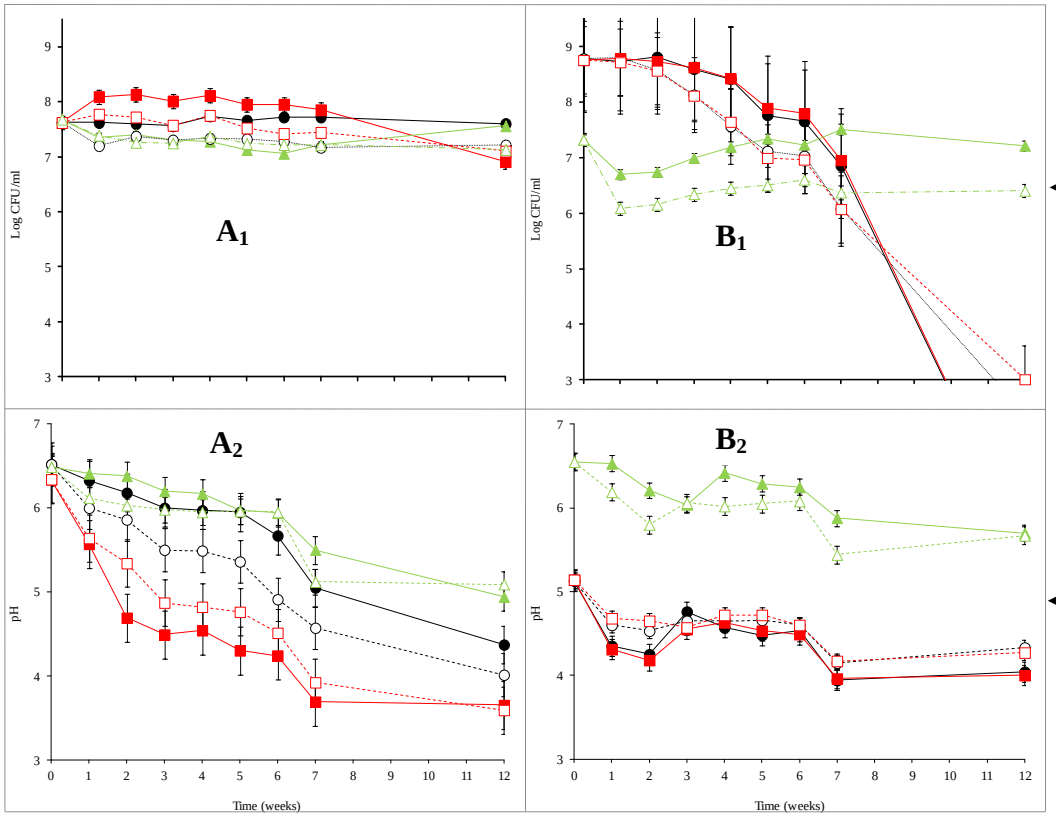


Fig. 7.7 Viable populations (row 1) and pH changes (row 2) of pure cultures. (panel A) *Lb. plantarum* isolates: (black circle) isolate R2, outer crust; (red square) isolate B30, blue veins; (green triangle) isolate W8, white core. (panel B) *Lactococcus* strains: (black circle) *Lc. lactis* NCIMB 6681; (red square) *Lc. lactis* subsp. *lactis*; (green triangle) *Lc. lactis* NCIMB 9918. Strains were cultured in un-salted cows' milk (solid lines, closed symbols) or in salted cows' milk (broken lines, open symbols) for 12 weeks at 18^{o}C. Enumeration was performed on Rogosa (for lactobacilli) and difference in counts between Rogosa and MRS agar (for lactococci). Points are means of nine technical replicates obtained from three independent experiments and error bars are ± standard errors (SE) of the means, $p = 0.05$.

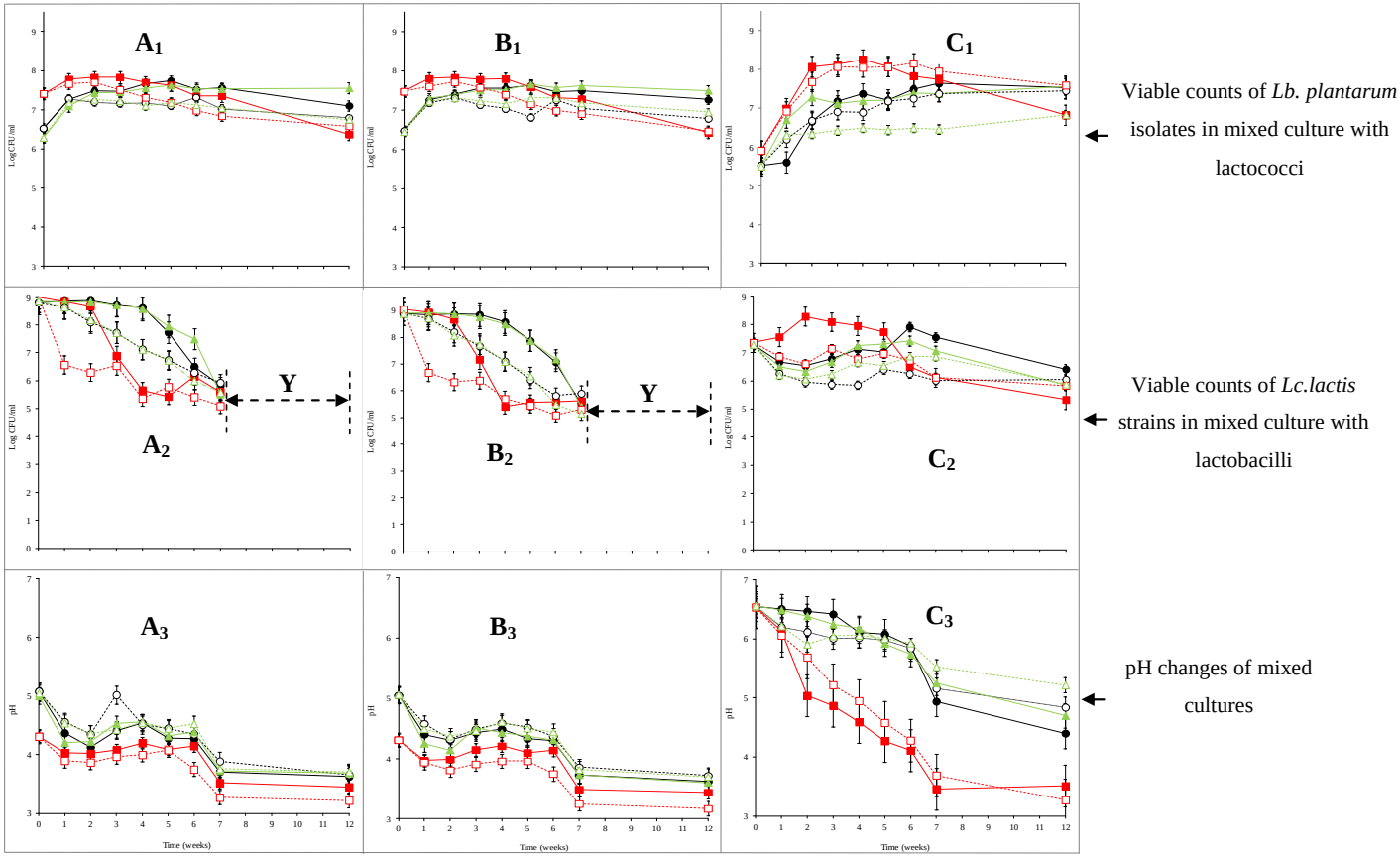


Fig. 7.8 Viable populations (A_1-C_1 and A_2-C_2) and pH changes (A_3-C_3) of mixed cultures. (A_1-C_1) *Lb. plantarum* isolates obtained from Stilton cheese: (black circle) isolate R2, outer crust; (red square) isolate B30, blue veins; (green triangle) isolate W8, white core. (A_2-C_2) *Lactococcus* count from the respective *Lactobacillus* co-culture: (A_2) *Lc. lactis* NCIMB 6681, (B_2) *Lc. lactis* subsp. *lactis*, (C_2) *Lc. lactis* NCIMB 9918. Strains were grown in un-salted cows' milk (solid lines, closed symbols) or in salted cows' milk (broken lines, open symbols) for 12 weeks at 18^{o}C. Enumeration was performed on Rogosa (for lactobacilli) and difference in counts between Rogosa and MRS agar (for lactococci). Points are means of nine technical replicates obtained from three independent experiments and error bars are ± SE, $p = 0.05$. (**Y**) tiny colonies of *Lc. lactis* could not be enumerated on agar plates (in mixed culture) below the dilution level of 10^{-3} due to confluence of the large *Lactobacillus* colonies.

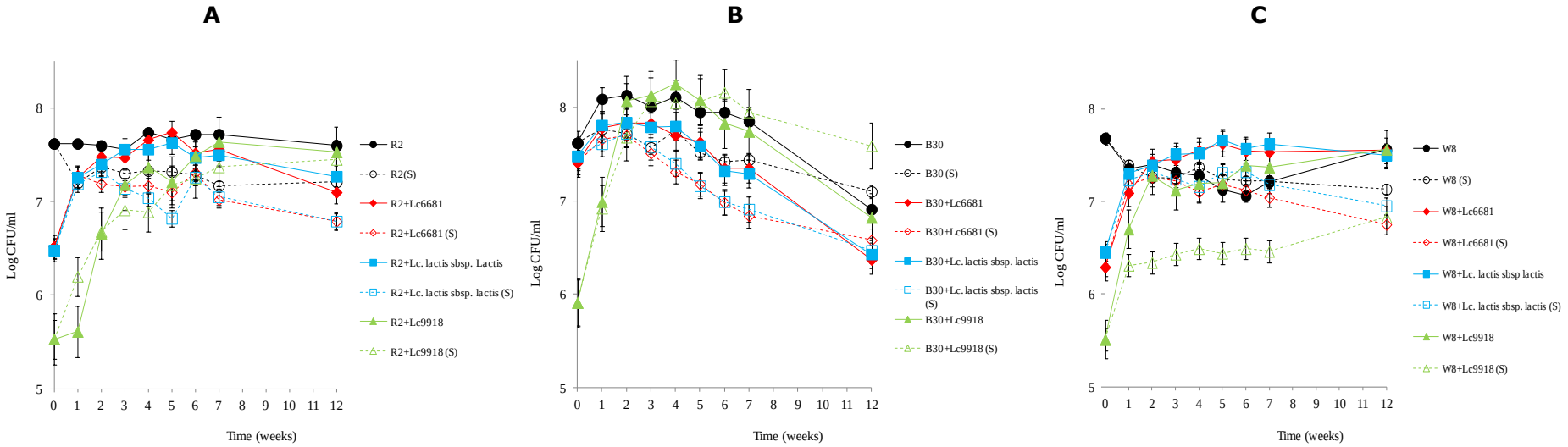


Figure 7.9 Replotting of data from Figs. 7.7A$_1$ and 7.8A$_1$-C$_1$ showing comparative plots of viable populations of *Lb. plantarum* isolates. (A) isolate R2 from the outer crust, (B) isolate B30 from the blue veins, and (C) isolate W8 from the white core. The isolates were grown in un-salted cows' milk (solid lines, closed symbols) or in salted cows' milk (broken lines, open symbols) for 12 weeks at 18^{o}C as pure cultures (black circle), or as co-cultures with: (red diamond) *Lc. lactis* NCIMB 6681, (blue square) *Lc. lactis* subsp. *lactis*, (green triangle) *Lc. lactis* NCIMB 9918. Enumeration was performed on Rogosa (for lactobacilli) and difference in counts between Rogosa and MRS agar (for lactococci). Points are means of nine technical replicates obtained from three independent experiments and error bars are ± standard errors of the means, $p = 0.05$.

At 12 weeks, the presence of salt caused some reduction of the viable count of the non acid-producing lactococcal strain in pure culture compared with the unsalted sample (Fig. 7.7B_1). In mixed culture however, salt had no effect on this *Lactococcus* strain (Fig. 7.8C_2, p>0.05). Salt also showed some inhibition of the growth of acid-producing *Lc. lactis* strains; this effect was mainly evident at 2-6 weeks in pure culture (Fig. 7.7B_1, p<0.05), and at 1-6 weeks in mixed culture (Fig. 7.8A_2-B_2, p<0.05). However, the main factor which influenced *Lactococcus* survival was acid production, as the acid producers showed much poorer survival. Hence pH, and not salt, appears to be the main factor causing *Lactococcus* starter die off in maturing cheeses.

As evident in Figs. 7.7 and 7.8, none of the *Lactobacillus* isolates had a marked effect on the growth patterns of lactococcal strains in mixed culture regardless of salt addition or not (p>0.05). This is due to the fact that the overall growth pattern of the latter *Lactococcus* strains in pure culture was similar to that of the mixed culture and followed a decreasing trend after 2-4 weeks in both the salted and unsalted milk, with and without co-inoculation with *Lb. plantarum* isolates.

However, variable results were obtained for *Lb. plantarum* isolates when they were co-cultured with the different strains of *Lc. lactis* in presence or absence of salt (Fig. 7.9A-C). In the case of pure and mixed cultures in unsalted milk, there was no significant difference in the final cell density of *Lactobacillus* isolates R2 and W8 obtained from the outer crust and white core, respectively (p>0.05; Fig. 7.9A & C). In salted milk however, *Lb. plantarum* R2 had poorer growth when co-cultured with *Lc. lactis* NCIMB 6681 and *Lc. lactis* subsp. *lactis* (acid producers) compared with the non acid-producing *Lc. lactis* NCIMB 9918 (Fig. 7.9A). Salt addition also led to poorer growth in the pure culture of *Lb. plantarum* W8 as well as in all its co-inoculations with *Lc. lactis* strains (Fig. 7.9C, p<0.05). For *Lb. plantarum* B30 (blue veins), salt addition led to better growth when it was co-cultured with *Lc. lactis* NCIMB 9918 (p<0.05); for the rest of the samples, there was no significant difference in the final cell density of salted and unsalted cultures of this *Lactobacillus* isolate (p>0.05; Fig. 7.9B). Overall at 1-4 weeks, *Lb. plantarum* B30 grew better both in pure and mixed cultures than the other *Lactobacillus* isolates as evident from Fig. 7.9.

Similar with the results of HPLC (Section 7.3.1), pH changes at 18^{o}C showed significant differences in acidification capabilities of the lactobacilli isolates and lactococcal strains (p<0.05). In pure culture, *Lb. plantarum* R2 (outer crust) and W8 (white core) produced significantly less acid than isolate B30 (blue veins) (p<0.05; Fig. 7.7A_2). *Lc. lactis* subsp. *lactis* and *Lc. lactis* NCIMB 6681 in pure cultures showed more pH reduction than *Lc. lactis* NCIMB 9918 (Fig. 7.7B_2). Salt addition had no significant effect on the pH changes of all pure culture inoculations (p>0.05). In mixed cultures however, the co-culture of the *Lb. plantarum* isolates and acid-producing lactococcal strains (Fig. 7.8A_3-B_3) produced more acid than that of *Lb. plantarum* isolates with *Lc. lactis* NCIMB 9918 (Fig. 7.8C_3). Co-culture of *Lc. lactis* NCIMB 9918 and *Lb. plantarum* B30 produced significantly more acid (p<0.05) than with isolates R2 and W8 (Fig. 7.8C_3). Salt addition had no significant effect on pH changes of the co-cultures (p>0.05). In general, pH drop during long term fermentation was faster in mixed cultures, highlighting the synergistic nature of some of the LAB species combinations examined. This synergism was demonstrated by a larger pH drop in the co-cultures compared with the additive effects of the individual pure cultures constituting the co-cultures. The microbial strains were subsequently evaluated for proteolysis and lipolysis (Section 7.3.3) and then examined for aroma profiles (Section 7.3.4).

7.3.3 Production of proteolytic and lipolytic enzymes

Lipolytic and proteolytic systems play an essential role in lipid and nitrogen metabolism of LAB in milk (Moulay *et al*., 2006). The process enhances bacterial growth and also contributes to flavour formation of the fermented milk (Liu *et al*., 2010). In cheese, these biochemical properties were previously attributed to endogenous milk enzymes but enzymes of fortuitous microorganisms and NSLAB have also been presumed to play a major role (Di Cagno *et al*., 2002). The objective of this part of the study was to identify *Lb. plantarum* isolates with lipolytic and proteolytic activity and to determine whether or not these properties could have an influence on the type and levels of volatile aroma compounds produced by the isolates in milk.

Different genotypes of *Lb. plantarum* were selected from the sampled sites (outer crust, 7 isolates; blue veins, 22 isolates; white core, 21 isolates; n=50) and screened for proteolytic and lipolytic activity on casein and tributyrin agar respectively, as described in §3.7. Isolates were grown in MRS broth overnight at 30^{o}C and 20 µl spotted on the agar

plates in triplicate. Plates were separately incubated for 1-3 weeks at 18, 30 and 37^{o}C, and examined for clear halos. Casein agar was formulated to contain 1% casein hydrolysate whereas tributyrin agar was constituted to contain 1% tributyrin. Clear halo formation was the result of hydrolysis of casein and tributyrin in the media. The diameter of the clear halos was considered as proportional to the extent of lipolytic/proteolytic activity of the isolates. *Yarrowia lipolytica* and *E. coli* were used as the positive and negative controls, respectively.

In all cases, *Lb. plantarum* isolates showed no enzymatic activity/clear halo formation implying the isolates did not possess either of these biochemical properties. Representative examples of clear halo formation on casein and tributyrin agar for *Yarr. lipolytica* are shown in Appendix 7.1. From this, it was evident that *Yarr. lipolytica* showed extensive proteolysis and lipolysis as demonstrated by pronounced hydrolysis of the media components. However, the results only became apparent after incubating the plates for 4-10 days even though yeast growth was evident on the agar plates within 1-2 days of incubation. This was probably due to degradation of the media components after exhaustion of carbon and nitrogen sources during the logarithmic growth phase of the yeast strain (Van den Tempel and Jakobsen, 2000).

7.3.4 Volatile aroma profile analysis

7.3.4.1 Early fermentation

The effect of growth interactions obtained from §7.3.2, on the volatile profiles of milk was examined further. Aliquots (5 ml) were taken at 24 and 48 h, transferred into 20 ml headspace vials and analysed for volatile profiles using SPME GC-MS as in §3.9.2. A range of compounds was detected in the headspace of milk samples inoculated with pure or mixed cultures of the *Lactobacillus* isolates (Appendix 7.7). The SPME GC-MS chromatograms consisted of 23 main peaks including six ketones, five alcohols, five esters, four carboxylic acids and three aldehydes as shown in Appendices 7.2-7.5.

At 24-48 h, the volatile profiles of pure cultures of *Lb. plantarum* strains and of mixed cultures with *Lc. lactis* NCIMB 9918 (Lc99) were similar to that of milk (Appendices 7.2-7.3). For the other samples, of the 23 compounds detected, only methyl aldehydes and methyl alcohols showed a significant increase relative to the control (Fig. 7.10). These compounds were mainly associated with the pure culture of *Lc. lactis* subsp. *lactis* (LcL).

The levels were amplified when the latter was co-cultured with the *Lb. plantarum* isolates. Co-culture of LcL with *Lb. plantarum* B30 from the blue veins gave the highest level of these branched volatiles compared with isolates R2 and W8 from the outer crust and white core, respectively. The dominant alcohol was 3-methyl butanol and was mostly detected ($6.2x10^7$ area units, AU) in the B30-LcL co-culture. Three-methyl butanal was the aldehyde present at the highest level ($1.3x10^8$ AU) and mainly associated with this mixed culture. Of all the ketones detected, only 3-hydroxy 2-butanone (acetoin) showed substantial increase and was mainly found in the pure culture of *Lc. lactis* NCIMB 9918 ($1.2x10^5$ AU) as well as its corresponding co-cultures with *Lb. plantarum* isolates (Appendix 7.2).

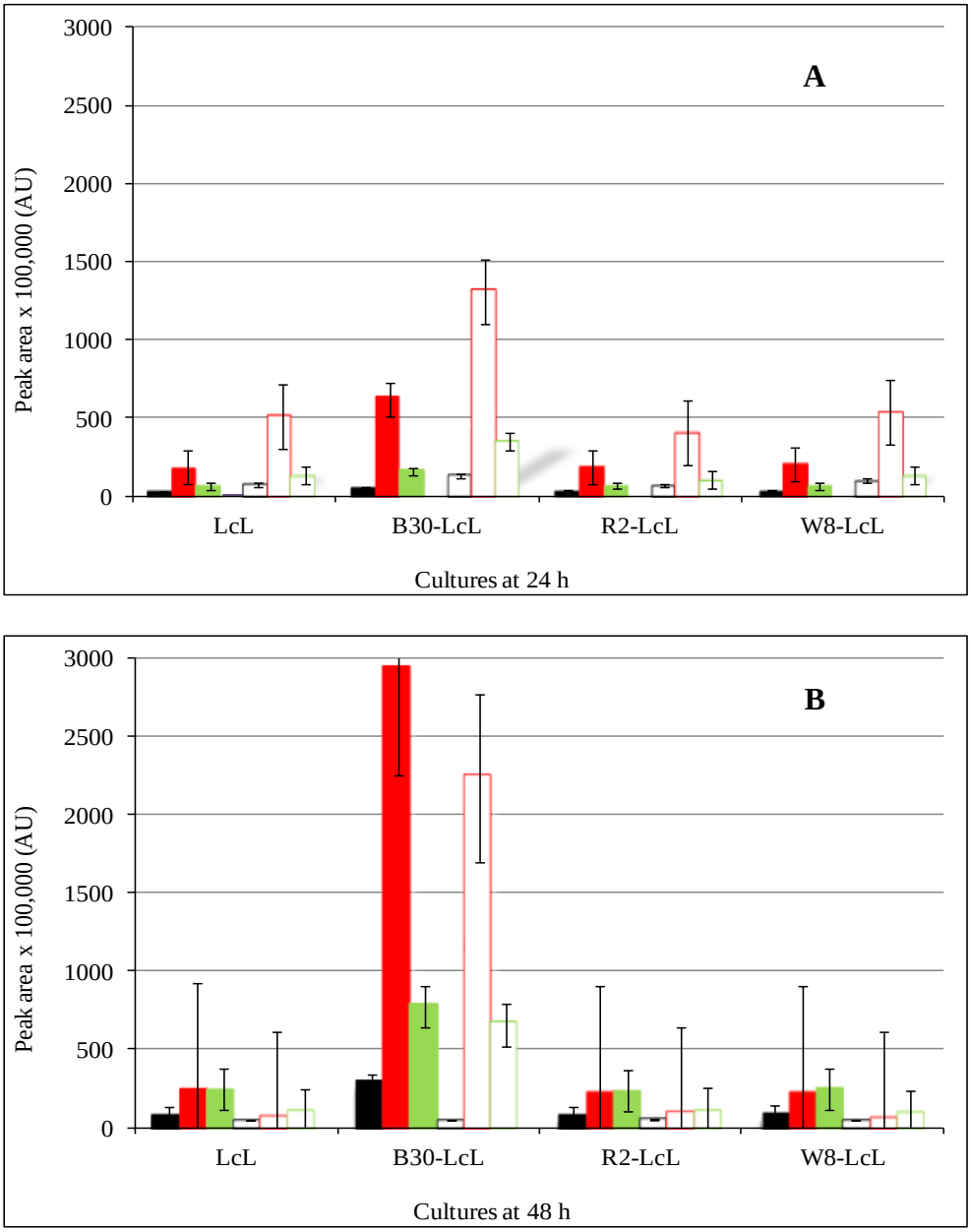


Figure 7.10 Levels of alcohols (closed bars) and corresponding aldehydes (open bars) detected in milk inoculated with pure or mixed cultures of *Lb. plantarum* B30 (blue veins), R2 (outer crust), W8 (white core), with *Lc. lactis* subsp. *lactis* (LcL). Samples were incubated at 30^{o}C for (A) 24 h, and (B) 48 h. Compounds: (black) 2-methyl propanol/ 2-methyl propanal, (red) 3-methyl butanol/ 3-methyl butanal, (green) 2-methyl butanol/ 2-methyl butanal. Values are means of three independent experiments and error bars are ± standard errors of the means, p = 0.05. The compounds were below threshold levels in the samples not shown on the plot.

At 48 h, these same samples showed an increase in levels of methyl alcohols and aldehydes (Fig. 7.10B). The B30-LcL co-culture had significantly higher levels of 3-methyl butanol ($2.9x10^8$ AU) and 2-methyl propanol ($2.9x10^7$ AU) compared with the pure culture of LcL or its co-culture with other *Lb. plantarum* isolates ($p<0.05$). Similarly, 2-methyl butanal ($6.6x10^7$ AU) and 3-methyl butanal ($2.2x10^8$ AU) increased in the B30-LcL co-culture at 48 h, and the levels were greater than for the pure culture of LcL or its co-culture with other *Lb. plantarum* isolates. However, the level of 2-methyl propanal ($4.6x10^6$ AU) in the B30-LcL co-culture, and that of 3-methyl butanal ($7.8x10^6$ AU) in the pure culture of LcL (Fig. 7.10B) decreased compared with that ($1.3x10^7$ and $5.1x10^7$ AU, respectively) found at 24 h (Fig. 7.10A). Butanoic ($3.1x10^5$ AU) and hexanoic acid ($8.1x10^5$ AU), and their corresponding methyl esters showed progressive increase in the pure culture of LcL or in its co-culture with *Lb. plantarum* B30 in comparison with the control and other samples (Appendix 7.3).

7.3.4.2 Long term ripening at 18^{o}C

7.3.4.2.1 Alcohols

Similar with the results obtained in Section 7.3.4.1, methyl propanol, 2-methyl butanol and 3-methyl butanol were the most abundant alcohols showing further increase during ripening at 18^{o}C (Fig. 7.11). At 4 and 7 weeks, these branched alcohols were mainly found in the pure cultures of *Lb. plantarum* B30 (blue veins) and W8 (white core) as well as in the pure culture of *Lc. lactis* subsp. *lactis* (LcL) and its co-inoculations with all *Lb. plantarum* isolates (Fig. 7.11). At 4 weeks (Fig. 7.11A), 2-methyl propanol was mostly detected in the pure culture of *Lb. plantarum* B30; but by 7 weeks, maximal levels of the compound were found in the B30-Lc99 co-culture. Levels of this compound at 4 weeks were reduced when the latter *Lactobacillus* isolate was co-cultured with the different strains of *Lc. lactis*. Salt presence had a marginal effect on the production of alcohols at 4 and 7 weeks in the pure culture of LcL and in its co-culture with all *Lb. plantarum* isolates (Fig. 7.11). Salt addition also suppressed ethanol synthesis in these cultures (Appendix 7.4-7.5).

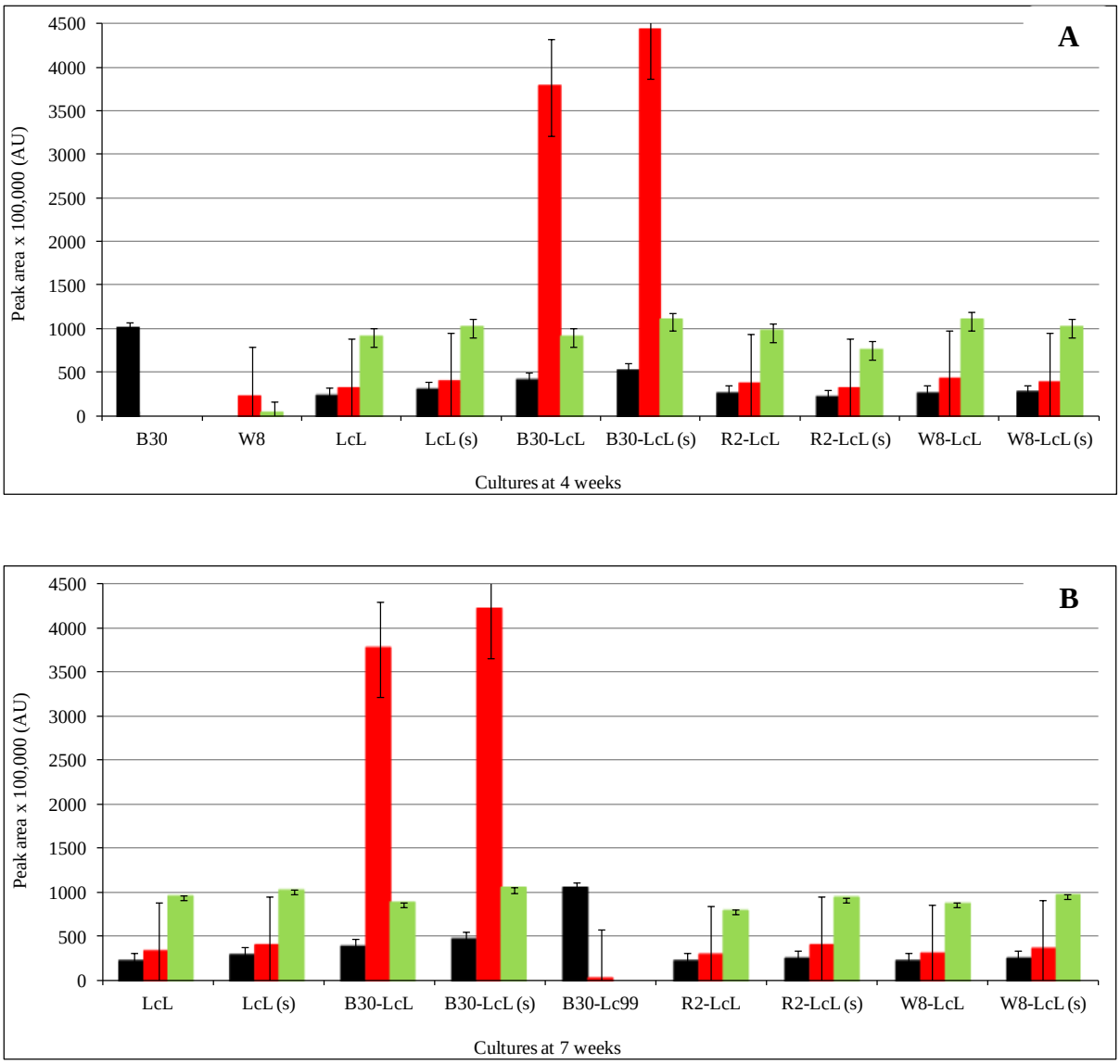


Figure 7.11 Levels of alcohols detected in milk inoculated with pure or mixed cultures of *Lb. plantarum* B30 (blue veins), R2 (outer crust), W8 (white core) with *Lc. lactis* subsp. *lactis* (LcL) or *Lc. lactis* NCIMB 9918 (Lc99). Samples were incubated at 30°C for 48 h and subsequently ripened at 18°C for (A) 4 weeks, and (B) 7 weeks. Compounds: (black) 2-methyl propanol, (red) 3-methyl butanol, (green) 2-methyl butanol. (s): salt (3.5%, w/v) was added to the sample at 48 h. Values are means of three independent experiments and error bars are ± standard errors of the means, p = 0.05. The compounds were below threshold levels in the samples not shown in the plot.

7.3.4.2.2 Carboxylic acids

The levels of organic acids showed an increase at 4-7 weeks compared to those obtained at 48 h. Analysis of pure culture data at 4 weeks revealed that acetic (Fig. 7.12) and hexanoic acid (Appendix 7.4) were the dominant organic acids produced. Acetic acid was mostly found in the pure culture of *Lb. plantarum* B30 (blue veins) as well as in all co-inoculations involving this *Lactobacillus* isolate, irrespective of the *Lactococcus* strain with which it was co-cultured (Fig. 7.12). Salt addition had no significant effect on acetic acid production in these cultures. Hexanoic acid was mainly detected in the pure culture of *Lb. plantarum* W8 (white core) but salt addition suppressed synthesis (Fig. 7.12). The substantially high level of hexanoic ($1.7x10^7$ AU) and butanoic acid ($1.2x10^7$ AU) recorded from the pure culture of *Lb. plantarum* W8 in unsalted milk at 4 weeks (Appendix 7.4) rapidly declined to low levels by the end of the ripening period, probably due to biochemical conversion into corresponding ketones and esters (Lawlor *et al*., 2003).

At 7 weeks, acetic (Fig. 7.12) and octanoic acid (Appendix 7.5) were the dominant acids detected. Acetic acid was mainly detected in samples co-inoculated with *Lb. plantarum* isolates and either of the *Lc. lactis* strains. With the exception of *Lb. plantarum* B30 (blue veins), all pure culture inoculations had low levels of the compound. The greatest acetic acid synthesis ($1.4x10^8$ AU) was recorded from the pure culture of *Lb. plantarum* B30 and in the co-culture of this *Lactobacillus* isolate with *Lc. lactis* NCIMB 9918 ($1.1x10^8$ AU) as evident in Fig. 7.12. Salt addition reduced acetic acid production in the pure culture of *Lb. plantarum* B30 and in all co-inoculations involving *Lb. plantarum* isolates and *Lc. lactis* NCIMB 9918. Octanoic acid was mainly found in pure cultures of *Lb. plantarum* R2 ($2.9x10^7$ AU) and W8 ($1.8x10^9$ AU), and in their corresponding co-cultures with *Lc. lactis* NCIMB 9918 (Lc99) (Appendix 7.5). Salt addition reduced octanoic acid synthesis in the co-cultures as well as in the pure culture of *Lb. plantarum* R2.

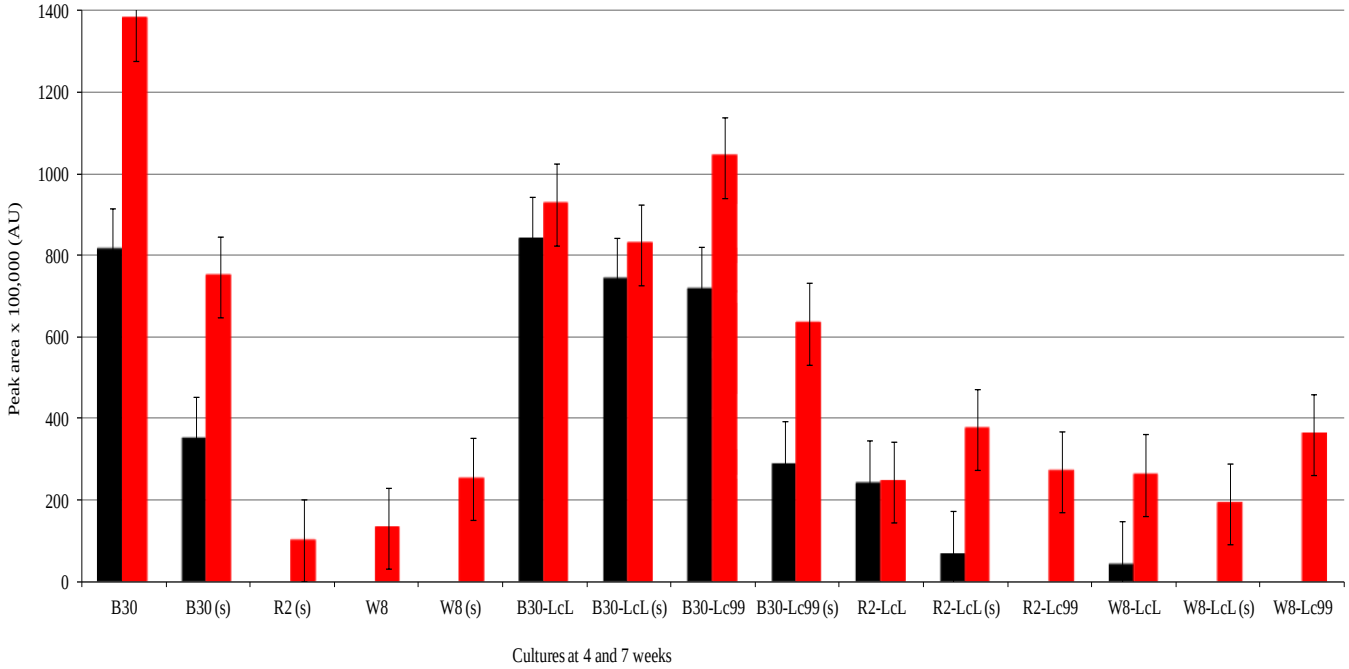


Figure 7.12 Changes in levels of acetic acid in milk inoculated with pure or mixed cultures of *Lb. plantarum* B30 (blue veins), R2 (outer crust), W8 (white core) with *Lc. lactis* subsp. *lactis* (LcL) or *Lc. lactis* NCIMB 9918 (Lc99). Samples were incubated for 48 h at 30°C and subsequently ripened at 18°C. Acetic acid was detected at (■) 4 weeks, and (■) 7 weeks. (s): salt (3.5%, w/v) was added to the sample at 48 h. Values are means of three independent experiments and error bars are ± standard errors of the means, $p = 0.05$. The compound was below threshold levels in the samples not shown in the plot.

7.3.4.2.3 Ketones

Similar to the results of early fermentation (Section 7.3.4.1), acetoin was the dominant ketone at 4 and 7 weeks (Fig. 7.13). In pure culture, the compound was mainly detected in *Lb. plantarum* B30 (blue veins) compared with *Lc. lactis* strains and the other *Lactobacillus* isolates. Co-culture of *Lb. plantarum* isolates with *Lc. lactis* NCIMB 9918 (Lc99) was associated with greater acetoin synthesis than with *Lc. lactis* subsp. *lactis* (LcL). With the exception of the pure culture of Lc99 and the B30-Lc99 co-culture, levels of acetoin were higher at 7 weeks than at 4 weeks. Salt addition reduced acetoin synthesis in pure and mixed cultures, except in the R2-LcL ($1.1x10^8$ AU) or B30-Lc99 ($3.3x10^8$ AU) co-cultures where increased levels were observed.

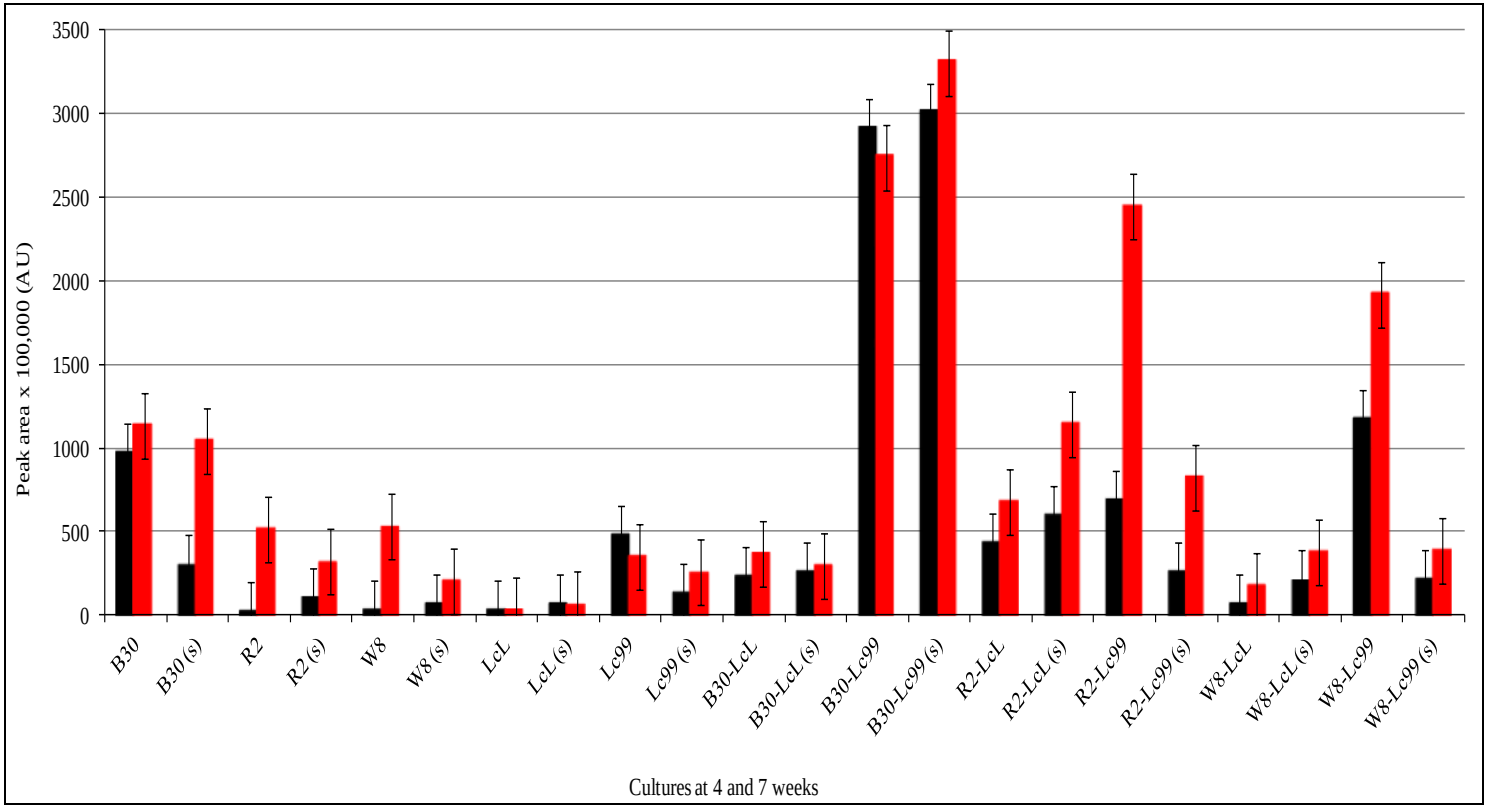


Figure 7.13 Changes in levels of acetoin in milk inoculated with pure or mixed cultures of *Lb. plantarum* B30 (blue veins), R2 (outer crust), W8 (white core) with *Lc. lactis* subsp. *lactis* (LcL) or *Lc. lactis* NCIMB 9918 (Lc99). Samples were incubated for 48 h at 30°C and subsequently ripened at 18°C. Acetoin was detected at (■) 4 weeks, and (■) 7 weeks. (s): salt (3.5%, w/v) was added to the sample at 48 h. Values are means of three independent experiments and error bars are ± standard errors of the means, $p = 0.05$. The compound was below threshold levels in the samples not shown in the plot.

In addition, 2-pentanone, 2-hexanone, 2-heptanone and 2-nonanone characterised the pure culture of *Lb. plantarum* W8 and its co-culture with Lc99 (Appendix 7.5). Salt addition stimulated synthesis of these ketones in pure cultures, whereas it was inhibitory in the co-cultures. At 4 and 7 weeks, 2-hydroxy 3-pentanone was mainly detected in pure cultures of *Lb. plantarum* R2 and W8 relative to other samples. Salt addition stimulated synthesis of the compound in the pure culture of W8 ($2.3x10^7$ AU), whereas it was inhibitory for strain R2 ($8.1x10^5$ AU) in pure culture (Appendix 7.5). These data correspond with the high salt tolerance of the *Lactobacillus* isolates as observed in Chapter 5 and support the suggestion for their growth in cheese to produce a variety of compounds as already presented in Sections 7.3.4.1-7.3.4.2.

7.3.4.2.4 Aldehydes

The relatively higher level of aldehydes found in the pure culture of *Lc. lactis* subsp. *lactis* (LcL) and its co-culture with *Lb. plantarum* isolates at 24 h (Fig. 7.10A) was reduced to marginal levels at 4-7 weeks (Appendices 7.4-7.5), probably due to biochemical reduction into corresponding alcohols (Vítová *et al*., 2006), as these were found to increase at 7 weeks.

7.3.4.2.5 Volatile esters

Although early fermentation was characterised by less synthesis of volatile esters, a substantial amount of these compounds was found in some samples at 4 and 7 weeks with no apparent change in detectable levels obtained at the latter data points (Appendices 7.4-7.5). Butanoic and hexanoic acid ethyl esters were mainly found in the pure culture of *Lb. plantarum* W8 (white core), whereby salt addition stimulated synthesis of both esters. In comparison, butanoic acid 2-methyl ester was mainly detected in the pure culture of LcL and in its co-culture with the *Lb. plantarum* isolates, with no apparent sensitivity to salt addition. At 4 weeks, propanoic acid methyl ester was mainly detected in unsalted pure culture of *Lb. plantarum* R2 ($1.2x10^6$ AU) but rapidly declined to marginal levels by the end of the ripening period, probably due to biochemical reduction into propanol (Lawlor *et al*., 2003), as the latter showed a 2-fold increase between 4 and 7 weeks.

7.3.4.3 Principal component analysis (PCA)

The PCA scores and loadings bi-plot of the mean peak area data for the 23 volatiles that showed significant variability among the samples at 7 weeks is shown in Fig. 7.14. The plot showed that two principal components (PC1 and PC2) accounting for 46% of the explained variance were needed to describe the differences in volatile compounds between the samples. PC1 accounted for 29% of the variation whereas PC2 was explanatory for only 17% of the observed total variation. The plot had good discrimination for branched primary alcohols (2-methyl butanol, 3-methyl butanol and 2-methyl propanol), ketones (2-pentanone, 2-hexanone, 2-heptanone and 2-nonanone) and aldehydes (2-methyl propanal, 3-methyl butanal and 2-methyl butanal). These groups of volatiles generally influenced the distribution of compounds on the plot. Carboxylic acids were mainly plotted on PC2. The salted pure culture of *Lb. plantarum* W8 and co-culture of the latter with Lc99 were outliers and mainly produced ketones and alkyl esters of butanoic acid (PC1). Whereas acetoin was a major component of the volatile profiles detected (Fig. 7.13), the compound was not comparatively more dominant on the PCA plot. The B30-Lc99 co-culture in salted or unsalted milk was the major acetoin producer and clustered as an acetoin producer but the R2-Lc99 co-culture which produced high levels of the compound as evidenced in Fig. 7.13 was not grouped with the other acetoin producers (cluster 2) on the PCA. This could be attributed to a stronger influence of other volatiles produced by this culture rather than this sole compound (acetoin). For example, R2-Lc99 produced relatively higher levels of ethanol, octanoic acid, pentanone and butanoic acid ethyl ester in addition to acetoin (Appendix 7.5), when compared with other acetoin producers.

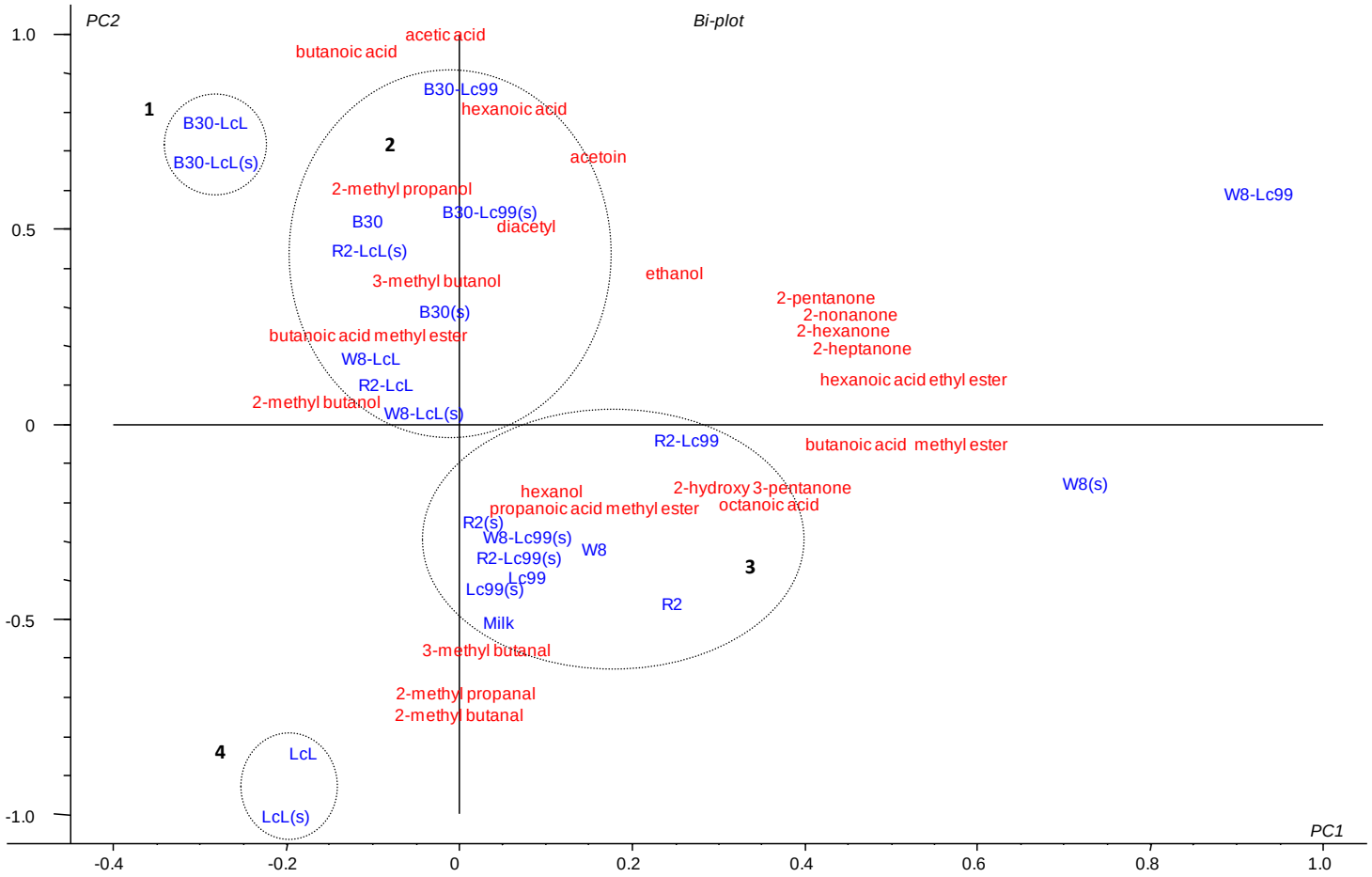


Figure 7.14 PCA plot of the aroma compounds obtained from SPME GC-MS peak area data after headspace analysis of milk samples inoculated with pure and mixed cultures of *Lb. plantarum* B30 (blue veins), R2 (outer crust), W8 (white core); *Lc. lactis* subsp. *lactis* (LcL; acid producer); and *Lc. lactis* NCIMB 9918 (Lc99; non acid-producer). The cultures were incubated for 48 h at 30^{o}C and subsequently ripened for 7 weeks at 18^{o}C in salted (s) or unsalted milk.

Four clusters (1-4) were delineated. Most volatiles were associated with clusters 2 and 3. The pure culture of *Lc. lactis* subsp. *lactis* (LcL) was associated with minimal production of volatiles probably due to cell death (Section 7.3.2). The volatile profiles of pure cultures of *Lc. lactis* NCIMB 9918 (Lc99), and *Lb. plantarum* R2 (outer crust) and W8 (white core) were generally similar to that of the milk control (cluster 3) and mainly comprised hexanol, 2-hydroxy 3-pentanone, octanoic acid, 3-methyl butanal and 2-methyl butanal (Fig. 7.14). These cultures produced substantial levels of acetoin (Fig. 7.13) but the compound was not plotted with cluster 3 as shown in Fig. 7.14. Co-culture of *Lb. plantarum* isolates with LcL, and pure or mixed cultures of *Lb. plantarum* B30 with Lc99 were mainly associated with synthesis of alcohols and organic acids (clusters 1 and 2). The dominant acids associated with the latter combination include acetic, butanoic and hexanoic acid. The plot also demonstrated that salt addition had minimal effect on the distribution of volatiles except for the pure culture of *Lb. plantarum* W8 and its co-culture with *Lc. lactis* NCIMB 9918 (W8-Lc99) (Fig. 7.14).

7.3.4.4 Overall changes in volatile profiles

Pure or mixed cultures of *Lb. plantarum* isolates and *Lc. lactis* produced various groups of volatile compounds in milk at the different stages of fermentation (30^{o}C) and subsequent ripening at 18^{o}C. The level of alcohols, carboxylic acids and ketones increased with time whereas aldehydes and esters decreased. Co-culture of the organisms increased the levels of most volatiles demonstrating the possible symbiotic (mutuality) nature of these LAB species. The symbiotic effect was highlighted by an overall increase (p<0.05) in levels of the volatiles in mixed cultures compared with the sum of levels of individual volatiles detected in pure culture inoculations that comprised the mixed culture. Symbiosis was mainly evident in the production of acetic acid (97-17,964% increase), 3-methyl butanol (1,010% increase), 2-pentanone and 2-hexanone (1,403-1,820% increase), and butanol 3-methyl acetate (1,515% increase) (p<0.05). In this context, acid production was mostly enhanced in co-cultures of *Lb. plantarum* R2 (outer crust) and W8 (white core) with *Lactococcus* strains, whereas alcohol synthesis was enhanced in the co-culture of *Lb. plantarum* B30 (blue veins) and *Lc. lactis* strains. Ketone synthesis was enhanced in all co-cultures, while increased aldehyde production was mainly associated with the co-culture of *Lb. plantarum* B30 and *Lc. lactis* subsp. *lactis* (p<0.05).

Salt addition stimulated alcohol synthesis in all inoculations involving *Lc. lactis* subsp. *lactis* (acid producer) ($p<0.05$). However, it was inhibitory for acetoin and acetic acid production. Salt addition in the co-culture of *Lc. lactis* NCIMB 9918 (non acid-producer) and *Lb. plantarum* B30 and R2 stimulated acetoin synthesis ($p<0.05$) which highlights that such a combination would contribute to producing a buttery aroma (Vítová *et al*., 2006) in ripened cheese. Very volatile compounds such as dimethyl sulfide and acetone were detected in each sample with no apparent variation with the different experimental combinations examined (Appendix 7.7, $p>0.05$). Diacetyl (2,3-butanedione) and 2,3-pentanedione are major aroma compounds of fermented milk (Otto *et al*., 1999). These diketones were also detected in the current study but only a slight increase in synthesis ($p>0.05$) was noted in some co-cultures during ripening at 18^{o}C.

7.4 Discussion

7.4.1 Acid production

Batch production of lactic and acetic acid by the different genotypes of *Lb. plantarum* was evaluated in MRS broth at 30^{o}C in an effort to identify high acid-producing isolates that may have good compatibility with the starter cultures. The results indicated that lactic acid production occurred at stationary phase and the process was not inhibited by low pH, which is in agreement with other studies (Schepers *et al*., 2002). The greatest lactic acid production led to a pH drop of ~3 units and was largely dependent on the site from which the strains were isolated as demonstrated by the differences in pH levels obtained. The isolates obtained from the blue veins and white core produced more lactic acid than those from the outer crust. In Picon Bejes-Tresviso, a Spanish blue cheese, pronounced pH drop occurs at the beginning of ripening with an average pH value of 5.13±0.23 after salting (Prieto *et al*., 1999). It is well recognised that pH decrease in the cheese is a result of lactic acid production due to degradation of lactose (Olson, 1990; Prieto *et al*., 1999; Schepers *et al*., 2002).

Whereas *Lb. plantarum* is a facultative bacterium and can utilise oxygen as electron acceptor for cell growth and for product metabolism (due to generation of additional ATP), Fu and Mathews (1999) have reported that presence of oxygen inhibits lactic acid production. This has been attributed to inhibition of cell growth due to accumulation of superoxide and hydrogen peroxide (Murphy and Condon, 1984), given that lactate production is principally a growth associated process. In the current study, production of

lactic and acetic acid was associated with cell density and growth phase. This observation was in agreement with other studies whereby different strains of *Lb. plantarum* grown in MRS medium (like in this study) at pH 4.5 produced significant levels of lactic acid with low levels of acetic acid detected (Passos *et al*., 1994), highlighting the homofermentative nature of the organism. Murphy and Condon (1984) also reported that *Lb. plantarum* produces lactic acid during the initial stages of fermentation in a glucose-rich medium (such as MRS broth), irrespective of incubation conditions, which is similar with the data obtained from this study. However, prolonged fermentation under aerobic conditions resulted in accumulation of acetate, whereas for anaerobic catabolism, it was lactate. Similar results were obtained when lactose and galactose were used as substrates. Fu and Mathews (1999) noted that the organism could only produce acetate when grown anaerobically in alkaline medium (pH 7.5 or above), which may partly explain the low levels of the acid recorded at low pH in the present study.

Lb. plantarum is homofermentative for hexose sugars producing 2 moles of lactic acid for every mole of hexose sugars metabolised. Therefore, lactic acid production is sometimes defined as a function of the energy required to form new cell protoplasm and the energy for the normal metabolic activity irrespective of growth (Bergen, 1977; Passos *et al*., 1994). While several studies have confirmed the validity of the linear relationship between lactic acid production and growth rate in most lactobacilli (Narendranath and Power, 2005), interpretation of this relationship has been previously found to be sometimes questionable (Passos *et al*., 1994). Therefore, although the current study indicated positive correlation between cell density and lactic acid production in MRS broth, in the real cheese matrix, growth and accompanying production of the acid by *Lb. plantarum* isolates may show some variation depending on a number of factors: physiological state of cells, differences in carbon and nitrogen sources as well as the presence or absence of other growth factors (Cogan *et al*., 2007) and this is an issue which requires further investigation. Fu and Mathews (1999) reported that in a pH controlled synthetic lactose medium, *Lb. plantarum* is homolactic and acid production is primarily growth associated. Similar to this study, the researchers pointed out that the greatest acid production occurred when pH of the medium was controlled at 5-6, with anaerobic fermentation giving 2.3 times higher yield than aerobic fermentation. Singh *et al*. (2003) suggested that *Lb. plantarum* can metabolise lactic acid and other substrates in

foods following prolonged ripening in presence of air to produce acetic acid, ethanol and acetoin which may also relate with the results obtained in this study (Section 7.3.4.2).

In foods, lactic acid produced by LAB inhibits the growth of other microorganisms such as *Strep. cremoris* (Bibal *et al*., 1989) as well as most Gram-negative bacteria (Gill *et al*., 1982), which may partly explain the antimicrobial activity of the isolates obtained in Chapter 6 (Section 6.3.1). It has been suggested that the molecule mainly exerts antimicrobial effects by creating an acidic pH (Fayol-Messaoudi *et al*., 2005). These authors proposed that L-lactic acid displays greater antibacterial activity than D or DL lactic acid. As a consequence, the broad spectrum of antibacterial activity observed among *Lb. plantarum* isolates in Section 6.3.1 could be related in part, to a greater proportion of L-lactate. Numerous parameters (microorganism, carbon and nitrogen source, fermentation mode, pH and temperature) affect the isometry of lactic acid produced (Hofvendahl and Hahn-Hägerdal, 2000). These authors reported that fermentation using a defined LAB strain results in production of only one of the isomers in an optically pure form. Synthetic production always results in a racemic mixture of DL lactic acid which highlights the role microorganisms can play in producing isometrically pure forms of lactic acid.

Lactic acid also directly inhibits cell growth through its damaging effects on membrane potential (Ahmed *et al*., 2009). In this case, the un-dissociated form is more active against most of the sensitive organisms (Fu and Mathews, 1999). Dissociated lactic acid mainly inhibits growth of Gram-negative bacteria (Gill *et al*., 1982). The pKa for lactic acid is 3.86. The relationship between pH, dissociated (L^-) and un-dissociated lactic acid (HL) can be presented by the following Henderson-Hasselbalch equation:

$pKa = pH - \log [L^-]/ [HL]$ (Equation 7.1).

Considering the data from the current study, the highest concentration of lactic acid (21.40±1.45 g/L) was obtained from *Lb. plantarum* W30 (white core; Table 7.1). Therefore, the total molar concentration of the acid species can be given as:

$[L^-] + [HL] = 21.40$ g/L = 0.238 M (Equation 7.2).

From Eq. 7.1 and 7.2, at pH of 3.76 corresponding with total molar acid concentration 0.238 M, it can be given that 56% of total lactic acid occurred in MRS broth in an un-dissociated form making the medium inhibitory for the tested bacterial strains. This may also partly explain why at low pH (3.76-4.09) in the current study there was slow growth (24-48 h) for all the *Lb. plantarum* isolates examined. Fu and Mathews (1999) reported that at pH 5 in typical whey fermentations containing ~50 g/L lactic acid, 93% of total lactic acid is dissociated and only 7% is un-dissociated. However, when pH drops to ~4, un-dissociated lactic acid content reaches about 42% of the total acid concentration, which is close to the estimate from this study.

Some studies have indicated that *Lb. plantarum* remains viable by maintaining an internal pH gradient (4.6-4.8) down to external pH 3 regardless of the type of acidulant present in the medium (Fu and Mathews, 1999; Ahmed *et al.*, 2009). This may account for the observed ability for the organism to continue growing at pH 4 and below in the current study, albeit at a slower rate. *Ped. acidilactici* could not tolerate the harmful effect of low pH and started dying off after 24 h probably because it does not possess this protective mechanism.

In the majority of ripened cheeses (pH ~5) where the typical concentration of lactic acid is ~50 g/L (Schepers *et al.*, 2002), 93% of total lactic acid occurs in dissociated form and only 6.8% of the acid is un-dissociated (Fu and Mathews, 1999). Further pH drop to ~4 could elevate the level (>42%) of un-dissociated lactic acid offering more inhibitory protection against undesired bacteria in these cheeses (Fu and Mathews, 1999). However, in blue cheese, the pH increases after piercing (6 weeks) due to lactic acid metabolism by the moulds. This may partly account for the presence of acid sensitive bacteria such as *Staphylococcus* in the outer crust of Stilton cheese (where pH is highest, ~6), as observed in Chapter 4 in the current study.

7.4.2 Dynamics of viable population changes

In spite of increasing interest in studies on the microbiological diversity of ripened cheeses (Fitzsimons *et al*., 1999; Ercolini *et al*., 2003; Coda *et al*., 2006), until recently, little work has been conducted on examining the possible role of non-starter lactobacilli in ripening of Stilton cheese. Moreover, information about the impact of non-starter LAB on quality characteristics is regarded as crucial to the cheese making process (Cogan *et al*., 2007). In the case of Stilton cheese, this could benefit growth of local industries and enhance marketing of products with consistent quality characteristics. The current work has investigated the impact of different genotypes of *Lb. plantarum* as the dominant NSLAB in Stilton cheese on the dynamics of growth and survival of different strains of *Lc. lactis* under simulated milk fermentation and cheese ripening conditions. Changes in pH and total viable counts were monitored during early fermentation (0-48 h) at 30^{o}C and throughout the 12 weeks of ripening in cows' milk at 18^{o}C with the addition of salt.

At 48 h, the viable counts of *Lb. plantarum* isolates (5.8-7.5 $\log_{10}$ CFU/ml) in pure and in mixed cultures were lower than *Lc. lactis* (7.4-9 $\log_{10}$ CFU/ml). The latter corresponds to the number (>10^{9} CFU/ml) usually found in a freshly prepared cheese curd (Broadbent *et al*., 2003). During the subsequent 12 weeks of ripening at 18^{o}C, the viable counts of *Lb. plantarum* isolates in co-culture with the acid-producing *Lc. lactis* strains remained stable at 7 $\log_{10}$ CFU/ml whereas the population of these lactococci rapidly declined to undetectable levels. In comparison, *Lb. plantarum* isolates co-cultured with the non acid-producing *Lc. lactis* maintained viability and continued to grow up to 7 weeks, whereas viable counts of this *Lactococcus* strain remained stable at 7 $\log_{10}$ CFU/ml. Salt addition had minimal effect on growth of the *Lactobacillus* isolates in pure culture but had variable effects in mixed culture. Salt also reduced the growth of acid and non acid-producing lactococci in pure culture at 1-6 weeks (p<0.05). However, at 1-6 weeks in mixed culture, its inhibitory effect was mainly evident on the growth characteristics of acid-producing lactococcal strains, and not for the non acid-producing *Lc. lactis*.

These results were in agreement with data from other researchers. Martin-Platero *et al*. (2008) reported that bacterial strains present at the beginning of cheese ripening are usually different from those at the end with *Lactobacillus* spp. being persistent throughout the process, which is similar to this study. Changes in environmental parameters such as relative humidity, redox potential, salt concentration, pH, presence or absence of proteins,

fatty acids and free amino acids are regarded as major factors that explain the microbial succession dynamics during cheese ripening (Martley and Crow, 1993; Martin-Platero *et al*., 2008). The same observation has been reported for Cheddar cheese, whereby the initial number of *Lc. lactis* starter bacteria seldom exceeds 10^9 CFU/g, but the harsh cheese ripening environment (no residual lactose, pH 5-5.3, 4-6% salt in moisture and 5-13^{o}C ripening temperature) causes the lactococci starter culture viability to decline as maturation progresses (Broadbent *et al*., 2003). These researchers have shown that the NSLAB populations, typically dominated by mesophilic facultative heterofermentative lactobacilli whose initial numbers are typically below 10^3 CFU/g, begin to grow and eventually plateau at cell densities of 10^7-10^9 CFU/g after 3-9 months of ageing. This is similar to the results obtained from this work.

This study demonstrated that by the end of ripening at 18^{o}C, the different genotypes of *Lb. plantarum* dominated the microflora of fermented milk which may partly account for the results obtained in Sections 4.2.1-4.2.2. The data also confirmed the hypothesis that acid-producing strains of *Lc. lactis* normally included in cheese ripening as the primary starter culture virtually disappear from the fermentation profile by the end of ripening. The major contribution of LAB cultures in ripening of Stilton cheese is thought to occur prior to piercing (6-8 weeks), after which *Pen. roqueforti* sporulates and dominates in the cheese matrix up to the end of ripening (12 weeks) (Ercolini *et al*., 2003). This study has shown that at 7 weeks, *Lc. lactis* strains and *Lb. plantarum* isolates remained viable.

Although the LAB isolates used in the current study were found to be neither proteolytic nor lipolytic (Section 7.3.3), blue cheeses are frequently colonised by lipolytic and proteolytic yeast species including *Yarr. lipolytica* and *Deb. hansenii* which occur as contaminants (Van den Tempel and Nielsen, 2000; Gkatzionis, 2010). In this case, the cheese matrix would support the growth and metabolic activity of *Lb. plantarum* due to increased provision of less complex nutrients (Martley and Crow, 1993). Also, sporulation of the proteolytic/lipolytic *Pen. roqueforti* after piercing (6 weeks) would further provide essential nutrients that could enhance the growth and metabolism of *Lb. plantarum* during cheese ripening to impart desired effects on the quality characteristics of the product (if any).

The growth interaction presented in the current work may be defined as amensalism, whereby acid-producing lactococci strains were repressed by the acidic environment produced by the mixed culture but was tolerated by *Lb. plantarum* strains (Flambard *et al*., 1997). As it is a common phenomenon that microorganisms in mixed culture usually interact in more than one way (Flambard *et al*., 1997), other possible explanations for the observed growth suppression of acid-producing lactococci strains in mixed culture may include competition for nutrients and senescence (Martley and Crow, 1993), both of which normally increase susceptibility of cells to various antimicrobial compounds produced by lactobacilli. It has previously been established that the majority of *Lb. plantarum* isolates expressed a certain level of antimicrobial activity against *Lc. lactis* (Section 6.3.1). This effect may become pronounced in older cells of the latter when subjected to various stress conditions. In Parmigiano-Reggiano, a PDO cheese produced in Northern Italy, survival of *Lactobacillus* spp. including *Lb. rhamnosus, Lb. casei* and *Lb. plantarum* during later (24 months) stages of ripening was attributed to derivation of energy from autolysis of starter culture lactococci followed by DNA degradation providing nutrients for the cells in the particularly hostile nutritional environment occurring during this period of ripening (Gatti *et al*., 2008). Ramírez-Nuñez *et al*. (2011) proposed that strains of *Lc. lactis* used in cheese should autolyse at early stages of ripening to ensure faster and higher production of aroma compounds. The process releases an array of enzymes which interact with substrates present in the cheese matrix to generate desired flavours and aromas and is enhanced by low salt concentration (0.17 M), temperature, water activity and acidic pH (5.4). This may explain the observed rapid cell death of acid-producing lactococci during 4-7 weeks in the current study.

It has been recognised that the net dynamics of growth, survival and biochemical activity of microorganisms in fermented food systems is the result of stress reactions in response to changes in physical and chemical conditions in their microenvironment (e.g. changes in gradients of pH, oxygen, water activity, salt and temperature) as the process progresses (Martley and Crow, 1993). On the basis of this, it can be predicted that the *Lb. plantarum* isolates examined in this study could have been more adapted to changes in the physical-chemical properties of Stilton cheese at later stages of ripening (given their characteristics presented in Chapter 5) than *Lc. lactis*. This has also been supported by other authors (Broadbent *et al*., 2003; Crow *et al*., 2001). However, the current study was conducted using simple UHT models. Hence, there is the need to ascertain the behaviour of the

isolates in more complex models such as the cheese matrix. In a real cheese ecosystem, various microbial populations interact to give a community. However, this is not actively dictated by the cheese producer, and as such, there can be different microbial species and/or strains in cheeses obtained from one producer to another and from batch-to-batch. None-the-less, in cheese, cells are generally immobilised and localised in high densities. Therefore, cheese ripening or degradation is rarely the result of activities of an individual but that of a group of microorganisms (Giraffa, 2004). Thus, the actual growth, survival and activity of *Lb. plantarum* isolates *in situ* in Stilton cheese may be determined by co-presence of other microorganisms and *in situ* cell-to-cell ecological interactions which often happen in a solid phase system (Giraffa, 2004; Giraffa and Carminati, 2008), which could deviate from the current data.

7.4.3 Volatile profiles production

Data from the current work have shown that, in contrast to the usual description of *Lb. plantarum* as homofermentative, the isolates examined were facultative heterofermentative and produced alcohols and lactic acid. The heterofermentative process normally occurs in presence of low oxygen (Årskold *et al*., 2008), which corresponds with the conditions (sealed vials) under which the current study was conducted. However, some air was frequently (every week) introduced into the cultures when collecting sample aliquots for analysis. Presence of oxygen usually leads to formation of hydrogen peroxide. In presence of air, *Lb. plantarum* sometimes utilises manganese as pseudo catalase to reduce oxygen concentration enabling the organism to utilise various carbon sources in an aerotolerant environment (Archibald and Fridovich, 1981), which may partly account for the various groups of compounds observed.

The study has shown that co-culture of different genotypes of the *Lb. plantarum* with different strains of *Lc. lactis* results in production of a wide variety of compounds with different functional groups. Previous studies on blue cheese varieties manufactured from pasteurised milk have found high levels of carbonyl compounds, methyl ketones, alcohols, carboxylic acid and aldehydes at the end of ripening (Lawlor *et al*., 2003; Vítová *et al*., 2006; Gkatzionis *et al*., 2009). As most of these compounds have low sensory threshold levels in cheese and oil models (Appendix 7.6), this study showed that *Lb. plantarum* isolates can contribute substantially to their associated aroma notes during cheese ripening. The levels of the volatiles were partly dependent on the sites from which

Lb. plantarum isolates were obtained showing they are genetically different strains. The study has shown that while the total concentration of alcohols, organic acids and carbonyls increased with time among some cultures, the levels of the majority of individual ketones at progressive stages of ripening showed some fluctuation and in some cases inversely correlated with their corresponding alcohols and aldehydes, probably due to biochemical inter-conversion (Madkor *et al*., 1987[b]).

Most high molecular weight ketones recorded from the study are products of free fatty acid metabolism. Therefore, inclusion of *Lb. plantarum* isolates as starter culture adjuncts in Stilton cheese undergoing extensive proteolysis and lipolysis (Madkor *et al*., 1987[a, b]) could have the concentration of these compounds intensified with substantial contribution to aroma characteristics of the cheese. Vítová *et al*. (2006) applied SPME GC-MS and identified 54 compounds in Niva, a soft blue cheese manufactured from pasteurised milk in Czech Republic. The compounds comprised three hydrocarbons, five aldehydes, three esters, 11 ketones, 18 alcohols, 10 fatty acids and four sulphur compounds, which is similar to this study. Lawlor *et al*. (2003) also reported that the dominant compounds in Stilton cheese include 2-methyl butanol, butanal, pentanal, methyl hexanoate, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone and 2-nonanone, which is generally compatible with the current results.

The most recent major study on the volatile profile of Stilton cheese focussing on differences between cheese sections (outer crust, blue veins and white core) and producer dairies was reported by Gkatzionis *et al*. (2009). The authors evaluated the aroma profiles of Stilton cheese samples from different creameries using SPME GC-MS, solvent extraction GC-MS and atmospheric pressure chemical ionisation - mass spectrometry (APCI-MS). In concordance with Coda *et al*. (2006), these authors reported that although SPME GC-MS was semi-quantitative, the sensitivity of the technique was high and had better discrimination for the compounds detected in the different cheese sections and producer dairies. Their study summarised that ketones were the major compounds found in all the cheese samples and sections with nonanone, heptanone, pentanone and the alcohol 3-methyl butanol dominating. In addition, they found three times more alcohols in the white core than in the blue veins and outer crust. Blue veins had the highest amount of volatiles. This account is generally compatible with other blue cheese varieties including Gorgonzola (Moio *et al*., 2000), Roquefort (Gallois and Langlois, 1990) and Danablu

(Lawlor *et al*., 2003). The current study has demonstrated that *Lb. plantarum* isolates can contribute to the production of these compounds whereby levels of most of the volatiles increased as ripening progressed. However, synthesis and levels of specific volatiles was largely dependent the geno-group of the *Lactobacillus* isolate examined (as elaborated in the next paragraph) and co-presence of other microflora (strain of *Lc. lactis* in the case of this study); salt addition played a minor effect.

The dominant volatiles at 7 weeks were ketones (principally acetoin), organic acids (acetic, hexanoic and butanoic acid), as well as the branched alcohols including 3-methyl butanol and 2-methyl propanol. In pure culture, these compounds were mainly produced by *Lb. plantarum* B30 as already presented on the PCA plot (Section 7.3.4.3). Gkatzionis *et al*. (2010) analysed the aroma volatiles from different sections of Stilton cheese and reported that all sections were dominated by ketones (outer crust, 72%; blue veins, 75%; and white core, 55%,). These authors also showed that there were more alcohols (three times higher) in the white core than in the outer crust (10%) or the blue veins (10%), but reported nothing about the presence and levels of organic acids in the cheese. Blue veins contained the highest amount of volatiles but levels were comparatively closer to those in the outer crust. Comparing these findings with the results obtained from the current study, it can be suggested that *Lb. plantarum* B30 could contribute to the production of acetoin, 2-methyl butanol and 3-methyl butanol in the blue veins from where it was isolated. In fact, co-culture of this *Lactobacillus* isolate with *Lc. lactis* NCIMB 9918 (non acid-producer) and salt addition led to a greater production these volatiles. As the compounds are commonly characterised by fruity, alcoholic and malty attributes (Lawlor *et al*., 2003; Gkatzionis *et al*., 2009), this suggests that *Lb. plantarum* B30 has the potential to contribute to these aroma notes in the blue veins. However, co-culture of the other *Lb. plantarum* isolates: R2 (outer crust) and W8 (white core) with the acid-producing *Lc. lactis* subsp. *lactis* was also associated with the some increase in acetoin and alcohol production demonstrating their potential to contribute the afore-mentioned notes in the respective sites from which they were isolated. The overall results of this study were in accordance with the model by Gkatzionis (2010) in which Lc99 was grown in UHT milk for 10 days at 25^{o}C as pure or as mixed culture with *Pen. roqueforti*. The author observed that in pure culture, Lc99 produced considerable amounts of ethanol and acetoin. Its co-culture with *Pen. roqueforti* increased ethanol and acetoin synthesis by four times.

The results presented in Section 7.3.2 revealed that viable counts of *Lc. lactis* subsp. *lactis* (LcL) which is normally included in cheese production had markedly declined at 7 weeks compared with that of the non acid-producing *Lc. lactis* NCIMB 9918 (Lc99) or *Lb. plantarum* isolates. This suggests that LcL produces volatiles during primary fermentation (24-48 h) and early stages of ripening when it is still actively growing. From this point of view, the results from this study suggested that the volatiles produced by LcL could persist in the model and some of them were amplified when the organism was co-cultured with *Lb. plantarum* isolates. This was the case for 2-methyl propanol, 2-methyl butanol and 3-methyl butanol. Since Lc99 remained viable in pure or in mixed cultures with *Lb. plantarum* isolates, the study suggested that the major volatiles (acetoin, ethanol and octanoic acid, particularly in co-culture) associated with Lc99 may be the result of continued metabolic activity or symbiotic association with *Lb. plantarum*, especially with lactobacilli isolates obtained from the blue veins as exemplified by strain B30. This suggests different enzyme activities leading to distinct metabolic pathways influencing the final flavour of the fermented milk in different ways depending on the types and levels of the compounds synthesised (Obodai, 2006).

7.5 Conclusions

It has been established that the different genotypes of *Lb. plantarum* obtained from Stilton cheese were acid tolerant (Section 5.3.2). The final concentration of lactic acid produced in MRS broth by the *Lactobacillus* isolates was found to be a function of the geno-group and cell numbers achieved during the growth phase. The growth and survival of the isolates was insensitive to lactic acid end product inhibition. The high acid tolerance of *Lb. plantarum* isolates as evidenced from Chapter 5 (Section 5.3.2) coupled with these results is indicative of its potential for industrial application.

During early fermentation in milk or in MRS broth, acetic acid production was not strongly growth associated. However, during later stages of ripening in milk at 18^{o}C, *Lb. plantarum* isolates synthesised acetic acid probably from lactic acid metabolism. Acetic acid synthesis was enhanced by co-culture with the non acid-producing *Lc. lactis* NCIMB 9918. In the co-cultures involving the *Lb. plantarum* isolate obtained from the white core, salt addition inhibited acetic acid synthesis. The study has demonstrated that *Lb. plantarum* isolates have the potential to produce lactic and acetic acid in the real cheese matrix as it is mainly constituted from milk. As well as having greater antimicrobial

activity (than lactic acid), acetic acid contributes to the sour/vinegar note in ripened cheese. Therefore, the results from this study suggest that co-culture of *Lactobacillus* isolates with *Lc. lactis* NCIMB 9918 would enhance these desirable properties in cheese. The effect of acetic acid in cheese would even be stronger in co-inoculations involving *Lb. plantarum* B30 from the blue veins; this isolate had the highest level of acetic acid synthesis compared with co-cultures involving the other *Lactobacillus* isolates.

The results of aroma studies have shown that some aroma compounds of *Lactobacillus*-*Lactococcus* cultures are already present in milk but a variety of others are synthesised during fermentation. Five groups of volatiles appeared during fermentation or were present in considerably increased amounts in the fermented milk compared to the control. Salt addition had a minimal effect, whereas pure and co-culture treatments had variable impacts on the volatile profiles synthesised at the different stages of fermentation and during ripening at 18^{o}C. SPME GC-MS headspace analysis was found to be fairly reproducible and accurate for semi quantification of volatiles in the fermented milk. As lactobacilli are fortuitously introduced organisms, this study suggested that aroma production is largely dependent on which isolates enter into the cheese during production. The process may also vary with the type of starter culture used. Overall, this would lead to variation in product characteristics from batch to batch.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Stilton cheese is one of the most popular traditional food products in the UK. The cheese is manufactured from pasteurised milk inoculated with single or a mixture of different strains of *Lc. lactis* (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* or *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*), *Ln. mesenteroides* subsp. *cremoris* and *Pen. roqueforti*. Although the processing steps have been standardised over a long period of time, commercial producers of Stilton cheese are often faced with the challenge of variation in organoleptic characteristics within batches and between dairies (Whitley, 2002; Gkatzionis *et al*., 2009). Crow *et al*. (2001) have summarised that the standard plate count of high quality pasteurised milk used for cheese-making seldom exceeds 10^5 CFU/ml, of which the number of organisms which survive pasteurisation (thermodurics) is normally less than 10^3 CFU/ml. The survivor organisms mainly comprise NSLAB which can gain entry into the cheese manufacturing equipment and become lodged in sites from where they are shed into the product during manufacture.

It is apparent from the above factors, that the total microbial population of Stilton cheese at the start of ripening can be derived from all contributing sources including deliberately added starter cultures, thermoduric organisms in milk and adventitious contaminants. Previous studies have demonstrated that at the onset of cheese ripening, the primary acid-producing starter culture (*Lc. lactis* or *Lc. cremoris*) dominates (10^7-10^9 CFU/ml) in the curd (Broadbent *et al*., 2003). However, as ripening continues over a period of weeks and months, the cheese bacterial flora becomes dominated by NSLAB (typically lactobacilli) which grow better under the prevailing conditions i.e., less than 40% moisture, 4-6% salt, pH 4-6 and temperature 5-18^{o}C. Secondary NSLAB can participate in a range of biochemical activities which can affect the cheese quality properties (Yim and Glover, 2003). There are no published reports which have conclusively demonstrated that metabolic activities of secondary NSLAB can directly contribute to the aroma attributes of Stilton cheese. This study aimed to examine the influence of different microenvironments (outer crust, blue veins and white core) on selection of different genotypes of the NSLAB that grow in Stilton cheese and examine how the interaction of

the latter with the primary starter culture (*Lc. lactis*) could affect the volatile aroma profile of the cheese.

This study has characterised the 80 bacterial isolates obtained from a ripened Stilton cheese. Few discrepancies were noted between colony characteristics on selective and non-selective media (phenotype), biochemical tests and genotypic identification protocols. The cheese sample was dominated by different genotypes of *Lb. plantarum*. The organism was recovered from all the sampled sites (outer crust, blue veins and white core) and co-existed with other species including *Lb. brevis*, *Staph. aureus*, *Psychrobacter* spp. and *En. faecalis* in the outer crust. None of these organisms is deliberately included during the cheese manufacture process. Therefore, their incidence in Stilton cheese could be a result of post-process contamination or survival of heat treatment (Briggiler-Marco *et al.*, 2007). The dominance of *Lb. plantarum* in Stilton cheese during ripening has also been reported by other researchers (Whitley, 2002; Ercolini *et al*., 2003; Hiscox *et al*., 2008).

8.1 Correspondence of phenotypic and genotypic characteristics

Examination of carbohydrate fermentation patterns (API 50 CHL) of lactobacilli in the five major clusters of the PFGE dendrogram revealed some variations in the sugar utilisation profiles of the *Lactobacillus* isolates belonging to the same species. The majority (80%) of the isolates identified as *Lb. plantarum* had an API biochemical profile 4; this was mostly revealed among the isolates obtained from the blue veins and white core which formed geno-group I (Appendix 8). A representative *Lb. plantarum* isolate from the blue vein isolates which clustered separately in cluster V (R28-R26) gave a different API profile. The outer crust lactobacilli gave different API profiles from other isolates and fell into two genotypes (clusters III-IV) with different biochemical profiles associated with each genotype (although in cluster III two different API profiles were evident). Only one isolate gave the same biochemical profile as a strain with which it did not cluster and that was the outlier R37 from the white core which gave the same API profile as a genotype IV crust strain. Despite that PFGE clustered *Lb. brevis* with *Lb. plantarum* geno-group III suggesting low compatibility of the methodologies employed (i.e. 16 S rDNA sequence analysis showed a different species), phenotypic characteristics were generally compatible with the genotyping data including PFGE profiling and 16S rDNA analysis which was applied for molecular speciation (Appendix 8).

However the presence of the plantaricin EF (plnEF) operons showed no association with PFGE genotyping clusters. For instance, whereas PFGE indicated that cluster I contained the highest number of genetically similar *Lb. plantarum* isolates suggesting they could have been clones of a single strain, plnEF data highlighted some variations within this cluster; even closely related isolates within subclusters isolated from the same site gave different results. For example, in geno-group I the first subcluster of four isolates which formed at a similarity level of ~67% contained the isolates B15/B30 which were negative for plnEF and R23/R42b which were positive for this operon. This generally indicated that PFGE was not discriminatory enough for these *Lactobacillus* isolates. However, identical genotypes did give the same plnEF operon results as evident for isolates B27/B28/W9 and W12/W13. Thus, from these data, a PFGE genogroup does not define a single identical strain. Overall, the study revealed that there were different strains (subpopulations) of *Lb. plantarum* growing in the different sites of the Stilton cheese sample examined; some strains could be found at more than one site but others were very site-specific.

Thus, the study showed high biodiversity among *Lactobacillus* isolates and confirmed the initial hypothesis that there were genotypically different strains of NSLAB in Stilton cheese. Girrafa and Neviani (1999) have also previously employed PFGE to demonstrate the presence of different strains (clusters) of *Lb. helveticus* in cheese. However, the method works with viable microbial isolates and may not find application for studying complex microbial communities *in situ* in the real cheese matrix.

8.2 Stress tolerance of *Lb. plantarum* isolates

The study has shown that different genotypes of *Lb. plantarum* had different thermal tolerances at 72^{o}C. The isolate obtained from the outer crust was more sensitive to heat treatment (50 s) than those from the blue veins and white core which survived the process for up to 70 s. The fact that the organism can adapt to survive the heat process, and that the D_{72} value used may result in insufficient process to destroy the organism, could explain why these bacteria dominate the non-starter bacterial flora of ripened Stilton cheese as evidenced from Section 4.2.2 and from other studies (Whitley, 2002; Ercolini *et al.*, 2003; Hiscox *et al.*, 2008). However, it should be pointed out that this study was not designed to determine the ability of commercial HTST milk pasteurisation to inactivate *Lb. plantarum* as the system could not be adequately simulated under the laboratory

conditions. Also, the cell numbers (10^7 CFU/ml) used in this study were much higher than the levels (10^2-10^3 CFU/ml) usually found in milk intended for cheese production. Therefore, there is need for evaluation of the isolates in the real cheese processing situation considering the fact that in the actual food systems, microbial strains may be less or more heat tolerant than in the conditions used in the laboratory.

The ability of the isolates to survive the acid and salt stresses was tested *in vitro* utilising MRS broth as the medium. These important prerequisites of technological and probiotic interest also need to be assessed *in situ* in Stilton cheese, where all these factors are co-present, in order to ascertain the real capacity of the organism to survive in the cheese matrix. Further research may focus on identification of acid stress-induced proteins coupled with a more in-depth understanding of their mode of action. This could lead to their exploitation towards identification of the most acid-resistant isolates that are more robust for use as starter culture adjuncts for blue cheese production.

Given that the relative humidity of cheese processing industries in the UK ranges between 75-90%, this study has demonstrated that *Lb. plantarum* isolates could survive for several days under desiccating conditions. The high survival rates in MRD imply that the isolates have the potential to persist in environments such as cheese production plants where protein, fats and other nutrients from milk contribute the bulk of organic soil. The isolate obtained from the outer crust was more desiccation tolerant than those obtained from the cheese interior suggesting this *Lactobacillus* isolate could have been introduced into the outer crust from the cheese production plant environment through handling or aerial contamination, and was able to survive in this cheese site due to its high desiccation and halotolerance as already shown in Chapter 5. Although it may not be possible to directly extrapolate the results of the desiccation experiment to the *in situ* situation in Stilton cheese, the behaviour of *Lb. plantarum* at different RH levels following slow drying in MRD at 30^{o}C may be a convenient and rapid tool for screening for drought-tolerant lactobacilli in cheese and the cheese processing plants. Quantitative differences between the tested isolates were large enough to permit this criterion. Attention should also be given to the isolates obtained from the cheese interior as these demonstrated that they are able to withstand variations in RH in presence and absence of MRD. Experiments at higher RH (such as >80%) should be of particular interest in regard to survival of *Lactobacillus* in cheese undergoing ripening. Further research is required to establish the

resistance of the organism to cleaning and disinfection procedures used in the cheese production plants and to model its survival on different food contact surfaces.

Overall, the study has demonstrated some properties of *Lb. plantarum* isolates which may be of importance to the cheese-making industry and highlighted that *Lactobacillus* in Stilton cheese represents a mixed flora with strains at different sites selected on the basis of their stress tolerances. These observations could be of interest in developing *Lactobacillus* starters and starter culture adjuncts with an improved resistance to multiple stresses for several applications including probiotics, where growth under harsh stress conditions would be an attribute. Use of *Lb. plantarum* as a starter culture adjunct for Stilton cheese would need to take the varying phenotypes into consideration. These results obtained at laboratory scale are a good estimation of stress tolerance that may be of importance in the cheese industry. Transcriptome and proteome studies coupled with microarray analysis may be applied to validate that stress tolerance of the isolates could be triggered by transient exposure of the organism to a wide range of sub-lethal factors including acid, salt, drying, oxidative stress and other conditions occurring at different gradients within the cheese matrix. Elucidation of these mechanisms would add to the understanding of how the organism survives the cheese ripening process and would enable a more targeted approach to non-starter culture adjunct selection for quality improvement, maintenance and reliability of Stilton cheese production. This may also improve the current industrial starter strains. In general, the results from these experiments concurred with data from Chapter 4 which indicated that *Lb. plantarum* isolates obtained from different sites in Stilton cheese had different biochemical and genotypic characteristics implying they were different strains. This may be the result of ecological selection following introduction into the cheese (Randazzo *et al.*, 2002; Duthoit *et al.*, 2003).

8.3 Antimicrobial activity of *Lb. plantarum* isolates

As most LAB produce bacteriocins, it was not surprising to find from this study that some *Lb. plantarum* isolates had the ability to produce the heat stable plantaricin EF bacteriocins. Although bacteriocin production was not a pronounced phenomenon and could only be demonstrable using *Lb. pentosus* as the sensitive organism, most of the isolates expressed a broad spectrum of antimicrobial activity against the various Gram-positive and Gram-negative organisms on solid medium (Section 6.3.1), due to

production of acid, and to a less extent, hydrogen peroxide as highlighted in Section 6.3.2. This observation was in accordance with the results of SPME GC-MS (Sections 7.3.1 and 7.3.4) which revealed that *Lb. plantarum* isolates produced lactic and acetic acid, acetoin and other metabolites. The combined effect of these substances normally results in pH drop which inactivates undesirable microflora, enhances biochemical conditions that contribute to organoleptic properties of the product and improves shelf life by inhibiting undesirable changes brought about by spoilage microorganisms (Gill and Newton, 1982; Essid *et al*., 2009). These factors may partly account for observed inhibition of some of the sensitive bacteria tested in the study.

As *Lb. plantarum* was found to be tolerant to acid and other stresses conditions typical of the microenvironments within Stilton cheese (Chapter 5), inclusion of the isolates in solid food matrices such as cheese could find application in bio-preservation of these foods against undesirable microorganisms. However, prospects for application of the isolates as an adjunct culture for cheese production could be undermined by the observed ability of some isolates to inhibit *Lc. lactis* on media plates. However, as the *Lactobacillus* cell-free supernatants could not show any inhibition of the latter, it was possible that inhibition on the media plates was the result of acid production due fermentation by both LAB species. This is an aspect which is supported by the results obtained from the co-culture studies presented in Chapter 7 (Section 7.3.2); it has been shown that *Lc. lactis* could survive up to 12 weeks in fermented milk co-inoculated with *Lb. plantarum* isolates as long as the lactococcal strains were not acid producers.

Selective use of bacteriocinogenic strains may find application in bio-control of spoilage and pathogenic bacteria in other fermented foods of dairy origin. Further research on the possible antimicrobial effects of the isolates against *Pen. roqueforti* is an issue of concern. Whitley (2002) conducted a detailed survey which associated good quality (sufficient blueing) Stilton cheese with the predominance of the homofermentative *Lb. plantarum* and *Lb. curvatus* and defective counterparts (poor blueing) with *Lb. brevis* as the dominant bacterial flora, as already elaborated in Chapter 2 (Section 2.3.1.2). From this account, it can be predicted with confidence that inclusion of *Lb. plantarum* isolates in the manufacture of Stilton cheese would enhance its flavour (as observed in Section 7.3.3), and blueing from *Pen. roqueforti* as well as offer antimicrobial protection against a variety of undesirable organisms. Further research should examine how this can be

affected by the complex (but normal) microflora of the cheese which comprises bacteria, yeasts and moulds. Future work may also look at in-depth evaluation of each of the isolates as a probiotic supplement for humans. Preliminary results from this work have shown that the isolates could tolerate up to 2% bile salts (data not shown) which is the approximate level present in the human gut. There is the need to determine the molecular mechanisms that may be used by these bacteria to exert probiotic effects, if any.

8.4 Volatile profiles production

It has been found from this study that *Lb. plantarum* isolates could, as expected, grow and remain viable in fermented milk for 12 weeks at low pH. Salt addition had minimal effect on the growth characteristics of *Lactobacillus* isolates irrespective of either the site of isolation or the strain of *Lc. lactis* with which they were co-cultured. The study has also indicated that in mixed culture, the acid-producing *Lc. lactis* (APL) strains were more salt tolerant and died out faster (1-4 weeks) after salting compared with the corresponding unsalted cultures and the non acid-producing *Lc. lactis* strain in single and mixed cultures (Chapter 7, Section 7.3.2.2). Reduced growth in presence of salt was even stronger when the APL were co-cultured with the most acid-producing *Lb. plantarum* isolate (B30) suggesting a combined antimicrobial effect on these lactococcal strains.

Lb. plantarum isolates produced lactic acid and low levels of acetic acid in MRS broth at 48 h (Section 7.3.1) and this depended on growth phase as well as the genotype of *Lactobacillus* examined. This is similar to results from the milk assay (Section 7.3.4). In fact, results of aroma analysis revealed marginal levels of acetic acid produced in milk between 24-48 h but synthesis was observed during further incubation (4-7 weeks) at 18^{o}C. From this account, it could be concluded that during early fermentation, *Lb. plantarum* isolates predominantly produced lactic acid. However, during long-term ripening in milk, the isolates metabolised the acid and other substrates to produce acetic acid, ethanol and acetoin (Singh *et al*., 2003) as evidenced in Section 7.3.4.2. As the latter volatiles have a positive contribution to the aroma profile of fermented milk (Vítová *et al*., 2006), *Lb. plantarum* isolates examined in this study may be considered to contribute positively to the fermentation and thus could be suitable for inclusion as starter culture adjuncts during the production of Stilton and other blue cheeses.

As is the case with its bacterial community structure (Ercolini *et al*., 2003), Stilton cheese has a complex fungal flora mainly comprising *Pen. roqueforti* and yeasts such as *Kluyveromyces lactis, Yarrowia lipolytica, Candida catenulate, Trichosporon ovoides* and *Debaromyces hansenii* (Whitley, 2002; Gkatzionis, 2010). Most of these organisms directly produce aroma compounds but also indirectly contribute to the cheese flavour through proteolysis and lipolysis (Lawlor *et al*., 2003; Gkatzionis, 2010). There is little evidence to suggest that the profile of compounds produced in Stilton cheese is a result of symbiotic interaction between *Lb. plantarum* isolates and these fungal communities. This aspect requires further investigation. There is also the need to determine the critical levels (or ratios) of the important microbial species whose interaction is of significance to the flavour properties of Stilton cheese. However, it was clear from this study that co-presence of *Lb. plantarum* isolates and *Lc. lactis* resulted in increased synthesis of alcohols, organic acids and ketones, and as such could be a major contributory factor to the differences in aroma profiles of cheeses manufactured within similar or different batches and/or creameries. It has been previously demonstrated that proteolytic strains of *Lc. lactis* stimulate growth and sporulation of *Pen. roqueforti* in cheese (Hansen and Jakobsen, 1997). The possible interactions between *Lc. lactis* and *Pen. roqueforti* usually determine the cheese maturation time (8-12 weeks), aroma profile, texture and appearance of the final cheese during ripening (van den Tempel and Nielsen, 2000). Therefore, further research on how the possible interactions between the different genotypes of *Lb. plantarum, Lc. lactis* and *Pen. roqueforti* could influence the aroma properties of Stilton cheese are justifiable.

Pen. roqueforti is the major mould in Stilton cheese and predominantly grows in the outer crust and blue veins but can be less active in the outer crust when inhibited by *Yarr. lipolytica* and other yeast species (Gkatzionis, 2010). High quality Stilton cheese is characterised by extensive blueing and growth of *Deb. hansenii* (van den Tempel and Jakobsen, 2000) and facultative homofermentative lactobacilli such as *Lb. plantarum* and *Lb. curvatus* (Whitley, 2002). Good quality Stilton is also associated with accumulation of alcohols in the white core and ketones in the blue veins (Gkatzionis *et al*., 2009). At 7 weeks in this study, pure cultures of *Lb. plantarum* isolates and *Lc. lactis* subsp. *lactis* produced significant levels of ketones and alcohols, respectively. Therefore, these bacterial species may be partly responsible for accumulation of these compounds in these respective cheese sites. Strikingly, alcohol and ketone production was even enhanced

when these LAB species were co-cultured as was the case for ethanol, 2-methyl propanol, 3-methyl butanol, 2-pentanone and acetoin (Section 7.3.4).

Enhanced alcohol and ketone production by co-culturing *Lb. plantarum* and *Lc. lactis* can be utilised to improve the aroma profile of Stilton cheese especially if this synergistic effect can be confirmed *in situ* in the real cheese matrix. The present work has only applied instrumental methods to evaluate the potential contribution of different genotypes of *Lb. plantarum* to aroma properties of Stilton cheese. Further research is required for measurement of sensory perception using the human sensory panel or techniques such as flash profile analysis (Gkatzionis, 2010) in order to complement these results. There is also the need to establish the biochemical pathways involved in microbial synergies observed. The enzymes responsible for production of desirable metabolites could be isolated, characterised and applied in enzyme modified cheeses.

As individual strains often pose profound effects on cheese flavour and body characteristics (Broadbent *et al*., 2003), the unpredictable and dynamic nature of presence of different strains of *Lb. plantarum* in Stilton cheese could be an important source of cheese flavour defects and production inconsistencies. This hypothesis has been supported by results of this study. Stringent control of undesirable non-starter organisms during cheese ripening in order to produce more consistent high quality products has been less successful (Broadbent *et al*., 2003). Use of *Lb. plantarum* isolates which have been evaluated in this study as adjunct cultures for Stilton cheese could provide one of the major strategies to accomplish this goal.

8.5 *Lb. plantarum* isolates with potential for use as starter culture adjuncts

The choice of *Lb. plantarum* isolates for application as starter culture adjuncts in Stilton cheese should be based on the following properties: good growth characteristics, antimicrobial activity towards undesirable microflora, acid production, compatibility with the cheese starter cultures, ability to tolerate the stress conditions in cheese and positive contribution to the volatile aroma profile of the cheese (Crow *et al*., 2001). On the basis of the above factors, there was conclusive evidence to show significant genotypic variability among the *Lb. plantarum* isolates examined and this could have arisen from selective pressures (stresses) present in the different sites of the cheese from where they were isolated.

Isolate B30 obtained from the blue veins was particularly found to have good growth characteristics and tolerated the stresses typical of Stilton cheese, inhibited the growth of spoilage and pathogenic microorganisms and consistently enhanced acid and aroma production in milk when co-cultured with different strains of *Lc. lactis*. As the isolate was well adapted to the microenvironments within Stilton cheese, *Lb. plantarum* B30 may be applied in the cheese without the need to modify its current traditional manufacturing process. The overall efficacy of enhancing flavour development of ripened cheese using NSLAB is strongly supported by other studies which have shown that starter culture adjuncts dominated by wild strains of lactobacilli play an important role during cheese ripening (Kocaoglu-Vurma *et al*., 2008) and, therefore, should be supported for this Stilton cheese *Lb. plantarum* isolate.

However, the dynamic environment of cheese ripening and the possible variability imparted by differences in cheese composition, strains of the added starter cultures and enzymes, and the cheese manufacturing and ripening regimes may offer some challenges for universal performance of the proposed isolate in other cheese types. For example, manufacture of Gouda, Colby and the stirred variety of Cheddar cheese usually involves a washing step to remove lactose and lactic acid from the curd, which gives the finished cheese a lower acid content and a slightly higher pH (Martley and Crow, 1993; Broadbent *et al*., 2003). Also, some varieties of Cheddar cheese can be aged for years. Thus, adaptability of this *Lactobacillus* isolate under these conditions may differ from the results obtained in the present study. None-the-less, the study has provided fundamental insights about the behaviour of genotypically different isolates of *Lb. plantarum* under different experimental conditions. The knowledge would support future studies aiming to utilise the organism as a starter culture adjunct in Stilton cheese in a commercial context. As dairy industries are often faced with problems of failed fermentations arising from starter culture inactivation by bacteriophages (Garneau and Moineau, 2011), there is a need to assess phage resistance of the proposed adjunct isolate.

In conclusion, there has been general concern that the dynamic and complex nature of the various microbial populations in Stilton cheese is a major source of quality defects and product inconsistencies (Whitley, 2002; Gkatzionis, 2010). The observed desirable properties of *Lb. plantarum* isolates would support future efforts to apply them as culture adjuncts in order to provide reliable and consistent quality characteristics of the cheese.

Lb. plantarum has been successfully applied as a culture adjunct in Swiss (Kocaoglu-Vurma *et al*., 2008) and Irish Cheddar cheeses (Crow *et al*., 2001). Additional research is required to establish the impact of the isolates on the quality characteristics of other ripened cheese varieties. This approach is supported by the current keen interest to explore new possibilities that would enhance the biodiversity of matured cheeses through searching for potential starter organisms. Ideal strains for this purpose are required to have rapid and reliable acid production and reproducible development of appropriate flavour compounds (Obodai, 2006). It has been demonstrated that *Lb. plantarum* B30 meets these criteria and further research on this isolate in line with the afore-mentioned approach needs to be considered. There is scant published literature about the microflora of Stilton cheese. This study has raised several questions which will serve as the gateway for further research using similar or different approaches on other properties of the cheese.

BIBLIOGRAPHY

Aarnikunnas, J. (2006) Metabolic engineering of lactic acid bacteria and characterization of novel enzymes for the production of industrially important compounds. PhD thesis submitted to the University of Helsinki, Finland. Pp. 10-17.

Adesokan, A., Ekanola, Y.A. and Okanlawon, B.M. (2010) Influence of cultural conditions on hydrogen peroxide production by lactic acid bacteria isolated from some Nigerian traditional fermented foods. *African Journal of Microbiology Research* 4, 1991-1996.

Adler-Nissen, J. and Demain, A.L. (1994) Aeration-controlled formation of acetic acid in heterolactic fermentations. *Journal of Industrial Microbiology* 13, 335-343.

Adnan, A.F.M. and Tan, I.K.P. (2003) Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia Pp. 1-6.

AHDB (2009) World Dairy Products Production. Retrieved from : http://www.dairyco.org.uk/links.aspx, December 2009.

Ahmed, N.M., Conner, D.E. and Huffman, D.L. (1995) Heat-resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. *Journal of Food Science* 60, 606-610.

Ahmed, S.A., Ibrahim, S.A., Kim, C. and Shahbazi, A. (2009) Significance of bile salt tolerant *Lactobacillus reuteri*. Proceedings of the 2007 National Conference on Environmental Science and Technology.

Albano, H., Oliveira, M., Aroso, R., ria Cubero, N., Hogg, T. and Teixeira, P. (2007) Anti-listerial activity of lactic acid bacteria isolated from ‘‘Alheiras’’ (traditional Portuguese fermented sausages): In situ assays. *Meat Science* 76, 796-800.

Alpert, P. (2005) The Limits and frontiers of desiccation-tolerant life. *Journal of Integrated and Complementary Biology* 45, 685-695.

Amin, M., Jorfi, M., Khosravi, A.D., Samarbafzadeh, A.R. and Sheikh, A.F. (2009) Isolation and identification of *Lactobacillus casei* and *Lactobacillus plantarum* from plants by PCR and detection of their antibacterial activity. *Journal of Biological Sciences* 9, 810-814.

Ammor, S., Tauveron, G., Dufour, E. and Chevallier, I. (2006) Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1: Screening and characterization of the antibacterial compounds. *Food Control* 17, 454-461.

Archibald, F.S. and Fridovich, I. (1981) Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. *Journal of Bacteriology* 145, 442-451.

Årskold, E., Lohmeier-Vogel, E., Cao, R., Roos, S., Rådstrom, P. and van Niel, E.W.J. (2008) Phosphoketolase pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis. *Journal of Bacteriology* 190, 206-212.

Attaie, R. (2009) Quantification of volatile compounds in goat milk Jack cheese using static headspace gas chromatography. *Journal of Dairy Science* 92, 2435-2443.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1992) Short protocols in molecular biology. A compendium of methods from current protocols in molecular biology. Second edition. Greene Publishing Associates and John Wiley & Sons, New York, USA. Pp. 2-4, 2-23.

Axelsson, L. (2004) Lactic acid bacteria: classification and physiology. In: Salminen, S., von Wright, A. and Ouwehand, A. (Eds). Lactic acid bacteria: Microbiological and functional aspects. 3rd Edition. Marcel Dekker Inc. Pp. 1-633.

Badel, S., Bernardi, T. and Michaud, P. (2011) New perspectives for lactobacilli exopolysaccharides. *Biotechnology Advances* 29, 54-66.

Bakermans, C., Ayala-del-Rio, H.L. Ponder, M.A., Vishnivetskaya, T., Gilichinsky, D., Thomashow, M.F. and Tiedje, J.M. (2006) *Psychrobacter cryohalolentis sp. nov*. and *Psychrobacter arcticus sp. nov*. isolated from Siberian permafrost. *International Journal of Systematic and Evolutionary Microbiology* 56, 1285-1291.

Balaban, N. and Rasooly, A. (2000) Staphylococcal enterotoxins. *International Journal of Food Microbiology* 61, 1-10.

Banks, J.G. (2006) Risk assessment of *L. monocytogenes* in UK retailed cheese. Project: B12006. Pp. 1-124.

Banks, J.M. and Williams, A.G. (2004) The role of non-starter lactic acid bacteria in Cheddar cheese ripening. *International Journal of Dairy Technology* 57, 145-152.

Belitz, H.D. and Grosch, W.P. (2009) Aroma compounds. In: *Food Chemistry*, Belitz, H.D., Grosch, W. and Schieberle, P. (Eds). 4th Edition. Springer-Verlag Berlin, Heidelberg. Pp. 340-402.

Benson, J.A. and Ferrieri, P. (2001) Rapid pulsed-field gel electrophoresis method for group B *Streptococcus* isolates. *Journal of Clinical Microbiology* 39, 3006-3008.

Beresford, T. and Cogan, T.M. (2000) Role of lactobacilli in flavour development of Cheddar. The Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. Pp. 1-15.

Beresford, T.P., Fitzsimons, N.A., Brennan, N.L. and Cogan, T.M. (2001) Recent advances in cheese microbiology. *International Dairy Journal* 11, 259-274.

Bergen, W.G. (1977) Factors affecting growth yields of micro-organisms in the rumen. *Tropical Animal Health and Production* 4, 13-20.

Bibal, B., Vayssier, Y., Tournou, M. and Pareilleux, A. (1989) Enhanced inhibitory effect of lactic acid on growth kinetics of *Streptococcus cremoris*during nutritional medium limitations. *Applied Microbiology and Biotechnology* 30, 630-635.

Blaiotta, G., Fusco, V., Ercolini, D. and Aponte, M. (2008) *Lactobacillus* strain diversity based on partial *hsp60* gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. *Applied and Environmental Microbiology* 74, 208-215.

Blaiotta, G., Moschetti, M., Simeoli, E., Andolfi, R., Villani, F. and Coppola, S. (2001) Monitoring lactic acid bacteria strains during 'Cacioricotta' cheese production by restriction endonuclease analysis and pulsed-field gel electrophoresis. *Journal of Dairy Science* 68, 139-144.

Bonade, A., Murelli, F., Vescovo, M. and Scolari, G. (2001) Partial characterisation of a bacteriocin produced by *Lactobacillus helveticus*. *Letters in Applied Microbiology* 33, 153-158.

Bora, N., Vancanneyt, M., Gelsomino, R., Swings, J., Brennan, N., Cogan,T.M., Larpin, S., Desmasures, N., Lechner, F.E., Kroppenstedt, R.M., Ward, A.C. and Goodfellow, M. (2007) *Agrococcus casei* sp. nov., isolated from the surfaces of smear-ripened cheeses. *International Journal of Systematic and Evolutionary Microbiology* 57, 92-97.

Boris, S., Jimenez-Diaz, R., Caso, J.L. and Barbes, C. (2001) Partial characterisation of a bacteriocin produced by *Lactobacillus delbrueckii* subsp. *lactis* UO004, an intestinal isolate with probiotic potential. *Journal of Applied Microbiology* 91, 328-333.

Bouton, Y., Guyot, P., Beuvier, E., Tailliez, P. and Grappin, R. (2002) Use of PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comte´ cheese ripening. *International Journal of Food Microbiology* 76, 27-38.

Briggiler-Marco, M., Capra, M.L., Quiberoni, A., Vinderola, G., Reinheimer, J.A. and Hynes, E. (2007) Nonstarter *Lactobacillus* strains as adjunct cultures for cheese making: In vitro characterization and performance in two model cheeses. *Journal of Dairy Science* 90, 4532-4542.

Broadbent, J.R., Houck, K., Johnson, M.E. and Oberg, C.J. (2003) Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. *Journal of Dairy Science* 86, 2773-2782.

Bromberg, R., Moreno, I., Zaganini, C.L., Delboni, R.R. and de Oliveira, J. (2004) Isolation of bacteriocin producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. *Brazilian Journal of Microbiology* 35,137-144.

Buchin, S., Delague, V., Duboz, G., Berdague, J.L., Beuvier, E, Pochet, S. and Grappin, R. (1998) Influence of pasteurization and fat composition of milk on the volatile

compounds and flavour characteristics of a semi-hard cheese. *Journal of Dairy Science* 81, 3097-3108.

Bulleman, L.B. (1981) Public health significance of moulds and mycotoxins in fermented dairy products. *Journal of Dairy Science* 64, 2439-2452.

Buntin, N., Chanthachum, S. and Hongpattarakere, T. (2008) Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics. *Songklanakarin Journal of Science and Technology* 30, 141-148.

Bylund, G. (1995) Dairy Processing Handbook. Tetra Pak Processing Systems AB, Lund, Sweden. Pp. 57 and 233.

Cantor, M.D., van den Tempel, T., Hansen, T.K. and Ardo, Y. (2004) Blue cheese. In: Fox, P.F., McSweeney, P.L.H., Cogan, T.M. and Guinee, T.P. (Eds). Cheese: chemistry, physics and microbiology. 3rd edition, volume 2 (Major cheese groups). Elsevier Academic Press, London, UK. Pp. 1-438.

Catte, M., Gancel, F., Dzierszinski, F. and Tailliez, R. (1999) Effects of water activity, NaCl and smoke concentrations on the growth of *Lactobacillus plantarum* ATCC12315. *International Journal of Food Microbiology* 52, 105-108.

CDSC (2005) Enumeration of *Staphylococcus aureus.* Issued by Standards Unit, Evaluations and Standards Laboratory: Specialist and Reference Microbiology Division. Issue no. 1.4 . Pp. 8- 12.

Chamba, J.F. and Irlinger, F. (2004) Secondary and adjunct cultures. *Cheese: Chemistry, Physics and Microbiology* 1, 191-206.

Champagne, C.P., Laing, R.R., Roy, D. and Mafu, A.A. (1994) Psychrophs in dairy products: Their effects and their control. *Critical Reviews in Food Science and Nutrition* 34, 1-30.

Chen, M. and Alexander, M. (1973) Survival of soil bacteria during prolonged desiccation. *Journal of Soil Biochemistry* 5, 213-221.

Cho, G.S., Huch, M., Hanak, A., Holzapfel, W.H. and Franz, C.M.A.P. (2010) Genetic analysis of the plantaricin EFI locus of *Lactobacillus plantarum* PCS20 reveals an unusual plantaricin E gene sequence as a result of mutation. *International Journal of Food Microbiology* 141, 117-124.

Coda, R., Brechany, E., De Angelis, M., De Candia, S., Di Cagno, R. and Gobbetti, M. (2006) Comparison of the compositional, microbiological, biochemical, and volatile profile characteristics of nine Italian Ewes' milk cheeses. *Journal of Dairy Science* 89, 4126-4143.

Cogan, T.M., Beresford, T.P., Steele, J., Broadbent, J., Shah, N.P. and Ustunol, Z. (2007) *Invited Review:* Advances in starter cultures and cultured foods. *Journal of Dairy Science* 90, 4005-4021.

Collins, C.H., Lyne, P.M. and Graige, J.M. (1989) Microbiological Methods (6th Edn). Butterworth & Co.: London. Pp. 100-105.

Collins, Y.F., McSweeney, P.L.H. and Wilkinson, M.G. (2003) Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal* 13, 841-866.

Conesa, R., Andreu, S., Fernandez, P.S., Esnoz, A. and Palop, A. (2009) Nonisothermal heat resistance determinations with the thermoresistometer Mastia. *Journal of Applied Microbiology* 107, 506-513.

Constantiniu, S., Romaniuc, A., Iancu, L.S., Filimon, R. and Taraşi, I. (2004) Cultural and biochemical characteristics of *Acinetobacter* spp. strains isolated from hospital units. *The Journal of Preventive Medicine* 12, 35-42.

Coolbear, T., Crow, V., Harnett, J., Harvey, S., Holland, R. and Martley, F. (2008) Developments in cheese microbiology in New Zealand: Use of starter and non-starter lactic acid bacteria and their enzymes in determining flavour. Milestone Achievements in dairy science research and their current and future industrial applications - Proceedings of a Symposium Marking the Retirement. *International Dairy Journal* 18, 705-713.

Corsetti, A., Rossi, J. and Gobbetti, M. (2001) Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *International Journal of Food Microbiology* 69, 1-10.

Crow, V., Curry, B. and Hayes, M. (2001) The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *International Dairy Journal* 11, 275-283.

De Angelis, M., Di Cagno, R., Huet, C., Crecchio, C., Fox, P.F. and Gobbetti, M. (2004) Heat shock response in *Lactobacillus plantarum*. *Applied and Environmental Microbiology* 70, 1336-1346.

De Freitas, M.F.L., Luz, I.S., Silveira-Filho, V.M., Júnior, J.W.P., Stamford, T.L.M., Mota, R.A., de Sena, M.J., de Almeida, A.M.P., Balbino, V.Q. and Leal-Balbino, T.C. (2008) Staphylococcal toxin genes in strains isolated from cows with subclinical mastitis1. *Pesquisa Veterinária Brasileira* 28, 617-621.

De Lima, C.J.B., Cortezi, M., Lovaglio, R.B., Ribeiro, E.J., Contiero, J. and De Araújo, E.H. (2008) Production of rennet in submerged fermentation with the filamentous fungus *Mucor miehei* NRRL 3420. *World Applied Sciences Journal* 4, 578-585.

De Vuyst, L. and Leroy, F. (2007) Bacteriocins from lactic acid bacteria: production, purification and food applications. *Journal of Molecular Microbiology and Biotechnology* 13, 194-199.

Deetae, P., Spinnler, H.E., P. and Sandra Helinck, S. (2009) Growth and aroma contribution of*Microbacterium foliorum*, *Proteus vulgaris*and *Psychrobacter* sp. during ripening in a cheese model medium. *Applied Microbiology and Biotechnology* 82, 169-177.

Derzelle, S., Hallet, B., Ferain, T., Delcour, J. and Hols, P. (2003) Improved adaptation to cold-shock, stationary-phase, and freezing stresses in *Lactobacillus plantarum* overproducing cold-shock proteins. *Applied and Environmental Microbiology* 69, 4285-4290.

Di Cagno, R., De Angelis, M., Calasso, M., Vincentini, O., Vernocchi, P., Ndagijimana, M., De Vincenzi, M., Dessı`, M.R., Guerzoni, M.E. and Gobbetti, M. (2010) Quorum sensing in sourdough *Lactobacillus plantarum* DC400: Induction of plantaricin A (PlnA) under co-cultivation with other lactic acid bacteria and effect of PlnA on bacterial and Caco-2 cells. *Proteomics* 10, 2175-2190.

Di Cagno, R., De Angelis, M., Lavermicocca, P., De Vincenzi, M., Giovannini, C., Faccia, M. and Gobbetti, M. (2002) Proteolysis by Sourdough lactic acid bacteria: effects on wheat flour protein fractions and gliadin peptides involved in human cereal intolerance. *Applied and Environmental Microbiology* 68, 623-633.

Diep, D.B., Havarstein, L.S., Nissen-Meyer, J. and Nes, I.F. (1996) Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. *Journal of Bacteriology* 178, 4472-4483.

Diep, D.B., Straume, Kjos, M., Torres, C. and Nes, I.F. (2009) An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. *Peptides* 30, 1562-1574.

Doyle, M.E. (2007) Microbial food spoilage: Losses and control strategies. A brief review of the literature. *FRI Briefings: Microbial Food Spoilage: Losses and Control Strategies*, 1-16.

Duthoit, F., Godon, J.J. and Montel, M.C. (2003) Bacterial community dynamics during production of Registered Designation of Origin Salers cheese as evaluated by 16S rDNA gene single-strand conformation polymorphism analysis. *Applied and Environmental Microbiology* 69, 3840-3848.

Eijsink, V.G.H., Axelsson, L., Diep, D.B., Håvarstein, L.S., Holo, H. and Nes, I.F. (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek* 81, 639-654.

Ennahar, S., Aoude-Werner, D., Sorokine, O., van Dorsselaer, A., Bringel, F., Hubert, J.C. and Hasselmann, C. (1996) Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. *Applied and Environmental Microbiology* 62, 4381-4387.

Ercolini, D., Hill, P.J. and Dodd, C.E.R. (2003) Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology* 69, 3540-3548.

Erdogan, A., Gurses, M. and Sert, S. (2003) Isolation of moulds capable of producing mycotoxins from blue mouldy Tulum cheeses produced in Turkey. *International Journal of Food Microbiology* 85, 83-85.

Essid, I., Medini, M. and Hassouna, M. (2009) Technological and safety properties of *Lactobacillus plantarum* strains isolated from a Tunisian traditional salted meat. *Meat Science* 81, 203-208.

Excoffier, L., Smouse, P.E. and Quattro, J.M. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial DNA restriction data. *Genetics* 131, 479-491.

Farkye, N.Y. (2004) Cheese technology. *International Journal of Dairy Technology* 57, 91-98.

Fayol-Messaoudi, D., Berger, S.N., Coconnier-Polter, M.H., Moal, V.L. and Servin, A.L. (2005) pH, lactic acid and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enterica* serovar *Typhimurium*. *Applied and Environmental Microbiology* 71, 6008-6013.

Felis, G.E. and Dellaglio, F. (2012) Taxonomy of lactobacilli and Bifidobacteria. *Current Issues in Intestinal Microbiology* 8, 44-61.

Fernández, M.A.B., Mauriz, E., Gómez, A. and Martín, J.F. (2009) Proteolytic activity, Mycotoxins and Andrastin A in *Penicillium roqueforti* strains isolated from Cabrales, Valdeón and Bejes–Tresviso local varieties of blue-veined cheeses. *International Journal of Food Microbiology* 136, 18-25.

Fernandez-Garcia, E. and Mcgregor, J.U. (1994) Determination of organic acids during the fermentation and cold storage of yogurt. *Journal of Dairy Science* 11, 2934-2939.

Fernández-Salguero, J., Alcalá, M., Marcos, A. and Esteban, M.A. (1986) Measurement and calculation of water activity in blue cheese. *Journal of Dairy Research* 53, 639-644.

Filtenborg, O., Frisvad, J.C and Thrane, U. (1996) Moulds in food spoilage. *International Journal of Food Microbiology* 33, 85-102.

Fitzpatrick, S.C. (1971) The influence of the internal atmosphere upon the ripening of blue Stilton cheese. PhD thesis submitted to the Department of Applied Biochemistry and Nutrition, The University of Nottingham. P. 1.

Fitzsimons, N.A., Cogan, T.M., Condon, S. and Beresford, T. (1999) Phenotypic and genotypic characterization of non-Starter lactic acid bacteria in mature Cheddar cheese. *Applied and Environmental Microbiology* 65, 3418-3426.

Fitzsimons, N.A., Cogan, T.M., Condon, S. and Beresford, T. (2001) Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. *Journal of Applied Microbiology* 90, 600-608.

Food Safety Authority of Ireland (2003) Information note on protected designation of origin (PDO), protected geographical indication (PGI) and traditional speciality guaranteed (TSG). Retrieved from: https://www.fsai.ie/uploadedFiles/About_Us/forums/artisan/PDO_PGI_TSG_Info_Note.pdf, August 2013.

Flambard, B., Richard, J. and Juillard, V. (1997) Interaction between proteolytic strains of *Lactococcus lactis* influenced by different types of proteinase during growth in milk. *Applied and Environmental Microbiology* 63, 2131-2135.

Florez, A.B. and Mayo, B. (2006) PCR-DGGE as a tool for characterising dominant microbial populations in the Spanish blue-veined Cabrales cheese. *International Dairy Journal* 16, 1205-1210.

Floreza, A.B., Alvarez-Martin, P., Lopez-Diaz, T.M. and Mayo, B. (2007) Morphotypic and molecular identification of filamentous fungi from Spanish blue-veined Cabrales cheese, and typing of *Penicillium roqueforti* and *Geotrichum candidum* isolates. *International Dairy Journal* 17, 350-357.

Fontana, C., Cappa, F., Rebecchi, A. and Cocconcelli, P.S. (2010) Surface microbiota analysis of Taleggio, Gorgonzola, Casera, Scimudin and Formaggio di Fossa Italian cheeses. *International Journal of Food Microbiology* 138, 205-211.

Fox, P., Mcsweeney, P.L.H., Cogan, T.M. and Guinee, T.P. (2000) Fundamentals of cheese science. USA: Aspen Publishers Inc.

Franz, C.M.A.P., Holzapfel, W.H. and Stiles, M.E. (1999) Enterococci at the crossroads of food safety. *International Journal of Food Microbiology* 47, 1-24.

Fu, W. and Mathews, A.P. (1999) Lactic acid production from lactose by *Lactobacillus plantarum*: kinetic model and effects of pH, substrate, and oxygen. *Biochemical Engineering Journal* 3, 163-170.

Fukuda, K., Shi, T., Nagami, K., Leo, F., Nakamura, T., Yasuda, K., Senda, A., Motoshima, H. and Urashima, T. (2010) Effects of carbohydrate source on physicochemical properties of the exopolysaccharide produced by *Lactobacillus fermentum* TDS030603 in a chemically defined medium. *Carbohydrate Polymers* 79, 1040-1045.

Fusco, V., Quero, G.M., Morea, M., Blaiotta, G. and Visconti, A. (2011) Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (*egc*) and quantitative detection in raw milk by real time PCR. *International Journal of Food Microbiology* 144, 528-537.

Gala, E., Landi, S., Solieri, L., Nocetti, M., Pulvirenti, A. and Giudici, P. (2008) Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. *International Journal of Food Microbiology* 125, 347-351.

Gallois, A. and Langlois, D. (1990) New results in the volatile odorous compounds of French blue cheeses. Lait (Lyon) 70, 89-106.

Gálvez, A., Abriouel, H., López, R.L. and Omar, N.B. (2007) Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology* 120, 51-70.

Ganzle, M.G., Vermeulen, N. and Vogel, R.F. (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. *Food Microbiology* 24, 128-138.

Garde, S., Gaya, P., Medina, M. and Nuñez, M. (1997) Acceleration of flavour formation in cheese by a bacteriocin-producing adjunct lactic culture. *Biotechnology Letters* 10, 1011-1014.

Garneau, J.E. and Moineau, S. (2011) Bacteriophages of lactic acid bacteria and their impact on milk fermentations. *Garneau and Moineau Microbial Cell Factories* 10, 1-10.

Gatti, M., Lindner, J.D., De Lorentiis, A., Bottari, B., Santarelli, M., Bernini, V. and Neviani, E. (2008) Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. *Applied and Environmental Microbiology*, 74, 6161-6167.

Gelsomino, R., Vancanneyt, M., Cogan, T.M., Condon, S. and Swings, J. (2002) Source of enterococci in a farmhouse raw-milk cheese. *Applied and Environmental Microbiology* 68, 3560-3565.

Ghotbi, M. (2011) Identification of *L. pentosus*, *L. paraplan*tarum and *L. plantarum* in Lighvan cheese with 4 months ripening period by means of *rec*A gene sequence analysis. *2nd International Conference on Biotechnology and Food Science* vol 7, IACSIT Press, Singapore.

Gill, C.O. and Newton, K.G. (1982) Effect of lactic acid concentration on growth on meat of Gram negative psychrotrophs from a meatworks. *Applied and Environmental Microbiology* 43, 284-288.

Giraffa, G. (2003) Functionality of enterococci in dairy products. *International Journal of Food Microbiology* 2-3, 215-222.

Giraffa, G. (2004) Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiology Reviews* 28, 251-260.

Giraffa, G. and Carminati, D. (2008) In: Cocolin, L. and Ercolini, D. (Eds). Molecular techniques in food fermentation: Principles and applications. Springer Science and Business Media, Newyork, USA. Pp. 1-4.

Girrafa, G. and Neviani, E. (1999) Different *Lactobacillus helveticus* strain populations dominate during *Grana Padano* cheesemaking. *Food Microbiology* 16, 205-210.

Gkatzionis, K. (2010) Flavour production of Stilton blue cheese microflora. PhD thesis submitted to The University of Nottingham. Pp. 1-248.

Gkatzionis, K., Linforth, R.S.T. and Dodd, C.E.R. (2009) Volatile profile of Stilton Cheeses: Differences between zones within a cheese and dairies. *Food Chemistry* 113, 506-512.

Gonzalez, B., Arca, P., Mayo, B. and Suarez, J.E. (1994) Detection, purification, and partial characterization of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain of dairy origin. *Applied and Environmental Microbiology* 60, 2158-2163.

Grappin, R. and Beuvier, E. (1997) Possible implications of milk pasteurisation on the manufacture and sensory quality of ripened cheese. *International Dairy Journal* 7, 751-761.

Gunasekaran, S. and Mehmet, M. (2003) Cheese rheology and texture. CRC Press, Florida, USA. Pp. 1-30.

Hansen, T.K. and Jakobsen, M. (1997) Possible role of microbial interactions for growth and sporulation of *Penicillium roqueforti* in Danablu. *Lait* 77, 479-488.

Harris, L.J., Fleming, H.P. and Klaenhammer, T.R. (1992) Novel paired starter culture system for Sauerkraut, consisting of a nisin-resistant *Leuconostoc mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain. *Applied and Environmental Microbiology* 58, 1484-1489.

Hiscox, E.R., Harrison, J. and Wolf, J.Z. (1940) The microflora of Stilton cheese. *Journal of Applied Microbiology* (2008) 3, 23-26.

Hocking, A.D. and Faedo, M. (1992) Fungi causing thread mould spoilage of vacuum packaged Cheddar cheese during maturation. *International Journal of Food Microbiology* 16, 123-130.

Hofvendahl, K. and Hahn-Hägerdal, B. (2000) Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Technology* 26, 87-107.

Honeyman, A., Friedman, H. and Bendinelli, M. (2002) *Staphylococcus aureus* infection and disease. Kluwer Academic Publishers, London, UK. Pp. 122-126.

Hood, M.I., Jacobs, A.C., Sayood, K., Dunman, P.M. and Skaar, E.P. (2010). *Acinetobacter baumannii* increases tolerance to antibiotics in response to monovalent cations. *American Society of Microbiology* 3, 1029-1041.

Hurst, A. (1977) Bacterial injury: a review. *Canadian Journal of Microbiology* 23, 936-944.

Iacobellist, N.S. and Devay, J.E. (1986) Long-term storage of plant-pathogenic bacteria in sterile distilled water. *Applied and Environmental Microbiology* 52, 388-389.

Ibourahema, C., Dauphin, R.D., Jacqueline, D. and Thonart, P. (2008) Characterization of lactic acid bacteria isolated from poultry farms in Senegal. *African Journal of Biotechnology* 7, 2006-2012.

International Dairy Federation, IDF (1988) Fermented milks: Science and Technology. *Bulletin of the IDF* No. 227. Pp. 1-227.

International Dairy Foods Association, IDFA (2012) Cheese sales and trends. Retrieved from: http://www.idfa.org/news--views/media-kits/cheese/cheese-sales-and-trends/, July 2012.

Johnson, W.M., Tyler, S.D., Ewan, E.P., Ashton, F.E., Pollard, D.R. and Rozee, K.R. (1991) Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *Journal of Clinical Microbiology* 29, 426-430.

Jordan, K.N. and Cogan, T.M. (1999) Heat resistance of *Lactobacillus* spp. isolated from Cheddar cheese. *Letters in Applied Microbiology* 29, 136-140.

Jordan, S.J., Dodd, C.E.R., and Stewart, G.S.A.B. (1999) Use of conformation polymorphism analysis to examine the variability of the rpoS sequence in environmental isolates of *Salmonellae*. *Applied and Environmental Microbiology* 65, 3582-3587.

Kang, D.H. and Siragusa, G.R. (1999) Agar underlay method for recovery of sublethally heat-injured bacteria. *Applied and Environmental Microbiology* 65, 5334-5337.

Khalid, K. (2011) An overview of lactic acid bacteria. *International Journal of Biosciences* 1, 1-13.

Kieronczyk, A., Skeie, S., Langsrud, T. and Yvon, M. (2003) Cooperation between *Lactococcus lactis* and non-starter lactobacilli in the formation of cheese aroma from amino acids. *Applied and Environmental Microbiology* 69, 734-739.

King, K.Y., Horenstein, J.A. and Caparon, M.G. (2000) Aerotolerance and peroxide resistance in peroxidase and *PerR* mutants of *Streptococcus pyogenes*. *Journal of Bacteriology* 19, 5290-5299.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W., Stiekema, W., Lankhorst, R.M., Bron, P.A., Hoffer, S.M., Groot, M.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos W.M. and Siezen, R.J. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Science U.S.A*. 100, 1990-1995.

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

Kocaoglu-Vurma, N.A., Harper, W.J., Drake, M.A. and Courtney, P.D. (2008) Microbiological, chemical, and sensory characteristics of Swiss cheese manufactured with adjunct *Lactobacillus* strains using a low cooking temperature. *Journal of Dairy Science* 91, 2947-2959.

Kumar, C.G. and Anand, S.K. (1998) Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology* 42, 9-27.

Kunene, N.F., Geornaras, I., von Holy, A. and Hastings, J.W. (2000) Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented weaning food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. *Applied and Environmental Microbiology* 66, 1084-1092.

Lamprell, H., Villard, L., Chamba, J.F., Beuvier, E., Borges, E., Maurin, F., Mazerolles, G., Noel, Y. and Kodjo, A. (2004) Identification and biotyping of coagulase positive staphylococci (CPS) in ripened French raw milk cheeses and their *in vitro* ability to produce enterotoxins. *Revue Médicin Véterinnaire* 155, 92-96.

Lawlor, J.B., Delahunty, C.M., Sheehan, J. and Wilkinson, M.G. (2003) Relationships between sensory attributes and the volatile compounds, non-volatile and gross compositional constituents of six blue-type cheeses. *International Dairy Journal* 13, 481-494.

Lawrence, R.C., Creamer, L.K. and Gilles, J. (1987) Texture development during cheese ripening. *Journal of Dairy Science* 70, 1748-1760.

Le Loir, Y., Baron, F. and Gautier, M. (2003) *Staphylococcus aureus* and food poisoning. *Genetic and Molecular Research* online Journal,1676-5680. Retrieved from: http://www.funpecrp.com.br/gmr/year2003/vol1-2/sim0009_full_text.htm, October 2011.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C.J. (2008) Genes and molecules of lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reviews* 72, 728-764.

Ledenbach, L.H. and Marshall, R.T. (2009) Microbiological spoilage of dairy products. In: Sperber, W.H. and Doyle, M.P. (Eds.) *Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety* vol. 41. Springer Science and Business Media. Pp. 41-67.

Li, J.S., Bi, Y.T., Dong, C., Yang, J.F. and Liang, W.D. (2011) Transcriptome analysis of adaptive heat shock response of *Streptococcus thermophilus*. *PLoS ONE* 6, Retrieved from: doi:10.1371/journal.pone.002577. www.plosone.org, November 2011.

Liao, Q., Hang, X., Liu, X., Pan, J., Zhang, H. and Yang, H. (2010) The influence of pH on heat stress response by probiotic *Lactobacillus plantarum* LP-Onlly. *Annual Microbiology Reviews* 60, 341-348.

Linders, L.J.M., Wolkers, W.F., Hoekstra, F.A. and van't Riet, K. (1997) Effect of added carbohydrates on membrane phase behaviour and survival of dried *Lactobacillus plantarum*. *Cryobiology* 35, 31-40.

Litopoulou-Tzanetaki, E., Graham, D.C. and Beyatli, Y. (1989) Detection of pediococci and other non-starter organisms in American Cheddar cheese. *Journal of Dairy Science* 72, 854-858.

Little, C.L., Harris, J., Sagoo, S.K., Greenwood, M., Mithani, V., Grant, K., McLauchlin, J. and Food, Water and Environmental surveillance network (2005) Microbiological examination of cheese made from pasteurised milk from production establishments and retail premises in the United Kingdom. *European Commission co-ordinated programme for official control of foodstuffs*, 1-24.

Liu, M., Bayjanov, J.R., Renckens, B., Nauta, A. and Siezen, R.J. (2010)The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BioMed Central Genomics* 11, 1471-2164.

Liu, S.Q. (2003) Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *International Journal of Food Microbiology* 83, 115-131.

Longo, M.A. and Sanromán, M.A. (2006) Production of food aroma compounds: Microbial and enzymatic methodologies. *Food Technology and Biotechnology* 44, 335-353.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., Gheynst, J.V. and Mills, D.A. (2003) Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* 69, 6801-6807.

Lortal, S. and Chapot-Chartier, M.P. (2005) Role, mechanisms and control of lactic acid bacteria lysis in cheese. *International Dairy Journal* 15, 857-871.

Madkor, S., Fox, P.F., Shalabi, S.I. and Metwalli, N.H. (1987)[a] Studies on the ripening of Stilton cheese: Proteolysis. *Food Chemistry* 25, 13-29.

Madkor, S., Fox, P.F., Shalabi, S.I. and Metwalli, N.H. (1987)[b] Studies on the ripening of Stilton cheese: Lipolysis. *Food Chemistry* 25, 93-109.

Maicas, S., Ferrer, S. and Pardo, I. (2002) NAD(P)H regeneration is the key for heterolactic fermentation of hexoses in *Oenococcus oeni*. *Microbiology* 148, 325-332.

Maldonado, A., Jimenez-Díaz, R. and Ruiz-Barba, J.L. (2004) Induction of plantaricin production in *Lactobacillus plantarum* NC8 after co-culture with specific Gram-positive bacteria is mediated by an auto-induction mechanism. *Journal of Bacteriology* 186, 1556-1564.

Maldonado, A., Ruiz-Barba, J.L. and Jimenez-Díaz, R. (2003) Purification and genetic characterization of plantaricin NC8, a novel co-culture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. *Applied and Environmental Microbiology* 69, 383-389.

Maldonado-Barragán, A., Ruiz-Barba, J.L. and Jiménez-Díaz, R. (2009) Knockout of three component regulatory systems reveals that the apparently constitutive plantaricin-production phenotype shown by *Lactobacillus plantarum* on solid medium is regulated via quorum sensing. *International Journal of Food Microbiology* 130, 35-42.

Marathe, M.Y. and Ghosh, J.S. (2009) Study of proteinase activity of *Lactobacillus plantarum* NCIM 2083. *International Journal of Genetics and Molecular Biology* 1, 1-5.

Martin-Platero, A.M., Valdivia, E., Maqueda, M., Martin-Sanchez, I. and Martinez-Bueno, M. (2008) Polyphasic approach to bacterial dynamics during the ripening of Spanish Farmhouse cheese, using culture-dependent and independent methods. *Applied and Environmental Microbiology* 74, 5662-5673.

Martley, F.G. and Crow, V.L. (1993) Interactions between non-starter microorganisms during Cheese manufacture and ripening. *International Dairy Journal* 3, 461-483.

Mary, P., Ochin, D. and Tailliez, R. (1985) Rates of drying and survival of *Rhizobium meliloti* strains during storage at different relative humidities. *Applied and Environmental Microbiology* 50, 207-211.

Maus, J.E. and Ingham, S.C. (2003) Employment of stressful conditions during culture production to enhance subsequent cold and acid tolerance of bifidobacteria. *Journal of Applied Microbiology* 95, 146-154.

McCann, M.S., McGovern, A.C., McDowell, D.A., Blair, I.S. and Sheridan, J.J. (2006) Surface decontamination of beef inoculated with *Salmonella* Typhimurium DT104 or *Escherichia coli* O157 : H7 using dry air in a novel heat treatment apparatus. *Journal of Applied Microbiology* 101, 1177-1187.

McSweeney, P.L.H. (2004) Biochemistry of cheese ripening. *International Journal of Dairy Technology* 57, 127-144.

Meijer, W., Van De Bunt, B., Twigt, M., De Jonge, B., Smit, G. and Hugenholtz, J. (1998) Lysis of *Lactococcus lactis* subsp. *cremoris* SK110 and its nisin-immune transconjugant in relation to flavor development in cheese. *Applied and Environmental Microbiology* 64, 1950-1953.

Mehrotra, M., Wang, G. and Johnson, W.M. (2000) Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1 and methicillin resistance. *Journal of Clinical Microbiology* 38, 1032-1035.

Meng, X.C., Stanton, C., Fitzgerald, G.F., Daly, C. and Ross, R.P. (2008) Anhydrobiotics: the challenges of drying probiotic cultures. *Food Chemistry* 106, 1406-1416.

Messi, P., Bondi, M., Sabia, C., Battini, R. and Manicardi, G. (2001) Detection and preliminary characterisation of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. *International Journal of Food Microbiology* 64, 193-198.

Mikelsaar, M., Annuk, H., Shchepetova, J., Mandar, R.,1, Sepp, E. and Bjorksten, B. (2002) Intestinal lactobacilli of Estonian and Swedish children. *Microbial Ecology in Health and Disease* 14, 75-80.

Milesi, M.M., Wolf, V., Bergamini, C.V. and Hynes, E.R. (2010) Two strains of non-starter lactobacilli increased the production of flavor compounds in soft cheeses. *Journal of Dairy Science* 93, 5020-5031.

Mills, S., Stanton, C. and Ross, R.P. (2011) New developments and applications of bacteriocins and peptides in foods. *Annual Reviews in Food Science and Technology* 2, 299-329.

Moll, G.N., Van den Akker, E., Hauge, H.H., Nissen-Meyer, J., Nes, W.N.K. and Driessen, A.J.M. (1999) Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. *Journal of Bacteriology* 181, 4848-4852.

Moore, M.A. and Datta, A.R. (1994) DNA fingerprinting of *Listeria monocytogenes* strains by pulse-field gel electrophoresis. *Journal of Food Microbiology* 11, 31-38.

Morgan, C.A., Herman, N., White, P.A. and Vesey, G. (2006) Preservation of micro-organisms by drying; a review. *Journal of Microbiological Methods* 66, 183-193.

Morgana, S.M., O'Sullivana, L., Rossa, R.P. and Hill, C. (2002) The design of a three strain starter system for Cheddar cheese manufacture exploiting bacteriocin-induced starter lysis. *International Dairy Journal* 12, 985-993.

Moulay, M., Aggad, H., Benmechernene, Z., Guessas, B., Henni, D.E. and Kihal, M. (2006) Cultivable lactic acid bacteria isolated from Algerian raw goat's milk and their proteolytic activity. *World Journal of Dairy and Food Sciences* 1, 12-18.

Mounier, J., Gelsomino, R., Goerges, S., Vancanneyt, M., Vandemeulebroecke, K., Hoste, B., Scherer, S., Swings, J., Fitzgerald, G.F. and Cogan, T.M. (2005) Surface microflora of four smear-ripened cheeses. *Applied and Environmental Microbiology* 71, 6489-6500.

Mubarack, H.M., Doss, A., Dhanabalan, R. and Balachander, S. (2010) Microbial quality of raw milk samples collected from different villages of Coimbatore District, Tamilnadu, South India. *Indian Journal of Science and Technology* 3, 61-63.

Mullan, W.M.A. (2001) Microbiology of starter cultures. Retrieved from: http://www.dairyscience.info/cheese-starters/49-cheese-starters.html, April 2013.

Murphy, M.G. and S. Condon, S. (1984) Correlation of oxygen utilisation and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. *Archives of Microbiology* 138, 44-48.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rDNA. *Applied and Environmental Microbiology* 59, 695-700.

Naffa, R.G., Bdour, S.M., Migdadi, H.M. and Shehabi, A.A. (2006) Enterotoxicity and genetic variation among clinical *Staphylococcus aureus* isolates in Jordan. *Journal of Medical Microbiology* 55, 183-187.

Narendranath, N.V. and Power, R. (2005) Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and *Saccharomyces cerevisiae* during ethanol production. *Applied and Environmental Microbiology* 71, 2239-2243.

Nelson, J.H. (1970) Production of blue cheese flavour via submerged fermentation by *Penicillium roqueforti*. *Journal of Agriculture and Food Chemistry* 18, 567.

Nguyen, A.L., Tran-Dinh, D., Hocking, A. and Fleet, G. (2010) Effects of age on heat resistance of *Byssochlamys ascopores*. In: 22nd *International ICFMH Symposium Food Micro 2010*, P. 231.

Nissen-Meyer, J., Larsen, A.G., Sletten, K., Daeschel, M. and Nes, I.F. (1993) Purification and characterization of plantaricin A, a *LactobacifZus plantarum* bacteriocin

whose activity depends on the action of two peptides. *Journal of General Microbiology* 139, 1973- 1978.

Nissen-Meyer, J., Oppegard, C., Rogne, P., Haugen, H.S., Kristiansen, P.E. (2010) Structure and mode of action of the two peptide (class IIb) bacteriocins. *Probiotics and Antimicrobial Proteins* 2, 52-60.

Obadina, A.O., Oyewole, O.B., Sanni, L.O. and Tomlins, K.I. (2006) Bio-preservative activities of *Lactobacillus plantarum* strains in fermenting Casssava 'fufu'. *African Journal of Biotechnology* 5, 620-623.

Obodai, M.A.B. (2006) Characterisation of the dominant microbiota in *Nyamie*, a Ghananian fermented milk product. PhD thesis submitted to the University of Nottingham. Pp. 46-50.

Olaoye, O.A. And Dodd, C.E.R. (2010) Evaluation of bacteriocinogenic *Pediococcus acidilactici* as protective culture in the preservation of *tsire*, a traditional Nigerian stick meat. *Journal of Food Safety* 30, 867-888.

Olson, N.F. (1990) The impact of lactic acid bacteria on cheese flavour. *FEMS Microbiology Letters* 87, 131-147.

Ott, A., Germond, J.E. and Chaintreau, A. (2000) Vicinal diketone formation in yogurt: (13) C precursors and effect of branched-chain amino acids. *Journal of Agriculture and Food Chemistry* 48, 724-731.

Pacheco, F.P. and Galindo, A.B. (2010) Microbial safety of raw milk cheeses traditionally made at a pH below 4.7 and with other hurdles limiting pathogens growth. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, 1205-1213.

Pacova, Z., Urbanova, E. and Durnova, E. (2001) *Psychrobacter immobilis* isolated from foods: Characteristics and identification. *Veterinary Medicine-Czech Republic* 46, 95-100.

Pan, X., Chen, F., Wu, T., Tang, H. and Zhao, Z. (2009) The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. *Journal of Food Control* 20, 598-602.

Park, S.C., Baik, K.S., Kim, D. and Seong, C.N. (2009) *Maritimimonas rapanae* gen. nov., sp. nov., isolated from gut microflora of the veined rapa whelk, *Rapana venosa*. *International Journal of Systematic and Evolutionary Microbiology* 59, 2824-2829.

Passos, F.V., Fleming, H.P., Ollis, D.F., Felder, R.M. and Mcfeeters, R.F. (1994) Kinetics and modelling of lactic acid production by *Lactobacillus plantarum*. *Applied and Environmental Microbiology* 60, 2627-2636.

Patil, M., Pal, A., Pal, V. and Yaddula, R.K. (2007) Isolation of bacteriocinogenic lactic acid bacteria from rat intestine. *Journal of Culture Collections* 5, 58-63.

Peakall, R. and Smouse, P.E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6, 288-295.

Pedersen, T.B., Olsen, J.E. and Bisgaard, M. (2008) Persistence of Salmonella Senftenberg in poultry production environments and investigation of its resistance to desiccation. *Avian Pathology* 37, 421-427.

Pelinescu, D.R., Sasarman, E., Chifiriuc, M.C., Stoica, I., Nohit, A.M., Avram, I., Serbancea, F. and Dimov, T.V. (2009) Isolation and identification of some *Lactobacillus* and *Enterococcus* strains by a polyphasic taxonomical approach. *Romanian Biotechnology Letters* 14, 4225-4233.

Peterson, S.D. and Marshall, R. T. (1990) Nonstarter lactobacilli in Cheddar cheese: A review. *Journal of Dairy Science* 73, 1395-1410.

Pieterse, B., Leer, R.J., Schuren, F.H.J. and van der Werf, M.J. (2005) Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. *Microbiology* 151, 3881-3894.

Pitcher, D.G., Saunders, N.A. and Owen, R.J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology* 8, 151-156.

Platt, C.G. (1987) Fermented foods of the world: a dictionary and guide. London, Butterworths. ISBN: 0-407-00313-4.

PM Food and Dairy Consulting (2009) The global cheese market report: Challenges and opportunities in a volatile market. P. 1.

Powell, J.E., Witthuhn, R.C., Todorov, S.D. and Dicks, L.M.T. (2007) Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. *International Dairy Journal* 17, 190-198.

Prabhakara, V., Kocaoglu-Vurmaa, N., Harpera, J. and Rodriguez-Saona, L. (2011) Classification of Swiss cheese starter and adjunct cultures using Fourier transform infrared microspectroscopy. *Journal of Dairy Science* 94, 4374-4382.

Prieto, B., Urdiales, R., Franco, I., Tornadijo, M.E. Fresno, J.M. and Carballo, J. (1999) Biochemical changes in PicoÂ n Bejes-Tresviso cheese, a Spanish blue-veined variety, during ripening. *Food Chemistry* 67, 415-421.

Raccach, M., McGrath, R. and Daftarian, H. (1989) Antibiosis of some lactic acid bacteria including *Lactobacillus acidophilus* towards *Listeria monocytogenes*. *International Journal of Food Microbiology* 9, 25-32.

Ramírez-Nuñez, J., Romero-Medrano, R., Nevárez-Moorillón, G.V. and Gutiérrez-Méndez, N. (2011) Effect of pH and salt gradient on the autolysis of *Lactococcus lactis* strains. *Brazilian Journal of Microbiology* 42, 1495-1499.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M. and Vaughan, E.E. (2002) Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rDNA analysis. *Applied and Environmental Microbiology* 68, 1882-1892.

Rathnayake, I., Hargreaves, M. and Huygens, F. (2011) SNP diversity of *Enterococcus faecalis* and *Enterococcus faecium* in a South East Queensland waterway, Australia, and associated antibiotic resistance gene profiles. *BMC Microbiology* 11, 201. Retrieved from: http://www.biomedcentral.com/1471-2180/11/201, October 2011.

Recipe Goldmine (2009) Kitchen Charts. Retrieved from: http://www.recipegoldmine.com/kitchart/kitchart41.html, December 2009.

Rello, J. (1999). *Acinetobacter baumannii* Infections in the ICU: Customization is the Key. American College of Chest Physicians. Retrieved from: http://chestjournal.chestpubs.org/content/115/5/1226.full.html, April 2010.

Roberson, J.R., Fox, L.K., Hancock, D.D., Gay, J.M. and Besser, T.E. (1998) Sources of intramammary infections from *Staphylococcus aureus* in dairy heifers at first parturition. *Journal of Dairy Science* 81, 687-693.

Robert, H., Le Marrec, C., Blanco, C. and Jebbar, M. (2000) Glycine betaine, carnitine and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus halophila*. *Applied and Environmental Microbiology* 66, 509-517.

Robinson, R.K. (1990) Dairy microbiology: The microbiology of milk. 2nd Edn, vol 2. Elsevier Applied Sciences: London, UK. Pp. 1-386.
Robinson, R.K. and Tamime, A.Y. (2006) Types of fermented milks. In: Fermented milks. Tamime, A. (Ed). Blackwell Science, Oxford, UK. Pp. 1-8.

Rogers and Smith (1917) *Lactobacillaceae*. In: Whitman, W.B. (2009) Bergy's manual of systematic bacteriology. 2nd Edn, vol. 3: The Firmicutes. Springer Dordrecht Heidelberg, London. Pp. 465-471.

Rojo-Bezares, B., Sa´ enz, Y., Navarro, L., Zarazaga, M., Ruiz-Larrea, F. and Torres, C. (2007) Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23 isolated from grape must. *Food Microbiology* 24, 482-491.

Rollins, D.M. and Joseph, S.W. (2004) The Gram Stain. BSCI 424: *Pathogenic Microbiology*. Retrieved from: http://life.umd.edu/classroom/bsci424, March 2010.

Ruas-Madiedo, P., Alonso, L., de Llano, D.G., and de los Reyes-Gavilan, C.G. (1998) Growth and metabolic activity of a cheese starter in CO_2 acidified and non-acidified refrigerated milk. *Lebensm Unters Forsch A* 206, 179-183.

Russo, P., Mohedano, M.M., Capozzi, V., de Palencia, P.F., López, P., Spano, G. and Fiocco, D. (2012) Comparative proteomic analysis of *Lactobacillus plantarum* WCFS1 and Δ*ctsR* mutant strains under physiological and heat stress conditions. *International Journal of Molecular Science* 13, 10680-10696.

Ryan, M.P. and Adley, C.C. (2010) *Sphingomonas paucimobilis*: a persistent Gram negative nosocomial infectious organism. *Journal of Hospital Infection* 75, 153-157.

Rynne, N.M., Beresford, T.P., Kelly, A.L. and Guinee, T.P. (2007) Effect of milk pasteurisation temperature on age-related changes in lactose metabolism, pH and the growth of non-starter lactic acid bacteria in half-fat Cheddar cheese. *Food Chemistry* 100, 375-382.

Salminen, S., Von Wright, A. and Ouwehand, A. (2004) Lactic acid bacteria: Microbiological and functional aspects. 3rd Edn., USA: Marcel Dekker, Inc.

Sanchez, C., Neves, A.R., Cavalheiro, J., dos Santos, M.M., Garcıa-Quintans, N., Lopez, P. and Santos, H. (2008) Contribution of citrate metabolism to the growth of *Lactococcus lactis* CRL264 at low pH. *Applied and Environmental Microbiology* 74, 1136-1144.

Sanchez, I., Sesena, S. and Palop, L.L. (2004) Polyphasic study of the genetic diversity of lactobacilli associated with 'Almagro' eggplants spontaneous fermentation, based on combined numerical analysis of randomly amplified polymorphic DNA and pulsed-field gel electrophoresis patterns. *Journal of Applied Microbiology* 97, 446-458.

Santivarangkna, C., Higl, B. and Foerst, P. (2008) Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology* 25, 429-441.

Santos, A., San Mauro, M., Sanchez, A., Torres, J.M. and Marquina, D. (2003) The antimicrobial properties of different strains of *Lactobacillus* spp. isolated from Kefir. *Journal of Systematic and Applied Microbiology* 26, 434-437.

Sawitzki, M.C., Fiorentini, A.M., Bertol, T.M. and Sant'Anna, E.S. (2009) *Lactobacillus plantarum* strains isolated from naturally fermented sausages and their technological properties for application as starter cultures. *Ciência e Tecnologia de Alimentos* 29, 340-345.

Schepers, A.W., Thibault, J. and Lacroix, C. (2002) *Lactobacillus helveticus* growth and lactic acid production during pH controlled batch cultures in whey permeate/yeast extract medium. Part I. multiple factor kinetic analysis. *Enzyme and Microbial Technology* 30, 176-186.

Schmitz, F.J., Steiert, M., Hofmann, B., Verhoef, J., Hadding, U., Heinz, H.P. and Kohrer, K. (1998) Development of a multiplex-PCR for direct detection of the genes for enterotoxin B and C, and toxic shock syndrome toxin-1 in *Staphylococcus aureus* isolates. *Journal of Medicinal Microbiology* 47, 335-340.

Sedewitz, B., Schleifer, K.H. and Gotz, F. (1984) Physiological role of pyruvate oxidase in the aerobic metabolism of *Lactobacillus plantarum*. *Journal of Bacteriology* 160, 462-465.

Sharma, N.K., Rees, C.E.D. and Dodd, C.E.R. (2000) Development of a single-reaction multiplex PCR toxin typing assay for *Staphylococcus aureus* strains. *Applied and Environmental Microbiology* 66, 1347-1353.

Sharpe, M.E. and Brindley, M. (1956) Lactobacilli isolated from the surface of normal and slipcoat Stilton cheese. *Journal of Dairy Research* 23, 361-362.

Shaw, M.B. (2007) The manufacture of soft, surface mould, ripened cheese in France with particular reference to Camembert. *International Journal of Dairy Technology* 34, 131-138.

Singh, T.K., Drake, M.A. and Cadwallader, K.R. (2003) Flavour of Cheddar Cheese: a chemical and sensory perspective. *Comprehensive Reviews in Food Science and Food Safety* 2, 139-162.

Singh, S., Goswami, P., Singh, R. and Heller, K.J. (2009) Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: A review. *Food Science and Technology* 42, 448-457.

Sood, S., Peters, T., Ward, L.R. and Threlfall, E.J. (2002) Combination of pulsed-field gel electrophoresis (PFGE) and Single-enzyme amplified fragment length polymorphism (SAFLP) for differentiation of multiresistant *Salmonella enterica* serotype Typhimurium. *Journal of Clinical Microbiology and Infections* 8, 154-161.

Spano, G., Capozzi, C., Vernile, A. and Massa, S. (2004) Cloning, molecular characterization and expression analysis of two small heat shock genes isolated from wine *Lactobacillus plantarum*. *Journal of Applied Microbiology* 97, 774-782.

Steele, J, Broadbent, J. and Kok, J. (2013) Perspectives on the contribution of lactic acid bacteria to cheese flavour development. *Current Opinion in Biotechnology* 24, 135-141.

Stilton Cheese Makers Association, SCMA (2009) Retrieved from: http://www.stiltoncheese.com/stilton_facts, December 2009.

Stilton Cheese Makers Association, SCMA (2010) Retrieved from: http://www.stiltoncheese.com/stilton_facts, December 2010.

Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S. and Coppola, R. (2005) Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *FEMS Microbiology Letters* 244, 129-137.

Suwanjinda, D., Eames, C. and Panbangred, W. (2007) Screening of lactic acid bacteria for bacteriocins by microbiological and PCR methods. *Biochemistry and Molecular Biology Education* 35, 364-369.

Takemoto, L., Emmons, T. and Horwitz, J. (1993) The C-terminal region of α-crystallin: involvement in protection against heat-induced denaturation. *Biochemistry Journal* 294, 435-438.

Tamime, A.Y. and Robinson, R.K. (1999) Yoghurt: Science and Technology. Second edn. Woodhead Publishing Limited, England. P14.

Tanigawa, K. and Watanabe, K. (2011) Multilocus sequence typing reveals a novel subspeciation of *Lactobacillus delbrueckii*. *Microbiology* 157, 727-738.

Tao, Y., Drabik, K.A., Waypa, T.S., Musch, M.W., Alverdy, J.C., Schneewind, O., Chang, E.B. and Petrof, E.O. (2006) Soluble factors from *Lactobacillus GG* activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *American Journal of Cell Physiology* 290, 1018-1030.

Taskila, S., Neubauer, P., Tuomola, M., Breitenstein, A., Kronlöf, J. and Hillukkala, T. (2009) Improved enrichment cultivation of beer spoiling lactic acid bacteria by continuous glucose addition to the culture. *Journal of the Institute of Brewing* 115, 177-182.

Tavaria, F.K., Dahl, S., Carballo, F.J. and Malcata, F.X. (2002) Amino acid catabolism and generation of volatiles by lactic acid bacteria. *Journal of Dairy Science* 85, 2462-2470.

Temelli, S., Anar, S., Sen, C. and Akyuva, P. (2006) Determination of microbiological contamination sources during Turkish white cheese production. *Food Control* 17, 856-861.

Todorov, S.D. and Dicks, L.M.T. (2005) Bacteriocin production by *L. plantarum* ST194BZ. *Food Technology and Biotechnology* 43, 165-173.

Torriani, S., Felis, G.E. and Dellaglio, F. (2001) Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Applied And Environmental Microbiology* 67, 3450-3454.

Torkar, K.G. and Teger, S.G. (2008) The microbiological quality of raw milk after introducing the two day's milk collecting system. *Acta agriculturae Slovenica* 92, 61-74.

Tsau, J.T., Guffanti, A.A. and Montvill, T.J. (1992) Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. *Applied and Environmental Microbiology* 58, 891-894.

Tuma, S., Kucerova, K. and Ovà, M.P. (2008) Isolation of anti-clostridially active lactobacilli from semi-hard cheese. *Czech Journal of Food Science* 26, 324-332.

Turroni, F., Foroni, E., Pizzetti, P., Giubellini, V., Ribbera, A., Merusi, P., Cagnasso, P., Bizzarri, B., de'Angelis, G.L., Shanahan, F., van Sinderen, D. and Marco Ventura, M. (2009) Exploring the diversity of the Bifidobacterial population in the human intestinal tract. *Applied and Environmental Microbiology* 75, 1534-1545.

UK milk statistics (2009) Retrieved from: http://www.thecattlesite.com/articles/2035/uk-milk-statistics, September 2011.

USDA, Food and Agricultural Organization (2012) Cheese statistics. Retrieved from: http://www.statisticbrain.com/cheese-statistics April 2013.

Vallejo, M., Marguet, E.Y. and Etchechoury, V. (2008) Características de α-acetolactato sintetasa y producción de diacetilo por *Enterococcus faecium* ETw7 y *Enterococcus faecalis* ETw23. *Revista Peruana de Biología* 15, 97-100.

Van den Tempel, T. and Jakobsen, M. (2000) The technological characteristics of *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starters cultures for production of Danablu. *International Dairy Journal* 10, 263-270.

Van den Tempel, T. and Nielsen, M.S. (2000) Effects of atmospheric conditions, NaCl and pH on growth and interactions between moulds and yeasts related to blue cheese production. *International Journal of Food Microbiology* 57, 193-199.

Van Reenen, C.A., Dicks, L.M.T. and Chikindas, M.L. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* 84, 1131-1137.

Vancanneyt, M., Huys, G., Lefebvre, K., Vankerckhoven, V., Goossens, H. and Swings, J. (2006) Intraspecific genotypic characterization of *Lactobacillus rhamnosus* strains intended for probiotic use and isolates of human origin. *Applied and Environmental Microbiology* 72, 5376-5383.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematic. *Microbiological Reviews* 60, 407-438.

Varghese, S.K. and Mishra, H.N. (2008) Modelling of acidification kinetics and textural properties in *dahi* (Indian yogurt) made from buffalo milk using response surface methodology. *International Journal of Dairy Technology* 61, 284-289.

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T.R. and Zink, R. (2003) Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. *Applied and Environmental Microbiology* 69, 6908-6922.

Verluyten, J., Messens, W. and De Vuyst, L. (2004) Sodium chloride reduces production of curvacin A, a bacteriocin produced by *Lactobacillus curvatus* strain LTH 1174, originating from fermented sausage. *Applied and Environmental Microbiology* 70, 2271-2278.

Vítová, E., Loupancová, B., Zemanová, J., Štoudková, H., Březina, P. and Babák, L. (2006) Solid-Phase Microextraction for Analysis of mould cheese aroma. *Czech Journal of Food Sciences* 24, 268-274.

Vriezen, J.A.C., de Bruijn, F.J. and Nusslein, K. (2007) Responses of Rhizobia to desiccation in relation to osmotic stress, oxygen, and temperature. *Applied and Environmental Microbiology* 73, 3451-3459.

Wall, T., Båth, K., Britton, R.A., Jonsson, H., Versalovic, J. and Roos, S. (2007) The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. *Applied and Environmental Microbiology* 73, 3924-3935.

Watanabe,Y., Suzuki, N. and Kaiser, H.M. (1999) Predicting Japanese dairy consumption behaviour using qualitative survey data. *Agribusiness* 15, 71-79.

Welsh, D.T. (2000) Ecological significance of compatible solute accumulation by microorganisms: from single cells to global climate. *FEMS Microbiology Reviews* 24, 263-290.

Whetstine, M.E.C., Cadwallader, K.R and Drake, M. A. (2005) Characterization of aroma compounds responsible for the rosy/floral flavor in Cheddar cheese. *Journal of Agriculture and Food Chemistry* 53, 3126-3132.

White, S.R., Broadbent, J.R., Oberg, C.J. and McMahon, D.J. (2003) Effect of *Lactobacillus helveticus* and Propionibacterium *frefreudenrichii* ssp. *shermanii* combinations on propensity for split defect in Swiss cheese. *Journal of Dairy Science* 86, 719-727.

Whitley, E. (2002) The Microflora of blue Stilton cheese. PhD thesis submitted to The University of Nottingham. Pp. 1-10.

William, S. and Feil, H. (2004) Bacterial genomic DNA isolation using CTAB (Version 2). Standard operating procedure. Doe Joint Genome Institute. Retrieved from: http://www.docstoc.com/docs/640147/DNA-Isolation-Bacterial-CTAB-Protocol---General-Info, February 2011.

Williams, A.G. and Banks, J.M. (1997) Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. *International Dairy Journal* 7, 763-774.

Wilson, A.R., Sigee, D. and Epton, H.A.S. (2005) Anti-bacterial activity of *Lactobacillus plantarum* strain SK1 against *Listeria monocytogenes* is due to lactic acid production. *Journal of Applied Microbiology* 99, 1516-1522.

Wouters, J.T.M., Ayad, E.H.E., Hugenholtz, J. and Smit, G. (2002) Microbes from raw milk for fermented dairy products. *International Dairy Journal* 12, 91-109.

Yavuz, E., Gunes, H., Bulut, C., Harsa, S. and Yenidunya, A.F. (2004) RFLP of 16S-ITS rDNA region to differentiate lactobacilli at species level. *World Journal of Microbiology and Biotechnology* 20, 535-537.

Yeung, P.S.M., Kitts, C.L., Cano, R., Tong, P.S. and Sanders, M.E. (2004) Application of genotypic and phenotypic analyses to commercial probiotic strain identity and relatedness. *Journal of Applied Microbiology* 97, 1095-1104.

Yi, H., Zhang, L., Tuo, Y., Han, X. and Du, M. (2010) A novel method for rapid detection of class IIa bacteriocin-producing lactic acid bacteria. *Food Control* 21, 426-430.

Yim, G. and Glover, C. (2003). Food Microbiology: The basics and the details of cheese production. In: *The Science Creative Quarterly magazine* issue No. 4, 2009.

Zanon, P., Farrow, J.A.E., Phillips, B.A. and Collins, M.D. (1987) *Lactobacillus pentosus* (Fred, Peterson, and Anderson) sp. nov., norn, rev. *International Journal of Systematic Bacteriology* 37, 339-341.

Zapparoli, G., Torriani, S. and Dellaglio, F. (1998) Differentiation of *Lactobacillus sanfranciscensis* strains by randomly amplified polymorphic DNA and pulsed-field gel electrophoresis. *FEMS Microbiology Letters* 166, 325-332.

Zayed, G. and Roos, Y.H. (2004) Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. *Process Biochemistry* 39, 1081-1086.

Zeuthen, P. and Bùgh-Sùrensen, L. (2003) Food preservation techniques. Woodhead Publishing Limited, England. P. 115.

Zhang, J., Zhang, Y., Liu, S., Han, Y. and Zhou, Z. (2012) Modelling Growth and Bacteriocin Production by *Pediococcus acidilactici* PA003 as a Function of Temperature and pH value. *Applied Biochemistry and Biotechnology* 66, 1388-1400.

APPENDICES

Appendix 1 Composition of the media used in the study

1. **API 50 CHL medium (API 50 CHL)**: bovine/porcine polypeptone, 10 g; yeast extract, 5 g; Tween 80, 1 ml; dipotassium phosphate, 2 g; sodium acetate, 5 g; diammonium citrate, 2 g; magnesium sulphate, 0.2 g; manganese sulphate, 0.05 g; bromocressol purple, 0.17 g; water, 1 l; pH 6.7-7.1.
2. **AUX medium (API 20 NE)**: ammonium sulphate, 2 g; agar, 1.5 g; vitamin solution, 10.5 g; trace elements, 10 ml; monosodium phosphate, 6.24 g; potassium chloride, 1.5 g; water, 1 l; pH 7-7.2.
3. **Baird-Parker agar**: tryptone, 10 g; 'Lab-Lemco' powder, 5 g; yeast extract, 1 g; sodium pyruvate, 10 g; glycine, 12 g; lithium chloride, 5 g; agar, 20 g; egg yolk emulsion, 50 ml; potassium tellurite, 0.1 g; water, 1 l; pH 6.8±0.2.
4. **Brain heart infusion (BHI)**: calf brain infusion solids, 12.5 g; beef heart infusion solids, 5 g; proteose peptone, 100 g; glucose, 2 g; sodium chloride, 5 g; disodium phosphate, 2.5 g; water, 1 l; pH 7.4±0.2.
5. **Columbia blood agar**: proteose peptone, 15 g; liver digest, 2.5 g; yeast extract, 5 g; sodium chloride, 5 g; agar, 12 g; water, 1 l; pH 7.4±0.2.
6. **GP medium (API 20 Strep)**: L-cystine, 0.5 g; bovine/porcine tryptone, 20 g; sodium chloride, 5 g; sodium sulphite, 0.5 g; phenol red, 0.17 g; water, 1 l; pH 7.4-7.6.
7. **M17 agar**: tryptone, 5 g; soya peptone, 5 g; 'Lab-Lemco' powder, 5 g; yeast extract, 2.5 g; ascorbic acid, 0.5 g; magnesium sulphate, 0.25 g; disodium glycerophosphate, 19 g; agar, 11 g; water, 1 l; pH 6.9±0.2.
8. **MacConkey agar**: peptone, 20 g; lactose, 10 g; bile salts No. 3, 1.5 g; sodium chloride, 5 g; neutral red, 0.03 g; crystal violet, 0.001 g; agar, 15 g; water, 1 l; pH 7.1±0.2.
9. **Maximum recovery diluent**: peptone, 1 g; sodium chloride, 8.5 g; water, 1 l; pH 7.0±0.2.
10. **Mannitol salt agar**: Lab-Lemco' powder, 1 g; peptone, 10 g; mannitol, 10 g; sodium chloride, 75 g; phenol red, 0.025 g; agar, 15 g; water, 1 l; pH 7.5±0.2.

11. **de Man, Rogosa, Sharpe (MRS)**: peptone, 10 g; 'Lab-Lemco' powder, 8 g; yeast extract, 4 g; D(+) glucose, 20 g; 'Tween 80', 0.1% (vol/vol); dipotassium hydrogen phosphate, 2 g; sodium acetate, 5 g; tri-ammonium citrate, 2 g; magnesium sulphate.$7H_2O$, 0.2 g; manganese sulphate.$4H_2O$, 0.05 g; water, 1 l; pH 6.2±0.2.
12. **Normal physiological saline**: 0.85% sodium chloride, pH 7.4±0.2.
13. **Phosphate buffered saline**: sodium chloride, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.44 g; KH_2PO_4, 0.24 g; Tween 20, 2 ml; water, 1 l; pH 7.2±0.2.
14. **Potato dextrose broth**: Potato Infusion from 200 g, 4 g; dextrose, 20 g; pH 5.1±0.2.
15. **Rogosa agar**: tryptone, 10 g; yeast extract, 5 g; glucose, 20 g; 'Tween 80', 1 ml; potassium dihydrogen phosphate, 6 g; ammonium citrate, 2 g; sodium acetate (anhydrous), 17 g; magnesium sulphate, 0.575 g; manganese sulphate, 0.12 g; ferrous sulphate, 0.034 g; agar, 20 g; water, 1 l; pH 5.4±0.2.
16. **Rose Bengal chloramphenicol (RBC) agar base**: mycological peptone, 5 g; glucose, 10 g; dipotassium phosphate, 1 g; Magnesium sulphate, 0.5 g; Rose-Bengal, 0.05 g; agar, 15.5 g; pH, 7.2±0.2
17. ***Streptococcus* Kennar faecal agar**: enzymatic digest of animal tissue, 10 g; yeast extract, 10 g; sodium chloride, 5 g; sodium glycerophosphate, 10 g; maltose, 20 g; lactose, 1 g; sodium azide, 0.4 g; bromocressol purple, 0.015 g; triphenlytetrazolium chloride, 1%; agar, 20 g; water, 1 l; pH 7.2±0.2.

Appendix 4.1 Alignment of base sequences of 16S rDNA PCR amplicons (99% ID) of *Lactobacillus* isolates obtained from different sites in Stilton cheese. The base changes are highlighted in red.

R6: ATGGAGCACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACT
R28: GATGGAGCACGCCGCGTGAGTGAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACT
R45: TGATGGAGCACGCCGCGTGAGTGAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACT
R15: TGGAGCATGCCGCGTGAGTGAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGCTAACT

R6: ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGA
R28: ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGA
R45: ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGA
R15: ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGA

R6: AACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATAA
R28: AACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATAAA
R45: AACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATAAA
R15: AACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAAT

Key:
Lactobacillus isolates were identified as close relatives of: (R6, outer crust*) Lb. plantarum*, (R28, blue veins) *Lb. plantarum*, (R45, white core) *Lb. plantarum*, (R15, outer crust) *Lb. brevis*

Appendix 4.2 List of bacterial isolates obtained from Stilton cheese and their NCBI BLAST match identification

Isolate	Site	Close relative of	Primer	% ID	Genbank Accession Number
R1	crust	*Lb. plantarum*	V3	98	NC012984
R2	crust	*Lb. plantarum*	V6-V8	100	NC012984
R3	crust	*Lb. plantarum*	ND	ND	ND
R4	crust	*Lb. plantarum*	V3	99	NC012984
R5	crust	*Lb. plantarum*	V3	98	NC012984
R6	crust	*Lb. plantarum*	V6-V8	99	NC012984
R7	crust	*Lb. plantarum*	V3	99	NC012984
R16	veins	*Lb. plantarum*	ND	ND	ND
R17	veins	*Lb. plantarum*	ND	ND	ND
R19	veins	*Lb. plantarum*	V6-V8	100	NC012984
R20	veins	*Lb. plantarum*	V3	98	NC012984
R21	veins	*Lb. plantarum*	V6-V8	99	NC012984
R22	veins	*Lb. plantarum*	ND	ND	ND
R23	veins	*Lb. plantarum*	V6-V8	99	NC012984
R24	veins	*Lb. plantarum*	ND	ND	ND
R25	veins	*Lb. plantarum*	V3	99	NC012984
R26	veins	*Lb. plantarum*	V6-V8	100	NC012984
R27	veins	*Lb. plantarum*	V3	99	NC012984
R28	veins	*Lb. plantarum*	V6-V8	99	NC012984
R29	veins	*Lb. plantarum*	ND	ND	ND
R30	veins	*Lb. plantarum*	V6-V8	100	NC012984
B11	veins	*Lb. plantarum*	V6-V8	99	NC012984
B13	veins	*Lb. plantarum*	V6-V8	99	NC012984
B14	veins	*Lb. plantarum*	V6-V8	99	NC012984
B15	veins	*Lb. plantarum*	V6-V8	99	NC012984
B23	veins	*Lb. plantarum*	ND	ND	ND
B24	veins	*Lb. plantarum*	ND	ND	ND
B25	veins	*Lb. plantarum*	ND	ND	ND
B26	veins	*Lb. plantarum*	V6-V8	99	NC012984
B27	veins	*Lb. plantarum*	ND	ND	ND
B28	veins	*Lb. plantarum*	ND	ND	ND
B29	veins	*Lb. plantarum*	V6-V8	99	NC012984
B30	veins	*Lb. plantarum*	V6-V8	99	NC012984
R36	core	*Lb. plantarum*	V3	98	NC012984
R37	core	*Lb. plantarum*	V3	98	NC012984
R38	core	*Lb. plantarum*	ND	ND	ND
R39	core	*Lb. plantarum*	ND	ND	ND
R40	core	*Lb. plantarum*	V3	98	NC012984
R42	core	*Lb. plantarum*	V6-V8	99	NC012984
R43	core	*Lb. plantarum*	ND	ND	ND
R44	core	*Lb. plantarum*	V6-V8	100	NC012984
R45	core	*Lb. plantarum*	ND	ND	ND
W8	core	*Lb. plantarum*	V6-V8	99	NC012984
W9	core	*Lb. plantarum*	ND	ND	ND
W10	core	*Lb. plantarum*	ND	ND	ND
W11	core	*Lb. plantarum*	ND	ND	ND
W12	core	*Lb. plantarum*	ND	ND	ND

W13	core	*Lb. plantarum*	ND	ND	ND
W14	core	*Lb. plantarum*	V6-V8	99	NC012984
W15	core	*Lb. plantarum*	ND	ND	ND
W23	core	*Lb. plantarum*	ND	ND	ND
W24	core	*Lb. plantarum*	V6-V8	99	NC012984
W25	core	*Lb. plantarum*	ND	ND	ND
W26	core	*Lb. plantarum*	ND	ND	ND
W27	core	*Lb. plantarum*	ND	ND	ND
W28	core	*Lb. plantarum*	ND	ND	ND
W29	core	*Lb. plantarum*	ND	ND	ND
W30	core	*Lb. plantarum*	V6-V8	99	NC012984
R9	crust	*Lb. brevis*	V6-V8	99	NC008497
R15	crust	*Lb. brevis*	V6-V8	99	NC008497
W22	core	*Ac. baumanii*	V3	95	NC011586
Ou9	crust	*En. faecalis*	V3	98	NC004668
Ou8	crust	*Staph. aureus*	V3	97	NC013450
Ou10	crust	*Staph. aureus*	V3	97	NC013450
Ou21	crust	*Staph. aureus*	ND	ND	ND
Ou25	crust	*Staph. aureus*	ND	ND	ND
Ou30	crust	*Staph. aureus*	V3	97	NC013450
Ou6	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou7	crust	*Ps. cryohalolentis*	V3	95	NC007969
Ou8	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou11	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou12	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou14	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou15	crust	*Ps. cryohalolentis*	V3	95	NC007969
Ou22	crust	*Ps. cryohalolentis*	V3	96	NC007969
Ou23	crust	*Ps. cryohalolentis*	V3	95	NC007969
Ou26	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou28	crust	*Ps. cryohalolentis*	ND	ND	ND
Ou29	crust	*Ps. cryohalolentis*	V3	95	NC007969

(ND) not done

Appendix 4.3 (a) AMOVA for testing the significance of clustering of PFGE profiles of *Lactobacillus* isolates obtained from Stilton cheese.

Source of variation	df	SS	MS	Est. Var.	%
Among Populations	5	423.74	84.75	10.34	34%
Within Populations	53	1071.14	20.21	20.21	66%
Total	58	1494.89		30.55	100%
Stat	value	(rand >= data)			
PhiPT	0.338	0.010			

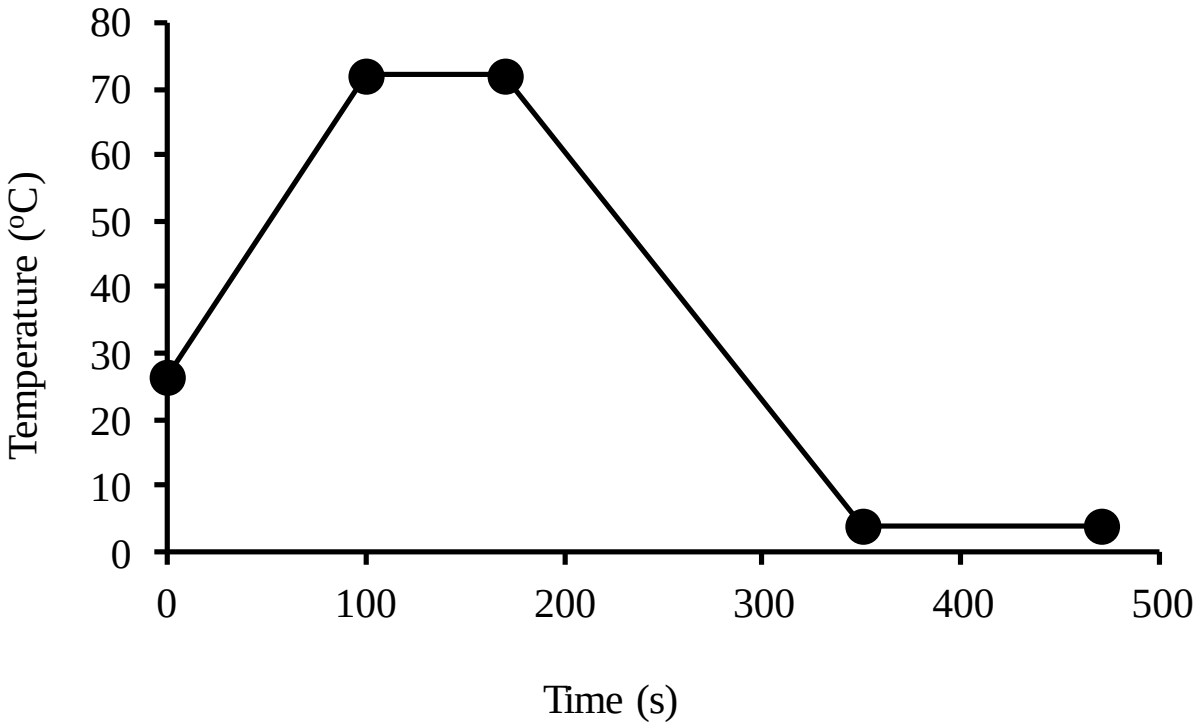
Probability, P (rand >= data), for PhiPT is based on permutation across the full data set.

Appendix 4.3 (b) Pair wise population PhiPT values from AMOVA analysis

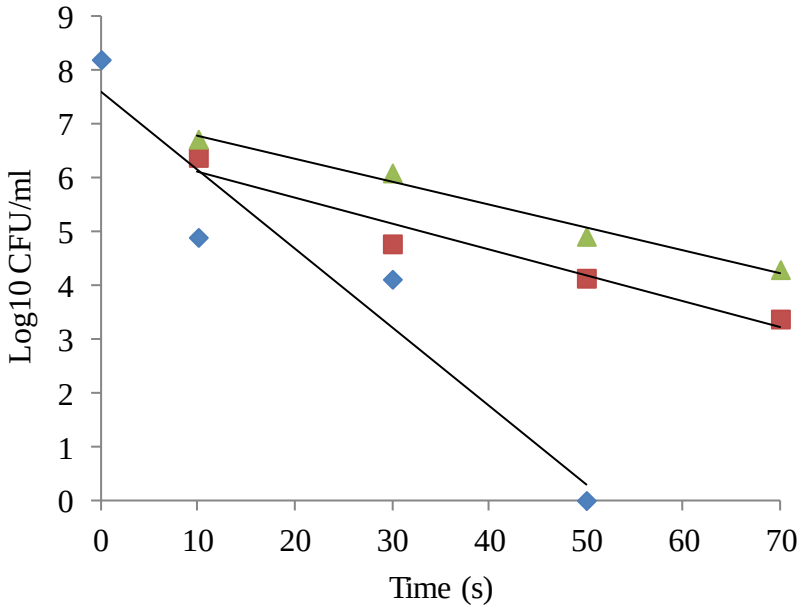
	1	2	3	4	5	6	
1	0.000	0.050	0.010	0.010	0.010	0.010	1
2	0.192	0.000	0.130	0.320	0.010	0.360	2
3	0.279	0.148	0.000	0.040	0.010	0.040	3
4	0.437	0.427	0.337	0.000	0.050	0.330	4
5	0.365	0.417	0.439	0.559	0.000	0.100	5
6	0.454	0.093	0.398	0.381	0.153	0.000	6
	1	2	3	4	5	6	

(1-6), clusters were the clusters delineated at 52% similarity level

Appendix 5.1 Representative thermal profile of cows milk during heating experiments.



Appendix 5.2 Linear regression plot of the data used for calculation of the D_{72} values after thermal inactivation of *Lb. plantarum* isolates obtained from Stilton and heated in milk at 72±1°C. Isolates: (▲) W30, white core (■) B14, blue veins (♦) R4, outer crust.



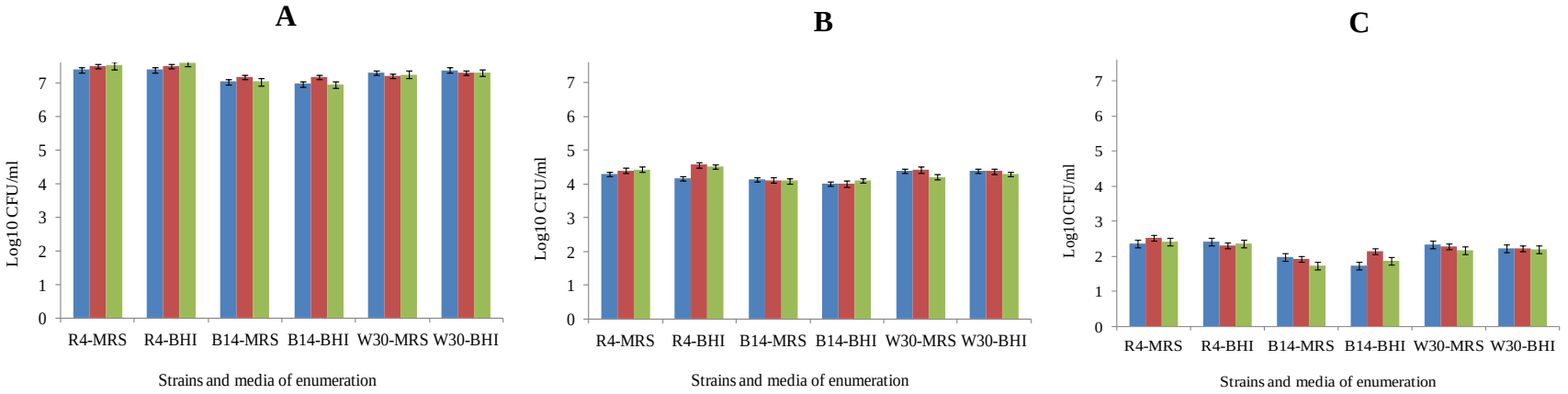
Linear regression equations:

R4 (outer crust): $y = -0.1458x + 7.5883$, $R^2 = 0.92$

B14 (blue veins): $y = -0.0484x + 6.6065$, $R^2 = 0.95$

W30 (white core): $y = -0.0424x + 7.2065$, $R^2 = 0.98$

Appendix 5.3 Behaviour of stationary phase cells of *Lb. plantarum* isolates R4 (outer crust), B14 (blue veins) and W30 (white core) in cows' milk at 4°C after incubating for: (■) 0, (■) 24 and (■) 48 h. Counts were taken from BHI agar after incubating anaerobically for 48 h at 30°C Values are means of two independent determinations ± standard errors of the means. The milk was spiked with a final cell concentration of: (**A**) 10^7; (**B**) 10^4; and (**C**) 10^2 $\log_{10}$ CFU/ml.



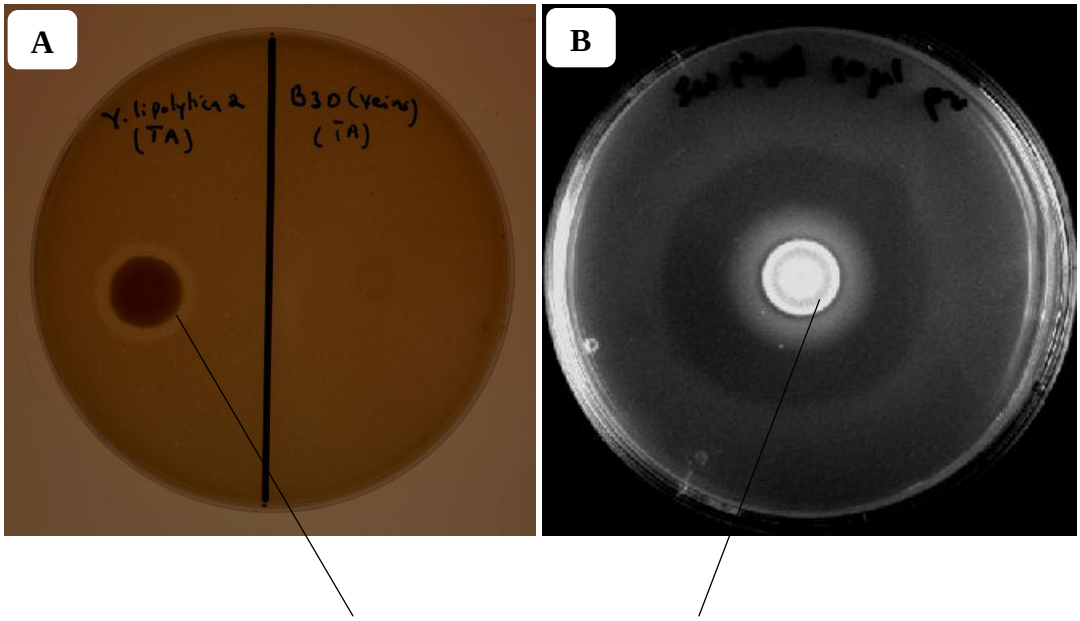
Appendix 6.1 Antimicrobial activity of different genotypes of *Lb. plantarum* obtained from Stilton cheese against the different indicator bacterial species. Activity was determined by the agar overlay method.

Isolate	site	Plantaricin EF operons	*Staph. aureus* NCTC12100	*L. monocyotgenes* NCTC11944	*E. coli* 0157 - stx	*Ps. aeruginosa* glaxo3	*Lc. lactis* NCIMB 4918	*Salm.* Typhimurium	*Cl. sporogenes*	*Staph. aureus* (Stilton)	*Lb. pentosus* NCIMB 8026
R1	crust	+	+	++	+++	++++	++	+	-	-	+++
R2	crust	+	+	++	+++	++++	+	+	-	+++	+++
R3	crust	-	+	+++	+++	++++	++	+	-	+++	++++
R4	crust	+	+	+++	+++	++++	++	+	-	+++	++++
R5	crust	+	+	++	+	++++	+	+	-	+	++++
R6	crust	+	+	+++	+	++++	+	+	-	+++	++++
R7	crust	+	+	++	+++	++++	+	+	-	++	++++
R16	veins	+	+	++	+++	++++	+	+	-	+++	++++
R17	veins	-	+	+	++	++++	+	+	-	++	++
R19	veins	+	+	++	+	+++	+	+	-	+++	++
R22	veins	+	+	+++	+++	++++	++	+	-	-	++++
R23	veins	+	+	++	+	++++	++	+	-	-	++++
R24	veins	-	+	++	+	++++	+	+	-	+++	++
R25	veins	+	+	+++	+++	++++	+	+	-	++++	++++
R26	veins	+	+	+++	+++	++++	+++	+	-	+++	++++
R28	veins	+	+	+++	+++	++++	+	+	-	++	++++
R29	veins	-	+	+++	++	++++	++	+	-	+++	++++
B11	veins	+	+	+++	+++	+++	+++	+	-	+++	++++
B13	veins	+	+	+++	+++	+++	++	+	-	+++	++++
B14	veins	-	+	++	+++	+++	++	+	-	+++	++
B15	veins	-	+	++	+++	+++	+++	+	-	+++	+++
B25	veins	+	+	++	+++	+++	+	+	-	+++	++
B26	veins	+	+	+	++	+++	+	+	-	+++	++
B28	veins	+	+	+++	+++	+++	+++	+	-	+++	+++
B29	veins	+	-	+++	+++	+++	+++	+	-	+++	++++
B30	veins	-	+	+++	+++	++++	+++	-	-	++++	+++
R36	core	+	+	++	+	++++	++	+	-	+++	++++

R37	core	+	+	++	++	++++	++	+	-	-	++++
R38	core	+	+	++	+++	++++	+	+	-	+++	++++
R39	core	+	+	++	++	++++	+	+	-	+++	++++
R40	core	+	+	++	++	++++	+	+	-	++	++++
R42	core	+	+	++	++	++++	+	+	-	+++	++++
R43	core	+	+	+++	++	++++	+	+	-	+++	++++
R45	core	+	+	+++	++	++++	+	+	-	+++	++++
W8	core	+	+	+++	++	++++	+++	+	-	+++	++
W9	core	+	+	+++	+++	++++	+++	+	-	+++	++
W10	core	+	+	++	++	++++	+++	+	-	++	+++
W11	core	+	+	++	+++	++++	++	+	-	++++	++
W12	core	-	+	+++	+++	++++	+++	+	-	+++	+++
W13	core	-	+	+++	+++	++++	+++	-	-	+++	++
W14	core	+	+	+++	+++	++++	++	+	-	++	++
W15	core	-	+	+++	+++	++++	+++	+	-	+++	++
W23	core	-	+	+++	+++	++++	+++	+	-	++++	++
W24	core	+	+	+++	+++	+++	+++	+	-	++++	+++
W25	core	+	+	+++	+++	+++	+++	+	-	++++	+++
W26	core	-	+	+++	+++	++++	+++	+	-	+++	++++
W27	core	-	+	+++	+++	++++	+++	+	-	++++	+++
W28	core	-	+	++	+++	++++	+++	+	-	+++	++++
W29	core	-	+	++	+++	++++	+++	+	-	++++	+++
W30	core	-	-	++	+++	+++	+++	+	-	++++	++++
Pedio			ND	++++	+++	++++	++	ND	-	+	ND

(+) Positive result; (-) negative result; (ND) not done; (Pedio) *Ped. acidilactici* NCIMB 700993. *Lb. plantarum* isolates obtained from: (R1-R7) outer crust; (R16-R29, B11-B30) blue veins; (R36- R45, W8-W30) white core. The number of +/- signs represents the intensity of reaction observed on the media plate.

Appendix 7.1 Photograph showing (A) lipolytic, and (B) proteolytic activity of *Yarrowia lipolytica* strain Y2 obtained from Stilton cheese. B30 is the *Lb. plantarum* isolate obtained from the blue veins of the cheese. Activity is demonstrated by the presence of a clear halo around the colony. Plates were incubated aerobically for 10 days at 25^{o}C



Colony of *Yarrowia lipolytica* surrounded by a clear halo

Appendix 7.2 Average SPME GC-MS signal intensities/peak areas (area units, AU) and standard deviations, SD ($x10^5$) for major compounds detected from headspace samples of model milk fermented at 30°C for 24 h. (B30, R2 and W8) *Lb. plantarum* isolates from the blue veins, outer crust and white core respectively. (LcL) *Lc. lactis* subsp. *lactis* (acid producer); (Lc99) *Lc. lactis* NCIMB 9918 (non acid-producer).

				Pure culture					Mixed culture					
				Lb. plantarum			*Lc. lactis*		*Lb. plantarum* + LcL			*Lb. plantarum* + Lc99		
Compound	LRI		Milk	B30	R2	W8	LcL	Lc99	B30-LcL	R2-LcL	W8-LcL	B30-Lc99	R2-Lc99	W8-Lc99
Alcohols														
		Mean	8	11	9	10	20	16	35	19	44	15	16	22
Ethyl alcohol (ethanol)		SD	6	10	3	2	9	5	21	10	14	10	17	21
		Mean	4	4	3	4	26	3	48	29	29	5	4	3
2-Methyl propanol	406	SD	2	0.53	0.56	1	18	2	14	25	2	0.93	1	0.37
		Mean	1	0.32	1	0.96	180	0.65	618	181	206	1	3	0.68
3-Methyl butanol	459	SD	0.97	0.08	2	0.38	104	0.26	145	102	51	0.21	4	0.38
		Mean	4	3	4	3	63	3	155	66	60	2	4	2
2-Methyl butanol	461	SD	3	1	1	2	44	1	51	57	9	0.37	2	0.31
		Mean	0.47	0.33	0.12	0.29	0.95	0.51	1	1	2	0.35	0.34	0.38
Hexanol	917	SD	0.42	0.12	0.00	0.12	0.77	0.12	0.60	0.91	1	0.20	0.15	0.24
Carboxylic acids														
		Mean	0.44	0.12	0.23	0.41	0.11	0.25	0.17	0.19	0.22	0.08	0.42	0.38
Acetic acid	399	SD	0.28	0.02	0.23	0.34	0.04	0.17	0.08	0.09	0.18	0.01	0.46	0.68
		Mean	1	0.19	0.17	1	0.10	0.12	0.13	0.12	0.18	0.13	0.22	0.13
Butanoic acid	479	SD	2	0.10	0.05	1	0.02	0.07	0.04	0.05	0.06	0.07	0.15	0.06
		Mean	1	0.11	0.10	7	0.09	0.38	0.12	0.11	0.18	0.08	0.20	0.12
Hexanoic acid	1157	SD	2	0.07	0.03	12	0.01	0.49	0.01	0.04	0.08	0.02	0.22	0.08
		Mean	0.52	0.08	0.10	0.22	0.06	0.09	0.10	0.09	0.11	0.07	0.19	0.12
Octanoic acid	1592	SD	0.73	0.01	0.02	0.19	0.01	0.04	0.04	0.02	0.02	0.03	0.24	0.06

Compound	LRI		Milk	Pure culture					Mixed culture					
				Lb. plantarum			*Lc. lactis*		*Lb. plantarum* + LcL			*Lb. plantarum* + Lc99		
				B30	R2	W8	LcL	Lc99	B30-LcL	R2-LcL	W8-LcL	B30-Lc99	R2-Lc99	W8-Lc99
Ketones														
		Mean	29	20	20	22	19	23	19	20	22	19	19	19
2-Pentanone	435	SD	15	2	5	5	3	4	2	4	5	2	0.67	4
		Mean	8	6	6	7	7	116	13	8	11	86	89	89
Acetoin	447	SD	5	2	2	2	3	34	1	5	5	10	3	15
		Mean	4	2	2	3	2	3	3	2	3	2	2	2
2-Hexanone	488	SD	2	1	0.47	1	0.70	0.93	0.45	0.49	1	0.62	0.29	0.70
		Mean	4	1	2	1	2	3	2	2	2	2	1	2
2-Hydroxy 3-pentanone	859	SD	3	0	1	0.76	0.73	0.42	0.39	1	1	1	0.39	0.69
		Mean	206	124	134	172	125	191	127	134	144	117	136	126
2-Heptanone	941	SD	118	25	49	70	22	24	31	50	38	25	3	38
		Mean	20	10	9	25	10	20	11	12	12	9	11	11
2-Nonanone	1410	SD	15	6	4	27	4	10	4	5	6	2	0.90	6
Aldehydes														
		Mean	1	0.83	0.79	0.97	70	1	126	66	95	0.75	0.68	0.80
2-Methyl propanal	305	SD	0.48	0.21	0.31	0.67	52	0.16	30	59	27	0.51	0.14	0.38
		Mean	5	3	4	4	511	3	1303	402	532	4	3	3
3-Methyl butanal	420	SD	5	2	2	2	92	0.72	388	100	17	1	1	2
		Mean	2	0.46	0.83	1	131	0.41	348	104	133	0.50	0.43	0.36
2-Methyl butanal	424	SD	1	0.14	0.39	0.29	37	0.08	110	39	8	0.24	0.22	0.05
Esters														
		Mean	0.53	0.33	0.23	0.28	1	0.44	2	2	2	0.26	0.30	0.20
Butanoic acid 2-methyl est	409	SD	0.49	0.20	0.07	0.15	1	0.20	0.47	2	0.12	0.08	0.08	0.06
		Mean	3	1	1	1	0.76	2	1	1	2	0.97	1	1
Butanoic acid ethyl ester	494	SD	3	0.31	0.66	0.65	0.39	0.81	0.61	0.42	0.74	0.21	0.20	0.34
		Mean	0.47	0.19	0.25	0.30	0.28	0.27	0.41	0.34	0.26	0.17	0.25	0.13
Butanol 3-methyl acetate	926	SD	0.41	0.02	0.10	0.14	0.13	0.08	0.20	0.11	0.08	0.04	0.03	0.07
		Mean	0.19	0.06	0.06	0.37	0.12	0.14	0.13	0.09	0.08	0.07	0.07	0.07
Hexanoic acid ethyl ester	1189	SD	0.17	0.03	0.02	0.48	0.05	0.08	0.07	0.04	0.02	0.03	0.04	0.04
		Mean	6	1	1	0.73	1	6	0.70	0.62	0.78	0.52	2	1
Propanoic acid methyl este	2000	SD	9	0.49	0.84	0.74	1	8	0.58	0.51	0.92	0.42	1	2

Appendix 7.3 Average SPME GC-MS signal intensities/peak areas (AU) and standard deviations, SD ($x10^5$) for major compounds detected from headspace samples of model milk fermented at 30°C for 48 h. (B30, R2 and W8) *Lb. plantarum* isolates from the blue veins, outer crust and white core respectively. (LcL) *Lc. lactis* subsp. *lactis* (acid producer); (Lc99) *Lc. lactis* NCIMB 9918 (non acid-producer).

				Pure culture					Mixed culture					
				Lb. plantarum			*Lc. lactis*		*Lb. plantarum* + LcL			*Lb. plantarum* + Lc99		
Compound	LRI		Milk	B30	R2	W8	LcL	Lc99	B30-LcL	R2-LcL	W8-LcL	B30-Lc99	R2-Lc99	W8-Lc99
Alcohols														
		Mean	8	8	13	9	12	17	18	15	27	18	23	30
Ethyl alcohol (ethanol)		SD	6	1	10	2	3	2	4	2	18	9	30	19
		Mean	4	3	4	3	78	4	289	84	88	4	5	5
2-Methyl propanol	406	SD	2	1	3	0.46	11	3	11	10	16	1	1	2
		Mean	1	1	2	1	248	3	2930	228	232	1	1	1
3-Methyl butanol	459	SD	0.97	0.25	1	0.33	74	5	180	19	31	1	0.45	0.31
		Mean	4	2	3	3	244	5	774	233	249	3	3	3
2-Methyl butanol	461	SD	3	1	2	1	43	3	22	58	48	2	2	1
		Mean	0.47	1	1	1	2	1	3	1	2	1	1	1
Hexanol	917	SD	0.42	0.33	1	0.21	1	1	0.38	0.23	1	0.22	0.43	1
Carboxylic acids														
		Mean	0.44	0.18	0.27	0.22	0.22	1	2	0.17	0.17	0.17	0.14	0.22
Acetic acid	399	SD	0.28	0.06	0.26	0.10	0.13	0.03	1	0.04	0.12	0.07	0.20	0.18
		Mean	1	0.11	0.31	0.11	3	0.26	8	0.14	0.15	0.13	0.18	0.46
Butanoic acid	479	SD	2	0.06	0.32	0.04	5	0.10	3	0.02	0.03	0.04	0.17	1
		Mean	1	0.09	0.20	0.17	8	0.13	2	0.09	0.26	0.15	0.13	0.20
Hexanoic acid	1157	SD	2	0.02	0.05	0.13	14	0.07	1	0.01	0.00	0.06	0.07	0.20
		Mean	0.52	0.12	0.15	0.14	0.42	0.14	0.19	0.15	0.09	0.09	0.10	0.14
Octanoic acid	1592	SD	0.73	0.01	0.07	0.05	1	0.00	0.07	0.07	0.03	0.02	0.07	0.15

				Pure culture					Mixed culture					
				Lb. plantarum			*Lc. lactis*		*Lb. plantarum* + LcL			*Lb. plantarum* + Lc99		
Compound	LRI		Milk	B30	R2	W8	LcL	Lc99	B30-LcL	R2-LcL	W8-LcL	B30-Lc99	R2-Lc99	W8-Lc99
Ketones														
		Mean	29	19	25	19	19	25	18	19	18	21	23	26
2-Pentanone	435	SD	15	1	11	2	4	10	1	1	1	6	7	14
		Mean	8	7	16	7	18	123	40	19	14	107	123	137
Acetoin	447	SD	5	1	8	3	5	53	3	9	2	39	59	82
		Mean	4	2	3	2	2	3	2	2	2	2	3	3
2-Hexanone	488	SD	2	1	1	1	1	2	0.25	0.29	0.31	1	2	3
		Mean	4	1	2	1	4	3	6	3	3	2	2	2
2-Hydroxy 3-pentanone	859	SD	3	0.39	1	1	2	2	1	1	0.44	0.44	0.49	1
		Mean	206	121	175	121	122	179	130	104	101	134	168	189
2-Heptanone	941	SD	118	12	85	25	42	88	12	10	8	48	68	141
		Mean	20	9	14	13	10	16	10	8	7	12	13	19
2-Nonanone	1410	SD	15	2	11	8	5	12	1	1	1	7	8	20
Aldehydes														
		Mean	1	1	1	ND	48	1	46	51	46	1	1	1
2-Methyl propanal	305	SD	0.48	0.31	0.34	ND	3	0.08	9	5	8	0.30	1	1
		Mean	5	1	4	3	78	2	2237	108	76	3	3	3
3-Methyl butanal	420	SD	5	0.49	0.40	0.21	4	1	126	44	14	1	3	2
		Mean	2	0.25	1	0.23	112	1	658	113	98	0.32	0.42	0.39
2-Methyl butanal	424	SD	1	0.10	1	0.10	9	1	63	17	11	0.25	0.36	0.14
Esters														
		Mean	0.53	0.26	1	0.18	4	0.39	16	5	6	0.20	0.34	0.36
Butanoic acid 2-methyl est	409	SD	0.49	0.11	1	0.02	1	0.34	1	1	1	0.15	0.14	0.33
		Mean	3	1	1	1	1	1	1	0.32	1	1	2	2
Butanoic acid ethyl ester	494	SD	3	0.28	1	0.41	1	1	0.33	0.25	0.42	0.45	1	2
		Mean	0.47	0.14	0.18	0.15	0.29	0.30	4	0.21	0.41	0.14	0.15	0.28
Butanol 3-methyl acetate	926	SD	0.41	0.08	0.13	0.06	0.18	0.30	1	0.09	0.13	0.05	0.05	0.20
		Mean	0.19	0.07	0.08	0.08	0.21	0.12	0.50	0.08	0.11	0.07	0.17	0.17
Hexanoic acid ethyl ester	1189	SD	0.17	0.07	0.02	0.05	0.22	0.08	0.02	0.01	0.06	0.05	0.12	0.15
		Mean	6	0.15	2	0.23	0.32	1	0.17	0.43	0.13	0.33	4	1
Propanoic acid methyl este	2000	SD	9	0.07	2	0.06	0.17	2	0.05	0.27	0.06	0.26	4	2

Appendix 7.4 Average SPME GC-MS signal intensities/peak areas (AU) and standard deviations, SD ($x10^5$) for major compounds detected from headspace samples of model milk ripened for 4 weeks at 18°C. (B30, R2 and W8) *Lb. plantarum* isolates from the blue veins, outer crust and white core respectively. (LcL) *Lc. lactis* subsp. *lactis* (acid producer); (Lc99) *Lc. lactis* NCIMB 9918 (non acid-producer); (s) salt (3.5%, w/v) added to the sample.

		Pure culture										Mixed culture											
		Lb. plantarum						*Lc. lactis*				*Lb. plantarum* + LcL						*Lb. plantarum* + Lc99					
Compound		B30	B30(s)	R2	R2(s)	W8	W8(s)	LcL	LcL(s)	Lc99	Lc99(s)	B30-LcL	B30-LcL(s)	R2-LcL	R2-LcL(s)	W8-LcL	W8-LcLs	B30-Lc99	B30-Lc99(s)	R2-Lc99	R2-Lc99(s)	W8-Lc99	W8-Lc99(s)
Alcohols																							
	Mean	18	27	10	26	9	14	17	20	39	21	19	23	18	17	38	37	241	41	36	14	30	29
Ethyl alcohol (ethanol)	SD	9	7	7	26	1	4	5	7	17	5	4	7	7	5	21	16	56	9	25	5	18	25
	Mean	989	7	4	4	13	29	239	301	4	3	411	515	261	217	260	272	20	6	18	16	3	4
2-Methyl propanol	SD	52	3	1	1	14	42	83	13	2	1	40	107	68	27	75	39	6	2	23	22	2	1
	Mean	1	1	1	1	240	3	330	405	2	1	3771	4417	384	329	432	398	9	1	4	1	1	1
3-Methyl butanol	SD	1	0.49	###	1	509	2	91	3	0.35	0.13	200	558	73	28	162	82	5	1	3	0.42	1	0.43
	Mean	2	2	2	2	49	4	899	1002	2	2	893	1078	954	748	1086	1006	2	4	2	2	4	3
2-Methyl butanol	SD	1	2	1	1	83	2	264	55	2	1	86	175	175	140	255	117	1	2	3	1	3	2
	Mean	1	5	4	4	23	5	5	4	2	1	4	4	7	3	8	5	7	4	3	2	2	3
Hexanol	SD	1	2	1	0.11	36	1	2	2	0.37	0.16	2	3	3	1	5	1	4	1	3	1	1	2
Carboxylic acids																							
	Mean	809	347	###	0.28	1	2	0.26	1	0.23	0.14	835	737	239	66	41	1	714	286	1	0.30	0.32	0.19
Acetic acid	SD	118	126	###	0.31	2	2	0.12	0.35	0.09	0.10	148	171	113	110	65	1	166	18	1	0.14	0.22	0.02
	Mean	30	28	###	0.19	121	5	16	9	0.19	0.16	48	60	34	19	39	17	42	14	23	0.22	14	0.26
Butanoic acid	SD	10	16	###	0.07	209	9	17	4	0.09	0.09	16	26	20	9	39	15	27	5	40	0.06	23	0.13
	Mean	3	4	1	0.11	170	2	5	3	0.06	0.10	9	20	11	4	32	6	14	2	19	0.09	102	0.13
Hexanoic acid	SD	2	4	1	0.02	294	3	8	2	0.01	0.02	6	24	16	2	52	8	20	1	33	0.06	176	0.02
	Mean	1	1	###	0.07	32	123	0.30	0.24	0.12	0.12	1	1	0.42	0.26	2	0.38	1	1	7	0.06	5	0.11
Octanoic acid	SD	1	0.47	###	0.01	55	214	0.19	0.09	0.06	0.07	0.25	1	0.34	0.13	2	0.28	0.25	0.30	12	0.05	8	0.03

Compound		Pure culture										Mixed culture											
		Lb. plantarum						*Lc. lactis*				*Lb. plantarum* + LcL						*Lb. plantarum* + Lc99					
		B30	B30(s)	R2	R2(s)	W8	W8(s)	LcL	LcL(s)	Lc99	Lc99(s)	B30-LcL	B30-LcL(s)	R2-LcL	R2-LcL(s)	W8-LcL	W8-LcLs	B30-Lc99	B30-Lc99(s)	R2-Lc99	R2-Lc99(s)	W8-Lc99	W8-Lc99(s)
Ketones																							
	Mean	17	24	21	22	21	171	19	22	21	21	19	24	22	20	23	23	23	22	20	22	695	23
2-Pentanone	SD	3	4	5	4	10	257	4	0	6	2	3	7	5	1	6	6	6	4	5	3	1173	1
	Mean	971	300	21	100	32	67	32	65	481	133	229	260	431	594	67	208	2907	2999	688	258	1172	214
Acetoin	SD	159	86	10	130	36	94	15	9	413	9	36	49	321	409	36	112	353	60	344	64	1517	45
	Mean	2	3	4	2	2	38	2	2	3	2	3	3	3	2	3	3	3	3	2	3	48	3
2-Hexanone	SD	###	1	2	1	1	61	1	0.26	1	0.24	1	1	1	0.42	1	1	1	1	1	0.41	80	1
	Mean	7	4	3	7	17	6	8	10	2	3	10	12	11	9	11	11	5	6	4	4	6	8
2-Hydroxy 3-pentanone	SD	2	1	2	1	27	4	4	1	1	1	4	4	4	1	6	6	1	2	1	3	7	3
	Mean	103	122	171	110	77	1358	111	120	183	112	138	143	141	107	171	128	160	131	96	121	1586	140
2-Heptanone	SD	20	28	30	11	50	2123	35	10	141	6	20	47	48	15	76	38	47	13	32	23	2570	10
	Mean	8	12	14	8	9	194	9	9	21	10	13	14	13	8	22	10	16	11	7	9	268	11
2-Nonanone	SD	2	2	7	0.26	6	320	4	1	24	2	2	6	6	1	20	4	9	3	3	2	452	1
Aldehydes																							
	Mean	###	0.13	###	0.28	4	0.20	18	30	1	1	0.45	0.31	2	0.45	6	1	0.24	0.10	0.26	1	0.24	0.44
2-Methyl propanal	SD	###	0.22	###	0.03	6	0.12	7	3	1	0.49	0.15	0.22	1	0.03	2	0.37	0.04	0.07	0.08	1	0.17	0.13
	Mean	11	7	1	3	149	1	24	30	4	2	11	8	5	3	9	5	7	4	6	2	2	2
3-Methyl butanal	SD	1	2	0	0	254	1	7	3	2	1	1	2	1	0.45	2	2	1	2	7	1	1	1
	Mean	1	1	1	0	42	0.32	37	47	0.24	0.23	2	1	5	1	16	5	1	0.20	1	0.27	0.18	0.45
2-Methyl butanal	SD	###	0.22	###	0.11	72	0.22	11	8	0.12	0.09	1	0.44	1	1	6	2	0.37	0.07	1	0.25	0.04	0.27
Esters																							
	Mean	###	0.24	###	0.19	0.46	0.25	13	17	0.13	0.25	24	31	15	12	15	14	0.29	0.23	0.20	0.21	0.17	0.25
Butanoic acid 2-methyl es	SD	###	0.06	###	0.10	0.50	0.05	5	1	0.03	0.02	4	9	4	2	5	3	0.13	0.11	0.04	0.07	0.00	0.15
	Mean	2	2	2	4	78	19	2	2	2	2	2	3	2	1	2	1	3	6	6	2	22	4
Butanoic acid ethyl ester	SD	###	0.30	1	1	135	25	0.49	0.40	2	1	0.20	1	1	1	1	0.37	1	1	9	1	35	2
	Mean	###	0.17	###	0.21	12	0.31	1	1	0.15	0.12	19	17	2	1	3	2	0.30	0.23	0.19	0.27	0.20	0.25
Butanol 3-methyl acetate	SD	###	0.03	###	0.01	20	0.09	0.47	0.27	0.10	0.04	4	6	1	0.18	1	0.26	0.09	0.05	0.06	0.17	0.09	0.05
	Mean	###	0.11	###	0.07	7	0.21	0.28	1	0.20	0.08	1	1	0.39	0.19	0.40	0.33	0.20	0.10	3	0.08	8	0.10
Hexanoic acid ethyl ester	SD	###	0.02	###	0.02	11	0.26	0.14	0.35	0.07	0.03	0.22	0.15	0.35	0.17	0.21	0.12	0.09	0.03	6	0.05	14	0.02
	Mean	###	0.15	12	0.32	0.20	0.19	0.17	0.12	0.10	1	0.15	0.15	0.18	1	0.25	0.22	0.28	0.10	1	0.34	0.11	0.45
Propanoic acid methyl este	SD	###	0.02	17	0.37	0.14	0.11	0.13	0.02	0.07	1	0.22	0.08	0.08	1	0.31	0.13	0.20	0.03	1	0.36	0.07	0.40

Appendix 7.5 Average SPME GC-MS signal intensities/peak areas (AU) and standard deviations, SD ($x10^5$) for major compounds detected from headspace samples of model milk ripened for 7 weeks at 18°C. (B30, R2 and W8) *Lb. plantarum* isolates from the blue veins, outer crust and white core respectively. (LcL) *Lc. lactis* subsp. *lactis* (acid producer); (Lc99) *Lc. lactis* NCIMB 9918 (non acid-producer); (s) salt (3.5%, w/v) added to the sample.

		Pure culture										Mixed culture											
		Lb. plantarum						*Lc. lactis*				*Lb. plantarum* + LcL						*Lb. plantarum* + Lc99					
Compound		B30	B30(s)	R2	R2(s)	W8	W8(s)	LcL	LcL(s)	Lc99	Lc99(s)	B30-LcL	B30-LcL(s)	R2-LcL	R2-LcL(s)	W8-LcL	W8-LcLs	B30-Lc99	B30-Lc99(s	R2-Lc99	R2-Lc99(s)	W8-Lc99	W8-Lc99(s)
Alcohols																							
	Mean	22	26	20	23	12	13	19	25	27	20	17	20	17	21	28	30	203	38	226	19	180	33
Ethyl alcohol (ethanol)	SD	8	4	15	16	0.40	2	2	8	1	1	3	4	5	5	14	13	32	9	173	6	26	26
	Mean	###	3	9	6	31	57	224	292	3	4	382	466	226	254	218	246	1019	8	21	26	11	4
2-Methyl propanol	SD	82	2	10	2	48	94	50	3	1	2	21	71	30	37	10	29	162	2	17	36	4	2
	Mean	2	1	19	5	2	2	337	412	2	5	3761	4194	305	406	314	367	32	2	14	4	32	3
3-Methyl butanol	SD	1	0.01	32	7	1	2	60	33	1	8	235	433	53	34	45	38	22	0.43	17	4	52	2
	Mean	2	1	5	3	4	2	931	1003	5	4	857	1020	772	912	852	949	8	2	9	4	8	3
2-Methyl butanol	SD	2	1	5	2	1	1	161	51	3	3	80	147	120	86	85	53	4	2	9	3	12	2
	Mean	5	8	8	11	5	13	8	6	8	2	5	5	6	5	7	6	5	6	10	5	7	8
Hexanol	SD	2	8	6	8	2	13	4	1	1	1	2	2	4	2	3	1	2	2	3	4	5	3
Carboxylic acids																							
	Mean	###	746	###	101	131	252	1	2	0.40	0.20	925	825	244	373	261	190	1040	632	269	1	361	1
Acetic acid	SD	158	192	###	175	227	436	0.19	1	0.40	0.11	124	108	31	130	62	40	214	55	464	1	407	1
	Mean	42	42	3	6	4	5	16	4	0.21	0.14	55	54	25	44	35	25	48	42	4	1	5	3
Butanoic acid	SD	14	17	5	10	6	8	8	1	0.04	0.04	23	10	11	20	15	6	22	12	3	1	5	4
	Mean	8	7	12	5	2	7	3	3	0.13	0.20	15	8	4	9	8	5	15	13	1	0.16	7	7
Hexanoic acid	SD	4	6	20	9	3	11	1	2	0.02	0.24	12	4	3	8	6	3	13	13	2	0.09	11	12
	Mean	1	1	286	0.41	107	183	0.23	0.26	0.12	0.08	1	1	0.30	1	0.43	1	1	1	101	0.11	104	1
Octanoic acid	SD	###	0.40	405	0.35	186	318	0.06	0.06	0.07	0.05	1	0.31	0.13	1	0.02	0.24	1	0.44	174	0.07	181	2

		Pure culture										Mixed culture											
		Lb. plantarum						*Lc. lactis*				*Lb. plantarum* + LcL						*Lb. plantarum* + Lc99					
Compound		B30	B30(s)	R2	R2(s)	W8	W8(s)	LcL	LcL(s)	Lc99	Lc99(s)	B30-LcL	B30-LcL(s)	R2-LcL	R2-LcL(s)	W8-LcL	W8-LcLs	B30-Lc99	B30-Lc99(s	R2-Lc99	R2-Lc99(s)	W8-Lc99	W8-Lc99(s)
Ketones																							
	Mean	21	21	93	29	60	226	18	20	23	22	18	22	17	24	19	21	19	20	82	25	1583	24
2-Pentanone	SD	2	3	127	12	61	357	2	3	4	3	3	5	2	4	3	2	4	3	108	3	2712	6
	Mean	###	1042	517	318	527	204	32	62	352	254	371	292	678	1141	176	375	2741	3303	###	824	1919	389
Acetoin	SD	269	421	853	454	878	324	7	5	11	208	37	29	316	587	60	83	181	330	593	699	1158	200
	Mean	2	3	7	4	7	52	2	3	4	3	3	3	3	2	2	2	3	2	9	4	165	4
2-Hexanone	SD	###	1	7	1	5	85	1	1	1	0.32	1	1	1	1	1	1	1	1	10	2	281	2
	Mean	7	2	120	8	7	227	9	9	7	5	10	9	9	12	9	10	5	4	7	7	16	9
2-Hydroxy 3-pentanone	SD	4	1	204	2	7	383	2	2	1	3	1	2	1	4	2	1	4	3	3	3	20	3
	Mean	133	105	167	145	211	1650	121	117	141	100	136	136	109	134	109	117	119	106	242	132	2602	158
2-Heptanone	SD	6	25	98	27	89	2659	29	12	29	3	29	37	31	26	17	16	31	23	195	20	4352	45
	Mean	13	9	20	13	29	300	10	9	12	6	12	17	9	12	11	9	12	9	16	9	685	16
2-Nonanone	SD	1	4	21	4	19	506	3	2	5	0.48	4	12	3	5	4	2	6	2	10	2	1172	10
Aldehydes																							
	Mean	###	0.13	1	1	0.43	0.19	15	24	0.50	1	0.38	0.14	0.36	0.28	1	0.19	0.27	0.20	1	0.50	1	1
2-Methyl propanal	SD	###	0.08	1	1	1	0.13	2	0.07	0.20	1	0.04	0.08	0.12	0.17	0.08	0.04	0.22	0.02	1	0.36	0.15	0.13
	Mean	8	4	17	5	8	4	24	20	2	4	9	6	3	3	2	2	6	5	11	4	4	2
3-Methyl butanal	SD	0	1	24	6	11	3	3	2	1	3	1	1	2	1	1	1	3	1	10	2	4	1
	Mean	1	1	5	1	6	0.40	26	35	0.34	1	1	0.42	1	1	1	0.47	1	1	2	0.44	1	1
2-Methyl butanal	SD	###	0.14	7	1	9	0.03	4	2	0.28	1	1	0.50	0.17	0.30	0.26	0.26	0.38	0.28	3	0.25	1	0.16
Esters																							
	Mean	###	0.20	###	0.10	1	0.21	12	16	0.30	0.31	22	25	13	14	11	13	0.30	0.36	1	0.25	0.13	0.21
Butanoic acid 2-methyl es	SD	###	0.09	###	0.05	2	0.09	3	0.50	0.26	0.12	3	4	1	3	0.31	1	0.10	0.05	1	0.03	0.03	0.11
	Mean	3	1	1	3	2	3	2	2	3	3	2	1	1	1	1	1	3	2	22	3	44	4
Butanoic acid ethyl ester	SD	1	0.08	###	2	0.19	0.79	1	0.29	1	1	1	1	1	1	1	0.13	0.41	1	26	2	74	2
	Mean	###	0.27	1	0.46	0.25	0.40	1	1	0.27	0.20	19	18	2	2	1	1	0.24	0.23	1	0.33	1	0.35
Butanol 3-methyl acetate	SD	###	0.16	1	0.25	0.10	0.35	0.48	0.34	0.10	0.03	4	5	0.34	0.46	1	0.26	0.17	0.10	0.43	0.12	1	0.48
	Mean	###	0.08	5	0.18	6	31	0.17	0.15	0.09	0.09	1	0.40	0.16	0.31	0.27	0.32	0.37	0.18	9	0.09	30	0.08
Hexanoic acid ethyl ester	SD	###	0.06	8	0.11	10	53	0.11	0.17	0.01	0.03	0.15	0.13	0.08	0.15	0.09	0.04	0.29	0.08	15	0.03	52	0.03
	Mean	###	0.21	1	0.10	1	1	0.19	0.16	0.19	0.12	0.24	1	0.27	0.21	0.24	0.23	0.19	0.15	0.17	0.09	1	2
Propanoic acid methyl este	SD	###	0.12	1	0.01	1	1	0.10	0.06	0.14	0.01	0.22	1	0.12	0.17	0.05	0.13	0.15	0.07	0.06	0.03	1	3

Appendix 7.6 Suggested odour notes of the major volatiles and their sensory threshold levels in different media. The compounds were detected from headspace samples of model milk fermented at 30°C for 24 h and subsequently ripened for 7 weeks at 18°C. The milk was inoculated with pure or mixed cultures of *Lb. plantarum* isolates B30 (blue veins), R2 (outer crust) and W8 (white core) with *Lc. lactis* subsp. *lactis* (acid producer) and *Lc. lactis* NCIMB 9918 (non acid-producer). Analysis was performed by using SPME GC-MS.

Compounds	Suggested odour note(s)	Sensory thresholds (ppm)	Reference
Alcohols			
Ethanol	Strong, ropy, fruity	200[a]	Lawlor *et al*. (2003)
2-methyl propanol	Fruity, alcoholic and malty	45[a]	Lawlor *et al*. (2003)
2-methyl butanol	Fruity, alcoholic and malty	7.5[a]	Lawlor *et al*. (2003)
3-methyl butanol	Fruity, alcoholic and malty	7.5[a]	Lawlor *et al*. (2003)
Organic acids			
Acetic acid	Vinegar, sour	36[a]	Whetstine (2005)
Butanoic acid	Rancid, cheesy	0.86[a]	Whetstine *et al*. (2005)
Hexanoic acid	Sweaty	2.8[a]	Whetstine (2005)
Octanoic acid		8.7[a]	
Ketones			
Diacetyl	Buttery	10[b]	Ott *et al*. (2000)
2-pentanone	mouldy, strong (ropy)	0.0001[c]	Lawlor *et al*. (2003)
Acetoin	Buttery	0.8[c]	Vítová *et al*. (2006)
2-hexanone		-	
2-hydroxy 3-pentanone		-	
2-heptanone	Mouldy, strong (ropy)	0.05[a]	Lawlor *et al*. (2003)
2-nonanone	Mouldy, strong (ropy)	0.05[a]	Lawlor *et al*. (2003)

Aldehydes			
2-methyl propanal	Malty	0.43[a]	
2-methyl butanal	Dark chocolate	0.19[a]	Lawlor *et al*. (2003)
3-methyl butanal	Malty, green-grassy	0.19[a]	Vítová *et al*. (2006)
Esters			
Butanoic acid 2-methyl ester	Fruity, floral	0.25[c]	Belitz and Grosch (2009)
Butanoic acid ethyl ester	Sweet	0.1[c]	Belitz and Grosch (2009)
Butanol 3-methyl acetate	Fruity, floral	3[c]	Lawlor *et al*. (2003)
Hexanoic acid ethyl ester	Fruity, floral	-	Lawlor *et al*. (2003)
Propanoic acid methyl ester	Fruity, floral	7[c]	Lawlor *et al*. (2003)

[a]Odour threshold of the compounds in goat milk Jack cheese model (Attaie, 2009)
[b]Odour threshold of the compounds dissolved in sunflower oil (Belitz and Grosch, 2009)
[c]Odour threshold of the compounds dissolved in water (Belitz and Grosch, 2009)

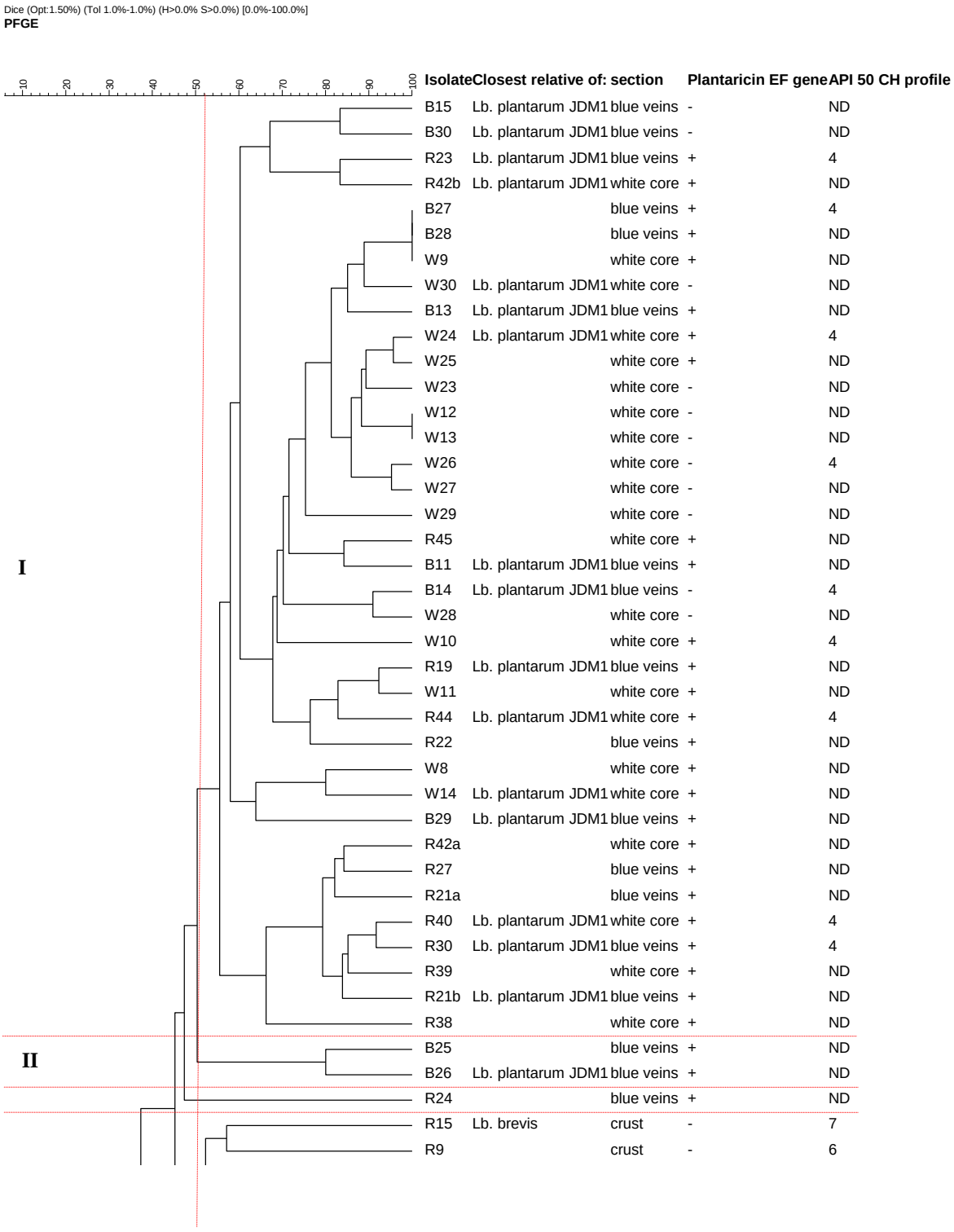
Appendix 7.7 List of compounds detected from headspace samples of model milk fermented for 48 h at 30°C and subsequently ripened for 7 weeks at 18°C. The milk was inoculated with pure or mixed cultures of *Lb. plantarum* isolates B30 (blue veins), R2 (outer crust) and W8 (white core) with *Lc. lactis* subsp. *lactis* (acid producer) and *Lc. lactis* NCIMB 9918 (non acid-producer). Analysis was performed by using SPME GC-MS.

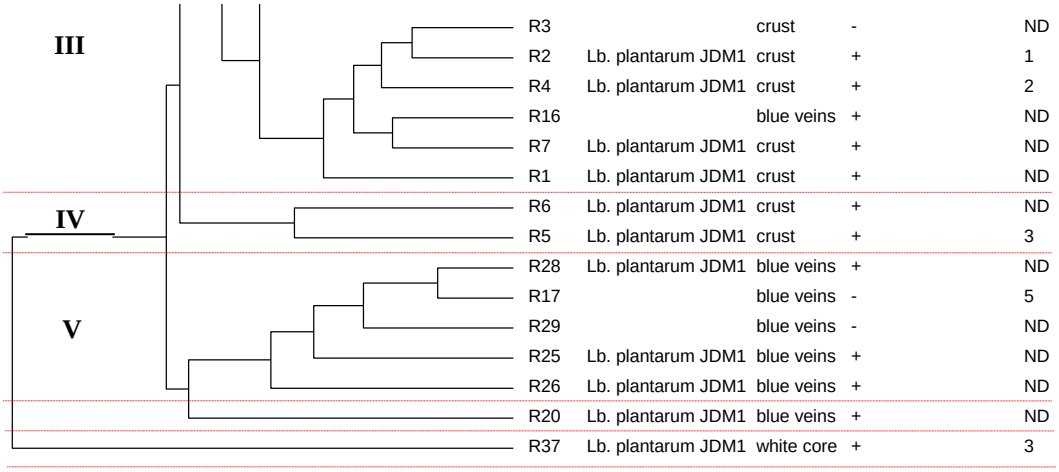
	Compound	Functional group	Molecular weight/molecular formula	CAS No.	Retention time (min)	Key ion (m/z)
1	Ethyl alcohol (ethanol)	alcohol	46, C_2H_6O	64-17-5	2.10	31
2	Acetone (2-propanone)	ketone	58, C_3H_6O	67-64-1	2.44	58
3	Dimethyl sulfide		62, C_2H_6S	75-18-3	2.79	47
4	2-Methyl propanal	aldehyde	72, C_4H_8O	78-84-2	3.28	72
5	2,3-Butanedione (diacetyl)		86, $C_4H_6O_2$	431-03-8	3.75	43
6	2-Butanone	ketone	72, C_4H_8O	78-93-3	3.90	72
7	Acetic acid	carbox. acid	60, $C_2H_4O_2$	64-19-7	4.12	60
8	Ethyl acetate	ester	88, $C_4H_8O_2$	141-78-6	4.25	61
9	2-Methyl propanol (isobutanol)	alcohol	74, $C_4H_{10}O$	78-83-1	4.47	43
10	Butanoic acid 2-methyl methyl ester	ester	116, $C_6H_{12}O_2$	868-57-5	4.59	57
11	3-Methyl butanal	aldehyde	86, $C_5H_{10}O$	590-86-3	5.08	44
12	2-Methyl butanal	aldehyde	86, $C_5H_{10}O$	96-17-3	5.30	57
13	2-Pentanone	ketone	86, $C_5H_{10}O$	107-87-9	5.78	86
14	2,3-Pentanedione	ketone	100, $C_5H_8O_2$	600-14-6	5.94	57
15	Pentanal	aldehyde	86, $C_5H_{10}O$	110-62-3	6.02	44
16	3-Hydroxy 2-butanone (acetoin)	diverse	88, $C_4H_8O_2$	513-86-0	6.32	45
17	2-Propenoic acid 2-methyl methyl ester	ester	100, $C_5H_8O_2$	80-62-6	6.37	69
18	3-Methyl butanol	alcohol	88, $C_5H_{12}O$	123-51-3	6.87	55

19	2-Methyl butanol	alcohol	88, $C_5H_{12}O$	34713-94-5	6.97	57
20	2,3 Dimethyl hexane	alkane	71, C8H18	584-94-1	7.33	71
21	Pentanol	diverse	88, $C_5H_{12}O$	71-41-0	7.64	55
22	Butanoic acid	acid	88, $C_4H_8O_2$	107-92-6	7.82	60
23	2-Hexanone	ketone	100, $C_6H_{12}O$	591-78-6	8.22	58
24	Butanoic acid ethyl ester	ester	116, $C_6H_{12}O_2$	105-54-4	8.47	71
25	Hexanal	aldehyde	100, $C_6H_{12}O$	66-25-1	8.49	56
26	2-Hydroxy 3-pentanone	ketone	102, $C_5H_{10}O_2$	5704-20-1	8.68	45
27	3-Methyl butanoic acid	acid	102, $C_5H_{10}O_2$	503-74-2	9.21	60
28	3,7,11-Trimethyl dodecanol	alcohol	228, $C_{15}H_{32}O$	6750-34-1	9.53	57
29	Butanoic acid, 2-ethyl-, 1,2,3-propanetriyl	acid	386, $C_{21}H_{38}O_6$	56554-54-2	9.60	88
30	Hexanol	alcohol	102, $C_6H_{14}O$	111-27-3	10.09	56
31	Pentanoic acid	acid	102, $C_5H_{10}O_2$	109-52-4	10.12	60
32	Butanol 3-methyl acetate	aldehyde	130, $C_7H_{14}O_2$	123-92-2	10.29	70
33	5-Hepten-2-one	ketone	112, $C_7H_{12}O$	6714-00-7	10.31	112
34	Octane	alkane	142, C10H22	15869-87-1	10.37	57
35	2-Heptanone	ketone	114, $C_7H_{14}O$	110-43-0	10.65	58
36	2-Heptanol	alcohol	116, $C_7H_{16}O$	543-49-7	10.84	45
37	Heptanal	aldehyde	114, $C_7H_{14}O$	111-71-7	10.92	70
38	2,5-Dimethyl 3-hexanol	alcohol	130, $C_8H_{18}O$	19550-07-3	12.05	69
39	Hexanoic acid	acid	116, $C_6H_{12}O_2$	142-62-1	12.34	60
40	2-Octanone	ketone	128, $C_8H_{16}O$	111-13-7	12.95	58
41	Hexanoic acid ethyl ester	ester	144, $C_8H_{16}O_2$	123-66-0	13.07	88
42	Heptanoic acid	acid	130, $C_7H_{14}O_2$	111-14-8	14.49	73
43	8-Nonen-2-one	ketone	140, $C_9H_{16}O$	5009-32-5	14.94	58
44	2-Nonanone	ketone	142, $C_9H_{18}O$	821-55-6	15.12	58

45	2-Nonanol	alcohol	144, $C_9H_{20}O$	628-99-9	15.27	45
46	Octanoic acid	carbox. acid	144, $C_8H_{16}O_2$	124-07-2	16.51	60
47	Octanoic acid ethyl ester	ester	172, $C_{10}H_{20}O_2$	106-32-1	17.15	88
48	Dodecane	alkane	170, C12H26	13475-82-6	17.26	170
49	2-Undecanone	ketone	170, $C_{11}H_{22}O$	112-12-9	19.05	58
50	3-Hydroxy dodecanoic acid	diverse	216, $C_{12}H_{24}O_3$	1883-13-2	19.12	55
51	Propanoic acid methyl ester	ester	216, $C_{12}H_{24}O_3$	74367-34-3	20.72	71

Appendix 8 PFGE dendrogram showing the variation in plantaricin EF genes and API 50 CHL profiles of the different clusters of *Lactobacillus* spp. obtained from different sites in Stilton cheese. The profiles were based on inability to metabolise a particular carbohydrate(s)





Key:

ND	Not done
Profile 1:	D-melezitose and α-keto-gluconate
Profile 2:	L-rhamnose and methyl- αD-mannoside
Profile 3:	L-rhamnose
Profile 4:	D-melezitose
Profile 5:	D-arabitol
Profile 6:	*Lb. brevis* isolate that metabolised α-keto-gluconate
Profile 7:	*Lb. brevis* isolate that could not metabolise D-galactose

Appendix 9 Summary of statistical analyses

ANOVA for Fig. 5.3 Mean viable counts (log_{10} CFU/ml) of *Lb. plantarum* isolates after incubation in MRS broth acidified to pH 4-6 using lactic acid.

Isolate	growth pH		Sum of Squares	df	Mean Square	F	Sig.
B14	pH 4.0	Between Groups	.216	5	.043	.942	.469
		Within Groups	1.377	30	.046		
		Total	1.593	35			
	pH 4.5	Between Groups	37.895	5	7.579	14.404	.000
		Within Groups	15.786	30	.526		
		Total	53.681	35			
	pH 5.0	Between Groups	92.748	5	18.550	1638.457	.000
		Within Groups	.340	30	.011		
		Total	93.087	35			
	pH 6.0	Between Groups	114.380	5	22.876	1318.771	.000
		Within Groups	.520	30	.017		
		Total	114.900	35			
R2	pH 4.0	Between Groups	1.872	5	.374	3.042	.024
		Within Groups	3.691	30	.123		
		Total	5.563	35			
	pH 4.5	Between Groups	42.709	5	8.542	45.930	.000
		Within Groups	5.579	30	.186		
		Total	48.288	35			
	pH 5.0	Between Groups	64.492	5	12.898	107.546	.000
		Within Groups	3.598	30	.120		
		Total	68.090	35			
	pH 6.0	Between Groups	85.647	5	17.129	137.479	.000
		Within Groups	3.738	30	.125		
		Total	89.385	35			
R25	pH 4.0	Between Groups	.434	5	.087	1.743	.155
		Within Groups	1.495	30	.050		
		Total	1.929	35			
	pH 4.5	Between Groups	47.908	5	9.582	225.344	.000
		Within Groups	1.276	30	.043		
		Total	49.184	35			
	pH 5.0	Between Groups	69.530	5	13.906	353.235	.000
		Within Groups	1.181	30	.039		
		Total	70.711	35			

	pH 6.0	Between Groups	89.688	5	17.938	527.492	.000
		Within Groups	1.020	30	.034		
		Total	90.709	35			
R40	pH 4.0	Between Groups	.040	5	.008	.049	.998
		Within Groups	4.899	30	.163		
		Total	4.939	35			
	pH 4.5	Between Groups	61.717	5	12.343	122.361	.000
		Within Groups	3.026	30	.101		
		Total	64.743	35			
	pH 5.0	Between Groups	96.696	5	19.339	222.104	.000
		Within Groups	2.612	30	.087		
		Total	99.308	35			
	pH 6.0	Between Groups	120.020	5	24.004	260.656	.000
		Within Groups	2.763	30	.092		
		Total	122.783	35			
R6	pH 4.0	Between Groups	12.050	5	2.410	17.551	.000
		Within Groups	4.119	30	.137		
		Total	16.169	35			
	pH 4.5	Between Groups	12.312	5	2.462	8.561	.000
		Within Groups	8.629	30	.288		
		Total	20.941	35			
	pH 5.0	Between Groups	71.619	5	14.324	301.801	.000
		Within Groups	1.424	30	.047		
		Total	73.043	35			
	pH 6.0	Between Groups	105.502	5	21.100	218.537	.000
		Within Groups	2.897	30	.097		
		Total	108.398	35			
W30	pH 4.0	Between Groups	1.099	5	.220	2.530	.050
		Within Groups	2.606	30	.087		
		Total	3.705	35			
	pH 4.5	Between Groups	57.545	5	11.509	70.820	.000
		Within Groups	4.875	30	.163		
		Total	62.421	35			
	pH 5.0	Between Groups	88.967	5	17.793	384.433	.000
		Within Groups	1.389	30	.046		
		Total	90.355	35			
	pH 6.0	Between Groups	111.113	5	22.223	466.425	.000
		Within Groups	1.429	30	.048		
		Total	112.543	35			

ANOVA for Fig. 5.4 Mean viable counts (log_{10} CFU/ml) of *Lb. plantarum* isolates after incubation in MRS broth containing different concentrations (0-10%, w/v) of NaCl.

Isolate	salt level applied		Sum of Squares	df	Mean Square	F	Sig.
B14	0 percent (control)	Between Groups	75.258	5	15.052	385.610	.000
		Within Groups	.468	12	.039		
		Total	75.727	17			
	3.5 percent	Between Groups	69.139	5	13.828	1465.851	.000
		Within Groups	.113	12	.009		
		Total	69.252	17			
	5.0 percent	Between Groups	51.434	5	10.287	164.339	.000
		Within Groups	.751	12	.063		
		Total	52.185	17			
	8.0 percent	Between Groups	.875	5	.175	4.638	.014
		Within Groups	.453	12	.038		
		Total	1.328	17			
	10.00	Between Groups	1.900	5	.380	18.580	.000
		Within Groups	.245	12	.020		
		Total	2.145	17			
R2	0 percent (control)	Between Groups	48.966	5	9.793	221.009	.000
		Within Groups	.532	12	.044		
		Total	49.498	17			
	3.5 percent	Between Groups	58.548	5	11.710	404.398	.000
		Within Groups	.347	12	.029		
		Total	58.895	17			
	5.0 percent	Between Groups	42.806	5	8.561	363.445	.000
		Within Groups	.283	12	.024		
		Total	43.088	17			
	8.0 percent	Between Groups	3.965	5	.793	19.842	.000
		Within Groups	.480	12	.040		
		Total	4.445	17			
	10.00	Between Groups	.067	5	.013	2.651	.077
		Within Groups	.061	12	.005		
		Total	.127	17			
R25	0 percent (control)	Between Groups	41.007	5	8.201	306.725	.000
		Within Groups	.321	12	.027		
		Total	41.328	17			
	3.5 percent	Between Groups	60.408	5	12.082	891.995	.000
		Within Groups	.163	12	.014		
		Total	60.570	17			

	5.0 percent	Between Groups	52.270	5	10.454	553.608	.000
		Within Groups	.227	12	.019		
		Total	52.496	17			
	8.0 percent	Between Groups	7.372	5	1.474	79.359	.000
		Within Groups	.223	12	.019		
		Total	7.594	17			
	10.00	Between Groups	.648	5	.130	1.311	.323
		Within Groups	1.187	12	.099		
		Total	1.835	17			
R40	0 percent (control)	Between Groups	96.104	5	19.221	219.096	.000
		Within Groups	1.053	12	.088		
		Total	97.157	17			
	3.5 percent	Between Groups	150.842	5	30.168	119.887	.000
		Within Groups	3.020	12	.252		
		Total	153.861	17			
	5.0 percent	Between Groups	115.949	5	23.190	134.321	.000
		Within Groups	2.072	12	.173		
		Total	118.020	17			
	8.0 percent	Between Groups	4.756	5	.951	1.703	.208
		Within Groups	6.703	12	.559		
		Total	11.459	17			
	10.00	Between Groups	.319	5	.064	4.592	.014
		Within Groups	.167	12	.014		
		Total	.486	17			
R6	0 percent (control)	Between Groups	96.451	5	19.290	271.458	.000
		Within Groups	.853	12	.071		
		Total	97.303	17			
	3.5 percent	Between Groups	115.629	5	23.126	315.400	.000
		Within Groups	.880	12	.073		
		Total	116.509	17			
	5.0 percent	Between Groups	69.115	5	13.823	229.810	.000
		Within Groups	.722	12	.060		
		Total	69.837	17			
	8.0 percent	Between Groups	6.548	5	1.310	16.710	.000
		Within Groups	.940	12	.078		
		Total	7.489	17			
	10.00	Between Groups	.536	5	.107	7.953	.002
		Within Groups	.162	12	.013		
		Total	.697	17			
W30	0 percent (control)	Between Groups	66.356	5	13.271	183.614	.000

	Within Groups	.867	12	.072		
	Total	67.223	17			
3.5 percent	Between Groups	70.520	5	14.104	240.182	.000
	Within Groups	.705	12	.059		
	Total	71.225	17			
5.0 percent	Between Groups	56.321	5	11.264	207.358	.000
	Within Groups	.652	12	.054		
	Total	56.973	17			
8.0 percent	Between Groups	1.026	5	.205	6.830	.003
	Within Groups	.360	12	.030		
	Total	1.386	17			
10.00	Between Groups	1.167	5	.233	2.954	.058
	Within Groups	.948	12	.079		
	Total	2.115	17			

ANOVA for Fig. 5.5 Mean viable counts (log_{10} CFU/ml) of *Lb. plantarum* isolates after incubation at different ERH levels (33 and 54%).

Isolate	relative humidity			Sum of Squares	df	Mean Square	F	Sig.
B14	33.00	Log CFU MRD	Between Groups	40.987	6	6.831	5.459	.004
			Within Groups	17.520	14	1.251		
			Total	58.507	20			
		Log CFU SDW	Between Groups	240.954	6	40.159	1262.861	.000
			Within Groups	.445	14	.032		
			Total	241.399	20			
	54.00	Log CFU MRD	Between Groups	175.581	6	29.264	95.894	.000
			Within Groups	4.272	14	.305		
			Total	179.853	20			
		Log CFU SDW	Between Groups	132.859	6	22.143	90.074	.000
			Within Groups	3.442	14	.246		
			Total	136.301	20			
R2	33.00	Log CFU MRD	Between Groups	27.523	6	4.587	9.633	.000
			Within Groups	6.667	14	.476		
			Total	34.189	20			
		Log CFU SDW	Between Groups	278.978	6	46.496	320.822	.000
			Within Groups	2.029	14	.145		
			Total	281.007	20			
	54.00	Log CFU MRD	Between Groups	188.481	6	31.414	258.740	.000
			Within Groups	1.700	14	.121		
			Total	190.181	20			
		Log CFU SDW	Between Groups	279.488	6	46.581	2961.580	.000
			Within Groups	.220	14	.016		
			Total	279.709	20			
R25	33.00	Log CFU MRD	Between Groups	39.664	6	6.611	52.832	.000
			Within Groups	1.752	14	.125		
			Total	41.415	20			
		Log CFU SDW	Between Groups	236.088	6	39.348	329.258	.000
			Within Groups	1.673	14	.120		
			Total	237.761	20			
	54.00	Log CFU MRD	Between Groups	170.747	6	28.458	205.062	.000
			Within Groups	1.943	14	.139		
			Total	172.690	20			
		Log CFU SDW	Between Groups	148.928	6	24.821	164.437	.000
			Within Groups	2.113	14	.151		

			Total	151.041	20			
R4	33.00	Log CFU MRD	Between Groups	5.782	6	.964	5.023	.006
			Within Groups	2.686	14	.192		
			Total	8.468	20			
		Log CFU SDW	Between Groups	257.247	6	42.875	211.921	.000
			Within Groups	2.832	14	.202		
			Total	260.080	20			
	54.00	Log CFU MRD	Between Groups	185.632	6	30.939	131.649	.000
			Within Groups	3.290	14	.235		
			Total	188.923	20			
		Log CFU SDW	Between Groups	157.627	6	26.271	33.503	.000
			Within Groups	10.978	14	.784		
			Total	168.605	20			
R40	33.00	Log CFU MRD	Between Groups	16.165	6	2.694	3.415	.027
			Within Groups	11.046	14	.789		
			Total	27.211	20			
		Log CFU SDW	Between Groups	275.000	6	45.833	305.876	.000
			Within Groups	2.098	14	.150		
			Total	277.098	20			
	54.00	Log CFU MRD	Between Groups	183.228	6	30.538	155.639	.000
			Within Groups	2.747	14	.196		
			Total	185.974	20			
		Log CFU SDW	Between Groups	194.486	6	32.414	43.453	.000
			Within Groups	10.444	14	.746		
			Total	204.929	20			
W30	33.00	Log CFU MRD	Between Groups	31.924	6	5.321	8.365	.001
			Within Groups	8.904	14	.636		
			Total	40.829	20			
		Log CFU SDW	Between Groups	239.780	6	39.963	100.584	.000
			Within Groups	5.562	14	.397		
			Total	245.342	20			
	54.00	Log CFU MRD	Between Groups	191.364	6	31.894	62.511	.000
			Within Groups	7.143	14	.510		
			Total	198.507	20			
		Log CFU SDW	Between Groups	76.926	6	12.821	24.848	.000
			Within Groups	7.224	14	.516		
			Total	84.150	20			

ANOVA for Table 7.1 Mean viable counts (log_{10} CFU/ml) of *Ped. acidilactici* NCIMB 700993 and the different genotypes of *Lb. plantarum* obtained from Stilton cheese in MRS broth assayed for lactic and acetic acid production.

Isolate		Sum of Squares	df	Mean Square	F	Sig.
B30	Between Groups	70.538	5	14.108	793.301	.000
	Within Groups	.213	12	.018		
	Total	70.751	17			
P. acidi	Between Groups	75.956	5	15.191	961.468	.000
	Within Groups	.190	12	.016		
	Total	76.146	17			
R2	Between Groups	72.033	5	14.407	641.082	.000
	Within Groups	.270	12	.022		
	Total	72.302	17			
R25	Between Groups	86.104	5	17.221	1259.039	.000
	Within Groups	.164	12	.014		
	Total	86.268	17			
R40	Between Groups	76.428	5	15.286	509.141	.000
	Within Groups	.360	12	.030		
	Total	76.788	17			
R6	Between Groups	72.900	5	14.580	118.221	.000
	Within Groups	1.480	12	.123		
	Total	74.380	17			
W30	Between Groups	77.357	5	15.471	1612.530	.000
	Within Groups	.115	12	.010		
	Total	77.472	17			

(P. acidi) *Ped. acidilactici*. Isolates of *Lb. plantarum* obtained from: (B30&R25) blue veins; (R2&R6) outer crust; (R40&W30) white core. All cultures were incubated aerobically for 48 h at 30^{o}C.

ANOVA for Table 7.1 Mean pH of MRS broth inoculated with *Ped. acidilactici* NCIMB 700993 and the different genotypes of *Lb. plantarum* obtained from Stilton cheese. The broths were assayed for lactic and acetic acid content

strain name		Sum of Squares	df	Mean Square	F	Sig.
B30	Between Groups	5.346	5	1.069	622.808	.000
	Within Groups	.010	6	.002		
	Total	5.356	11			
P. acidi	Between Groups	10.537	5	2.107	1708.667	.000
	Within Groups	.015	12	.001		
	Total	10.552	17			
R2	Between Groups	4.279	5	.856	863.061	.000
	Within Groups	.006	6	.001		
	Total	4.285	11			
R25	Between Groups	5.190	5	1.038	779.951	.000
	Within Groups	.009	7	.001		
	Total	5.200	12			
R40	Between Groups	4.287	5	.857	476.296	.000
	Within Groups	.011	6	.002		
	Total	4.297	11			
R6	Between Groups	4.033	5	.807	338.442	.000
	Within Groups	.014	6	.002		
	Total	4.047	11			
W30	Between Groups	7.064	5	1.413	623.291	.000
	Within Groups	.014	6	.002		
	Total	7.078	11			

(P. acidi) *Ped. acidilactici*. Isolates of *Lb. plantarum* obtained from: (B30&R25) blue veins; (R2&R6) outer crust; (R40&W30) white core. All cultures were incubated aerobically for 48 h at 30^{o}C.

ANOVA for Table 7.1 Mean lactic acid content of MRS broth inoculated with *Ped. acidilactici* NCIMB 700993 and the different genotypes of *Lb. plantarum* obtained from Stilton cheese.

strain name		Sum of Squares	df	Mean Square	F	Sig.
B30	Between Groups	476.948	5	95.390	716.198	.000
	Within Groups	1.598	12	.133		
	Total	478.546	17			
P. acidi	Between Groups	510.457	5	102.091	418.818	.000
	Within Groups	2.925	12	.244		
	Total	513.382	17			
R2	Between Groups	445.842	5	89.168	758.521	.000
	Within Groups	1.411	12	.118		
	Total	447.252	17			
R25	Between Groups	555.412	5	111.082	590.323	.000
	Within Groups	2.258	12	.188		
	Total	557.670	17			
R40	Between Groups	450.029	5	90.006	2419.871	.000
	Within Groups	.446	12	.037		
	Total	450.475	17			
R6	Between Groups	471.627	5	94.325	3227.867	.000
	Within Groups	.351	12	.029		
	Total	471.978	17			
W30	Between Groups	1130.947	5	226.189	459.185	.000
	Within Groups	5.911	12	.493		
	Total	1136.858	17			

(P. acidi) *Ped. acidilactici*. Isolates of *Lb. plantarum* obtained from: (B30&R25) blue veins; (R2&R6) outer crust; (R40&W30) white core. All cultures were incubated aerobically for 48 h at 30^{o}C.

ANOVA for Table 7.1 Mean acetic acid content of MRS broth inoculated with *Ped. acidilactici* NCIMB 700993 and the different genotypes of *Lb. plantarum* obtained from Stilton cheese.

strain name		Sum of Squares	df	Mean Square	F	Sig.
B30	Between Groups	1.626	5	.325	4.642	.014
	Within Groups	.841	12	.070		
	Total	2.467	17			
P. acidi	Between Groups	3.476	5	.695	15.283	.000
	Within Groups	.546	12	.045		
	Total	4.021	17			
R2	Between Groups	6.518	5	1.304	11.119	.000
	Within Groups	1.407	12	.117		
	Total	7.925	17			
R25	Between Groups	2.036	5	.407	9.939	.001
	Within Groups	.492	12	.041		
	Total	2.527	17			
R40	Between Groups	.847	5	.169	3.673	.030
	Within Groups	.554	12	.046		
	Total	1.401	17			
R6	Between Groups	3.568	5	.714	8.506	.001
	Within Groups	1.007	12	.084		
	Total	4.574	17			
W30	Between Groups	3.645	5	.729	21.451	.000
	Within Groups	.408	12	.034		
	Total	4.053	17			

(P. acidi) *Ped. acidilactici*. Isolates of *Lb. plantarum* obtained from: (B30&R25) blue veins; (R2&R6) outer crust; (R40&W30) white core. All cultures were incubated aerobically for 48 h at 30^{o}C.

ANOVA for Figs. 7.3-7.4 Viable population (log_{10} CFU/ml) and pH changes of cows' milk inoculated with single and mixed cultures of *Lb. plantarum* isolates and *Lc. lactis* strains and incubated for 48 h at 30^{o}C

culture type (single or mixed)			Sum of Squares	df	Mean Square	F	Sig.
Lc. lactis NCIMB6681	Log CFU/ml	Between Groups	87.888	6	14.648	981.522	.000
		Within Groups	.209	14	.015		
		Total	88.097	20			
	pH	Between Groups	109.117	6	18.186	21099.994	.000
		Within Groups	.012	14	.001		
		Total	109.129	20			
6681-*Lb. plantarum* B30 (veins)	Log CFU/ml	Between Groups	94.344	6	15.724	1068.621	.000
		Within Groups	.206	14	.015		
		Total	94.550	20			
	pH	Between Groups	108.002	6	18.000	68728.691	.000
		Within Groups	.004	14	.000		
		Total	108.006	20			
6681- *Lb. plantarum* R2 (crust)	Log CFU/ml	Between Groups	90.014	6	15.002	1215.933	.000
		Within Groups	.173	14	.012		
		Total	90.186	20			
	pH	Between Groups	108.677	6	18.113	37660.393	.000
		Within Groups	.007	14	.000		
		Total	108.684	20			
6681- *Lb. plantarum* W8 (core)	Log CFU/ml	Between Groups	89.989	6	14.998	2554.430	.000
		Within Groups	.082	14	.006		
		Total	90.071	20			
	pH	Between Groups	108.506	6	18.084	35163.948	.000
		Within Groups	.007	14	.001		
		Total	108.513	20			
Lc. lactis NCIMB9918	Log CFU/ml	Between Groups	63.071	6	10.512	279.994	.000
		Within Groups	.526	14	.038		
		Total	63.596	20			
	pH	Between Groups	112.030	6	18.672	5132.278	.000
		Within Groups	.051	14	.004		
		Total	112.081	20			
9918-B30	Log CFU/ml	Between Groups	67.494	6	11.249	507.035	.000
		Within Groups	.311	14	.022		
		Total	67.804	20			
	pH	Between Groups	112.411	6	18.735	27322.093	.000
		Within Groups	.010	14	.001		

		Total	112.420	20			
9918-R2	Log CFU/ml	Between Groups	67.635	6	11.272	109.058	.000
		Within Groups	1.447	14	.103		
		Total	69.082	20			
	pH	Between Groups	112.326	6	18.721	40530.069	.000
		Within Groups	.006	14	.000		
		Total	112.333	20			
9918-W8	Log CFU/ml	Between Groups	67.646	6	11.274	472.103	.000
		Within Groups	.334	14	.024		
		Total	67.980	20			
	pH	Between Groups	112.245	6	18.708	45156.234	.000
		Within Groups	.006	14	.000		
		Total	112.251	20			
B30	Log CFU/ml	Between Groups	46.489	6	7.748	736.925	.000
		Within Groups	.147	14	.011		
		Total	46.637	20			
	pH	Between Groups	113.848	6	18.975	33484.821	.000
		Within Groups	.008	14	.001		
		Total	113.856	20			
B30-6681	Log CFU/ml	Between Groups	40.514	6	6.752	873.148	.000
		Within Groups	.108	14	.008		
		Total	40.622	20			
	pH	Between Groups	108.002	6	18.000	68728.691	.000
		Within Groups	.004	14	.000		
		Total	108.006	20			
B30-9918	Log CFU/ml	Between Groups	16.608	6	2.768	184.650	.000
		Within Groups	.210	14	.015		
		Total	16.818	20			
	pH	Between Groups	112.411	6	18.735	27322.093	.000
		Within Groups	.010	14	.001		
		Total	112.420	20			
B30-*Lc. lactis* subsp. *lactis* (LLL)	Log CFU/ml	Between Groups	33.576	6	5.596	92.943	.000
		Within Groups	.843	14	.060		
		Total	34.419	20			
	pH	Between Groups	108.075	6	18.012	67546.851	.000
		Within Groups	.004	14	.000		
		Total	108.079	20			
LLL	Log CFU/ml	Between Groups	82.114	6	13.686	788.259	.000
		Within Groups	.243	14	.017		
		Total	82.357	20			

	pH	Between Groups	108.674	6	18.112	24860.087	.000
		Within Groups	.010	14	.001		
		Total	108.684	20			
LLL-B30	Log CFU/ml	Between Groups	92.493	6	15.415	756.544	.000
		Within Groups	.285	14	.020		
		Total	92.778	20			
	pH	Between Groups	108.075	6	18.012	67546.851	.000
		Within Groups	.004	14	.000		
		Total	108.079	20			
LLL-R2	Log CFU/ml	Between Groups	85.847	6	14.308	535.776	.000
		Within Groups	.374	14	.027		
		Total	86.221	20			
	pH	Between Groups	108.364	6	18.061	51253.171	.000
		Within Groups	.005	14	.000		
		Total	108.369	20			
LLL-W8	Log CFU/ml	Between Groups	94.558	6	15.760	401.738	.000
		Within Groups	.549	14	.039		
		Total	95.107	20			
	pH	Between Groups	108.240	6	18.040	17620.527	.000
		Within Groups	.014	14	.001		
		Total	108.255	20			
R2	Log CFU/ml	Between Groups	51.930	6	8.655	355.265	.000
		Within Groups	.341	14	.024		
		Total	52.271	20			
	pH	Between Groups	113.660	6	18.943	18943.311	.000
		Within Groups	.014	14	.001		
		Total	113.674	20			
R2-6681	Log CFU/ml	Between Groups	24.622	6	4.104	1133.895	.000
		Within Groups	.051	14	.004		
		Total	24.672	20			
	pH	Between Groups	108.677	6	18.113	37660.393	.000
		Within Groups	.007	14	.000		
		Total	108.684	20			
R2-9918	Log CFU/ml	Between Groups	14.282	6	2.380	85.770	.000
		Within Groups	.389	14	.028		
		Total	14.670	20			
	pH	Between Groups	112.326	6	18.721	40530.069	.000
		Within Groups	.006	14	.000		
		Total	112.333	20			
R2-LLL	Log	Between Groups	23.968	6	3.995	255.913	.000

	CFU/ml	Within Groups	.219	14	.016		
		Total	24.187	20			
	pH	Between Groups	108.364	6	18.061	51253.171	.000
		Within Groups	.005	14	.000		
		Total	108.369	20			
W8	Log CFU/ml	Between Groups	55.409	6	9.235	738.220	.000
		Within Groups	.175	14	.013		
		Total	55.584	20			
	pH	Between Groups	113.706	6	18.951	25842.331	.000
		Within Groups	.010	14	.001		
		Total	113.717	20			
W8-6681	Log CFU/ml	Between Groups	24.109	6	4.018	222.293	.000
		Within Groups	.253	14	.018		
		Total	24.362	20			
	pH	Between Groups	108.506	6	18.084	35163.948	.000
		Within Groups	.007	14	.001		
		Total	108.513	20			
W8-9918	Log CFU/ml	Between Groups	16.198	6	2.700	230.081	.000
		Within Groups	.164	14	.012		
		Total	16.362	20			
	pH	Between Groups	112.245	6	18.708	45156.234	.000
		Within Groups	.006	14	.000		
		Total	112.251	20			
W8-LLL	Log CFU/ml	Between Groups	25.294	6	4.216	68.830	.000
		Within Groups	.857	14	.061		
		Total	26.151	20			
	pH	Between Groups	108.240	6	18.040	17620.527	.000
		Within Groups	.014	14	.001		
		Total	108.255	20			

ANOVA for Figs. 7.5-7.6 Viable population (log_{10} CFU/ml) and pH changes of cows' milk inoculated with single and mixed cultures of *Lb. plantarum* isolates and *Lc. lactis* strains and incubated for 12 weeks at 18^{o}C with and without the addition of NaCl.

culture type (single or mixed)			Sum of Squares	df	Mean Square	F	Sig.
Lc. lactis NCIMB 6681 (6681)	Log CFU/ml, NO-SALT	Between Groups	189.627	8	23.703	301.754	.000
		Within Groups	1.414	18	.079		
		Total	191.040	26			
	Log CFU/ml, SALTED	Between Groups	96.928	8	12.116	41.056	.000
		Within Groups	5.312	18	.295		
		Total	102.240	26			
	pH, NO-SALT	Between Groups	3.152	8	.394	3.890	.008
		Within Groups	1.823	18	.101		
		Total	4.975	26			
	pH, SALTED	Between Groups	1.709	8	.214	9.499	.000
		Within Groups	.405	18	.022		
		Total	2.113	26			
6681-*Lb. plantarum* strain B30 (veins)	Log CFU/ml, NO-SALT	Between Groups	183.991	8	22.999	130.404	.000
		Within Groups	3.175	18	.176		
		Total	187.165	26			
	Log CFU/ml, SALTED	Between Groups	137.343	8	17.168	65.516	.000
		Within Groups	4.717	18	.262		
		Total	142.060	26			
	pH, NO-SALT	Between Groups	2.134	8	.267	8.790	.000
		Within Groups	.546	18	.030		
		Total	2.680	26			

	pH, SALTED	Between Groups	3.148	8	.393	13.266	.000
		Within Groups	.534	18	.030		
		Total	3.681	26			
6681-*Lb. plantarum* strain R2 (crust)	Log CFU/ml, NO-SALT	Between Groups	201.645	8	25.206	396.847	.000
		Within Groups	1.143	18	.064		
		Total	202.788	26			
	Log CFU/ml, SALTED	Between Groups	170.958	8	21.370	854.411	.000
		Within Groups	.450	18	.025		
		Total	171.408	26			
	pH, NO-SALT	Between Groups	4.503	8	.563	12.686	.000
		Within Groups	.799	18	.044		
		Total	5.301	26			
	pH, SALTED	Between Groups	5.099	8	.637	7.370	.000
		Within Groups	1.557	18	.086		
		Total	6.655	26			
6681-*Lb. plantarum* strain W8 (core)	Log CFU/ml, NO-SALT	Between Groups	203.463	8	25.433	421.074	.000
		Within Groups	1.087	18	.060		
		Total	204.550	26			
	Log CFU/ml, SALTED	Between Groups	175.101	8	21.888	474.634	.000
		Within Groups	.830	18	.046		
		Total	175.932	26			
	pH, NO-SALT	Between Groups	4.211	8	.526	8.992	.000
		Within Groups	1.054	18	.059		
		Total	5.265	26			

	pH, SALTED	Between Groups	4.103	8	.513	27.638	.000
		Within Groups	.334	18	.019		
		Total	4.437	26			
Lc. lactis NCIMB 9918 (9918)	Log CFU/ml, NO-SALT	Between Groups	1.805	8	.226	3.372	.015
		Within Groups	1.205	18	.067		
		Total	3.010	26			
	Log CFU/ml, SALTED	Between Groups	3.059	8	.382	2.782	.034
		Within Groups	2.474	18	.137		
		Total	5.534	26			
	pH, NO-SALT	Between Groups	2.017	8	.252	11.612	.000
		Within Groups	.391	18	.022		
		Total	2.408	26			
	pH, SALTED	Between Groups	2.415	8	.302	5.249	.002
		Within Groups	1.035	18	.058		
		Total	3.451	26			
9918- *Lb. plantarum* B30	Log CFU/ml, NO-SALT	Between Groups	24.246	8	3.031	8.789	.000
		Within Groups	6.207	18	.345		
		Total	30.453	26			
	Log CFU/ml, SALTED	Between Groups	5.470	8	.684	15.504	.000
		Within Groups	.794	18	.044		
		Total	6.264	26			
	pH, NO-SALT	Between Groups	27.324	8	3.415	15.266	.000
		Within Groups	4.027	18	.224		
		Total	31.351	26			

	pH, SALTED	Between Groups	28.118	8	3.515	137.098	.000
		Within Groups	.461	18	.026		
		Total	28.580	26			
9918- *Lb. plantarum* R2	Log CFU/ml, NO-SALT	Between Groups	5.812	8	.727	3.367	.015
		Within Groups	3.884	18	.216		
		Total	9.696	26			
	Log CFU/ml, SALTED	Between Groups	4.743	8	.593	1.268	.319
		Within Groups	8.418	18	.468		
		Total	13.161	26			
	pH, NO-SALT	Between Groups	13.865	8	1.733	9.055	.000
		Within Groups	3.445	18	.191		
		Total	17.311	26			
	pH, SALTED	Between Groups	6.779	8	.847	32.439	.000
		Within Groups	.470	18	.026		
		Total	7.249	26			
9918- *Lb. plantarum* W8	Log CFU/ml, NO-SALT	Between Groups	7.096	8	.887	1.546	.210
		Within Groups	10.326	18	.574		
		Total	17.421	26			
	Log CFU/ml, SALTED	Between Groups	4.887	8	.611	4.578	.004
		Within Groups	2.402	18	.133		
		Total	7.288	26			
	pH, NO-SALT	Between Groups	9.321	8	1.165	36.341	.000
		Within Groups	.577	18	.032		
		Total	9.898	26			

	pH, SALTED	Between Groups	3.551	8	.444	17.351	.000
		Within Groups	.461	18	.026		
		Total	4.012	26			
Lb. plantarum B30	Log CFU/ml, NO-SALT	Between Groups	3.571	8	.446	108.281	.000
		Within Groups	.074	18	.004		
		Total	3.645	26			
	Log CFU/ml, SALTED	Between Groups	1.069	8	.134	6.467	.001
		Within Groups	.372	18	.021		
		Total	1.441	26			
	pH, NO-SALT	Between Groups	17.863	8	2.233	67.001	.000
		Within Groups	.600	18	.033		
		Total	18.463	26			
	pH, SALTED	Between Groups	16.937	8	2.117	120.749	.000
		Within Groups	.316	18	.018		
		Total	17.253	26			
B30- 6681	Log CFU/ml, NO-SALT	Between Groups	5.061	8	.633	21.241	.000
		Within Groups	.536	18	.030		
		Total	5.597	26			
	Log CFU/ml, SALTED	Between Groups	3.467	8	.433	6.070	.001
		Within Groups	1.285	18	.071		
		Total	4.753	26			
B30-9918	Log CFU/ml, NO-SALT	Between Groups	15.098	8	1.887	19.467	.000
		Within Groups	1.745	18	.097		
		Total	16.842	26			

	Log CFU/ml, SALTED	Between Groups	13.189	8	1.649	32.655	.000
		Within Groups	.909	18	.050		
		Total	14.097	26			
B30-*Lc. lactis* subsp. *lactis* (LLL)	Log CFU/ml, NO-SALT	Between Groups	4.819	8	.602	23.902	.000
		Within Groups	.454	18	.025		
		Total	5.273	26			
	Log CFU/ml, SALTED	Between Groups	4.004	8	.500	8.135	.000
		Within Groups	1.107	18	.062		
		Total	5.111	26			
LLL	Log CFU/ml, NO-SALT	Between Groups	190.082	8	23.760	338.823	.000
		Within Groups	1.262	18	.070		
		Total	191.345	26			
	Log CFU/ml, SALTED	Between Groups	79.432	8	9.929	80.424	.000
		Within Groups	2.222	18	.123		
		Total	81.655	26			
	pH, NO-SALT	Between Groups	3.157	8	.395	8.720	.000
		Within Groups	.815	18	.045		
		Total	3.972	26			
	pH, SALTED	Between Groups	1.866	8	.233	11.802	.000
		Within Groups	.356	18	.020		
		Total	2.222	26			
LLL- *Lb. plantarum* B30	Log CFU/ml, NO-SALT	Between Groups	76.646	8	9.581	94.049	.000
		Within Groups	1.834	18	.102		
		Total	78.480	26			

	Log CFU/ml, SALTED	Between Groups	138.273	8	17.284	133.522	.000
		Within Groups	2.330	18	.129		
		Total	140.603	26			
	pH, NO-SALT	Between Groups	2.301	8	.288	10.353	.000
		Within Groups	.500	18	.028		
		Total	2.801	26			
	pH, SALTED	Between Groups	3.132	8	.392	22.311	.000
		Within Groups	.316	18	.018		
		Total	3.448	26			
LLL- *Lb. plantarum* R2	Log CFU/ml, NO-SALT	Between Groups	204.578	8	25.572	743.061	.000
		Within Groups	.619	18	.034		
		Total	205.198	26			
	Log CFU/ml, SALTED	Between Groups	175.012	8	21.877	624.450	.000
		Within Groups	.631	18	.035		
		Total	175.643	26			
	pH, NO-SALT	Between Groups	4.258	8	.532	8.560	.000
		Within Groups	1.119	18	.062		
		Total	5.377	26			
	pH, SALTED	Between Groups	3.833	8	.479	18.382	.000
		Within Groups	.469	18	.026		
		Total	4.302	26			
LLL- *Lb. plantarum* W8	Log CFU/ml, NO-SALT	Between Groups	204.805	8	25.601	308.111	.000
		Within Groups	1.496	18	.083		
		Total	206.300	26			

	Log CFU/ml, SALTED	Between Groups	180.454	8	22.557	705.961	.000
		Within Groups	.575	18	.032		
		Total	181.029	26			
	pH, NO-SALT	Between Groups	4.423	8	.553	9.038	.000
		Within Groups	1.101	18	.061		
		Total	5.524	26			
	pH, SALTED	Between Groups	3.984	8	.498	30.668	.000
		Within Groups	.292	18	.016		
		Total	4.276	26			
Lb. plantarum R2	Log CFU/ml, NO-SALT	Between Groups	.104	8	.013	.111	.998
		Within Groups	2.111	18	.117		
		Total	2.216	26			
	Log CFU/ml, SALTED	Between Groups	.433	8	.054	.191	.989
		Within Groups	5.102	18	.283		
		Total	5.535	26			
	pH, NO-SALT	Between Groups	10.976	8	1.372	8.357	.000
		Within Groups	2.955	18	.164		
		Total	13.931	26			
	pH, SALTED	Between Groups	14.680	8	1.835	9.837	.000
		Within Groups	3.358	18	.187		
		Total	18.038	26			
R2-6681	Log CFU/ml, NO-SALT	Between Groups	3.345	8	.418	13.851	.000
		Within Groups	.543	18	.030		
		Total	3.888	26			

	Log CFU/ml, SALTED	Between Groups	1.561	8	.195	1.077	.421
		Within Groups	3.263	18	.181		
		Total	4.824	26			
R2-9918	Log CFU/ml, NO-SALT	Between Groups	15.857	8	1.982	51.361	.000
		Within Groups	.695	18	.039		
		Total	16.552	26			
	Log CFU/ml, SALTED	Between Groups	9.268	8	1.158	21.020	.000
		Within Groups	.992	18	.055		
		Total	10.260	26			
R2-LLL	Log CFU/ml, NO-SALT	Between Groups	2.938	8	.367	17.021	.000
		Within Groups	.388	18	.022		
		Total	3.326	26			
	Log CFU/ml, SALTED	Between Groups	1.734	8	.217	1.139	.385
		Within Groups	3.424	18	.190		
		Total	5.158	26			
Lb. plantarum W8	Log CFU/ml, NO-SALT	Between Groups	.920	8	.115	1.680	.171
		Within Groups	1.232	18	.068		
		Total	2.152	26			
	Log CFU/ml, SALTED	Between Groups	.614	8	.077	12.438	.000
		Within Groups	.111	18	.006		
		Total	.725	26			
	pH, NO-SALT	Between Groups	6.012	8	.751	51.316	.000
		Within Groups	.264	18	.015		
		Total	6.276	26			

	pH, SALTED	Between Groups	4.991	8	.624	13.084	.000
		Within Groups	.858	18	.048		
		Total	5.849	26			
W8-6681	Log CFU/ml, NO-SALT	Between Groups	4.296	8	.537	29.287	.000
		Within Groups	.330	18	.018		
		Total	4.626	26			
	Log CFU/ml, SALTED	Between Groups	2.308	8	.289	7.279	.000
		Within Groups	.714	18	.040		
		Total	3.022	26			
W8-9918	Log CFU/ml, NO-SALT	Between Groups	9.246	8	1.156	7.149	.000
		Within Groups	2.910	18	.162		
		Total	12.157	26			
	Log CFU/ml, SALTED	Between Groups	3.007	8	.376	7.931	.000
		Within Groups	.853	18	.047		
		Total	3.860	26			
W8-LLL	Log CFU/ml, NO-SALT	Between Groups	3.253	8	.407	10.197	.000
		Within Groups	.718	18	.040		
		Total	3.970	26			
	Log CFU/ml, SALTED	Between Groups	1.851	8	.231	3.055	.023
		Within Groups	1.363	18	.076		
		Total	3.215	26			

Appendix 9 cont'd (a) Summary statistics showing the $\log_{10}$ CFU/ml (HPLC) pair wise comparison of the data based on the Turkey test

Analysis of variance, Log CFU/ml for HPLC data (Variable **R2** (**crust**):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	71.946	14.389	640.651	< 0.0001
Error	12	0.270	0.022		
Corrected T	17	72.215			

Computed against model Y=Mean(Y)

Summary of all pairwise comparisons of crust isolates for time (Tukey (HSD):

Category	LS means	Groups					
48	8.813	A				**R2-crust**	
24	8.317		B				
9	5.239			C			
6	4.713				D		
3	4.171					E	
0	3.696						F

Analysis of variance, Log CFU/ml for HPLC data (Variable **R6** (**crust**):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	72.947	14.589	119.047	< 0.0001
Error	12	1.471	0.123		
Corrected T	17	74.418			

Computed against model Y=Mean(Y)

Corrected T	17	74.418

Computed against model Y=Mean(Y)

Category	LS means	Groups				
48	8.958	A				**R6-crust**
24	7.631		B			
9	4.972			C		
6	4.362			C	D	
3	3.776				D	
0	3.686				D	

Analysis of variance, Log CFU/ml for HPLC data (Variable **B30** (veins):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	70.646	14.129	794.297	< 0.0001
Error	12	0.213	0.018		
Corrected T	17	70.860			

Computed against model Y=Mean(Y)

Summary of all pairwise comparisons of veins isolates for time (Tukey (HSD):

Category	LS means	Groups					
48	8.526	A				**B30-veins**	
24	8.427	A					
9	5.431		B				
6	4.711			C			
3	4.077				D		
0	3.581					E	

Analysis of variance, Log CFU/ml for HPLC data (Variable **R25** (veins):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	86.062	17.212	1269.297	< 0.0001
Error	12	0.163	0.014		
Corrected T	17	86.225			

Computed against model Y=Mean(Y)

Category	LS means	Groups					
48	9.008	A				**R25-veins**	
24	8.103		B				
9	4.969			C			
6	4.559				D		
3	3.739					E	
0	3.179						F

Analysis of variance, Log CFU/ml for HPLC data (Variable **R40** (core):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	77.298	15.460	1602.372	< 0.0001
Error	12	0.116	0.010		
Corrected T	17	77.414			

Computed against model Y=Mean(Y)

Analysis of variance, Log CFU/ml for HPLC data (Variable W3**0** (core):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	76.392	15.278	508.037	< 0.0001
Error	12	0.361	0.030		
Corrected T	17	76.753			

Computed against model Y=Mean(Y)

Analysis of variance, Log CFU/ml for HPLC data (Variable *Ped. acidilactici*):

Source	DF	Sum of squares	Mean square	F	Pr > F
Model	5	75.983	15.197	973.407	< 0.0001
Error	12	0.187	0.016		
Corrected T	17	76.170			

Computed against model Y=Mean(Y)

Summary of all pairwise comparisons of core isolates for time (Tukey (HSD):

Category	LS means	Groups				
48	9.128	A				**R40-core**
24	9.027	A				
9	5.850		B			
6	5.322			C		
3	4.465				D	
0	3.889					E

Category	LS means	Groups				
48	8.773	A				**W30-core**
24	7.898		B			
9	5.003			C		
6	4.557			C		
3	3.740				D	
0	3.313				D	

Category	LS means	Groups				
24	9.278	A				***Ped. acidilactici***
48	8.456		B			(control)
9	6.760			C		
6	5.585				D	
3	4.112					E
0	3.789					E

Appendix 9 cont'd (b) Summary statistics showing the pH (HPLC) pair wise comparison of the data based on the Turkey test

							Summary of all pairwise comparisons of crust isolates for time (Tukey (HSD):							
Analysis of variance, Log CFU/ml for HPLC data (Variable **R2** (**crust**):							Category	LS means	Groups					
							48	8.813	A				**R2-crust**	
Source	DF	ım of squar	Mean square	F	Pr > F		24	8.317		B				
Model	5	71.946	14.389	640.651	< 0.0001		9	5.239			C			
Error	12	0.270	0.022				6	4.713				D		
Corrected T	17	72.215					3	4.171					E	
Computed against model Y=Mean(Y)							0	3.696						F
Analysis of variance, Log CFU/ml for HPLC data (Variable **R6** (**crust**):							Category	LS means	Groups					
							48	8.958	A				**R6-crust**	
Source	DF	ım of squar	Mean square	F	Pr > F		24	7.631		B				
Model	5	72.947	14.589	119.047	< 0.0001		9	4.972			C			
Error	12	1.471	0.123				6	4.362			C	D		
Corrected T	17	74.418					3	3.776				D		
Computed against model Y=Mean(Y)							0	3.686				D		
Corrected T	17	74.418												
Computed against model Y=Mean(Y)														

Analysis of variance, pH for HPLC data (Variable **B30** (veins):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	8.045	1.609	1405.904	< 0.0001
Error	12	0.014	0.001		
Corrected	17	8.059			

Computed against model Y=Mean(Y)

Summary of all pairwise comparisons of veins isolates for time (Tukey (HSD):

Category	LS means	Groups				
0	5.790	A				**B30-veins**
9	5.590		B			
6	5.570		B			
3	5.453			C		
24	4.620				D	
48	3.913					E

Analysis of variance, pH for HPLC data (Variable **R25** (veins):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	7.648	1.530	1521.238	< 0.0001
Error	12	0.012	0.001		
Corrected	17	7.661			

Computed against model Y=Mean(Y)

Category	LS means	Groups				
9	5.803	A				
0	5.790	A				**R25-veins**
6	5.653		B			
3	5.437			C		
24	5.043				D	
48	3.940					E

Analysis of variance, pH for HPLC data (Variable **R40** (core):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	6.324	1.265	1054.030	< 0.0001
Error	12	0.014	0.001		
Corrected	17	6.339			

Computed against model Y=Mean(Y)

Summary of all pairwise comparisons of core isolates for time (Tukey (HSD):

Category	LS means	Groups				
0	5.790	A				**R40-core**
9	5.560		B			
6	5.553		B			
3	5.457			C		
24	5.263				D	
48	3.990					E

Analysis of variance, pH for HPLC data (Variable W3**0** (core):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	10.574	2.115	1399.482	< 0.0001
Error	12	0.018	0.002		
Corrected	17	10.592			

Computed against model Y=Mean(Y)

Category	LS means	Groups				
0	5.790	A				**W30-core**
9	5.593		B			
6	5.533		B			
3	5.403			C		
24	4.243				D	
48	3.757					E

Analysis of variance, pH for HPLC data (Variable *Ped. acidilactici*):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	10.537	2.107	1708.667	< 0.0001
Error	12	0.015	0.001		
Corrected	17	10.552			

Computed against model Y=Mean(Y)

Category	LS means	Groups				
0	5.790	A			***Ped. acidilactici***	
3	5.393		B		(control)	
6	5.360		B			
9	5.337		B			
48	3.977			C		
24	3.790				D	

Appendix 9 cont'd (c) Summary statistics showing the lactic acid production (HPLC) pair wise comparison of the data based on the Turkey test

Analysis of variance, lactic acid for HPLC data (Variable **R2** (**crust**):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	445.842	89.168	758.521	< 0.0001
Error	12	1.411	0.118		
Corrected	17	447.252			
Computed against model Y=Mean(Y)					

Summary of all pairwise comparisons of crust isolates for time (Tukey (HSD):

Category	LS means	Groups			**R2-crust**
48	13.633	A			
24	2.140		B		
6	0.030			C	
3	0.020			C	
9	0.020			C	
0	0.000			C	

Analysis of variance, lactic acid for HPLC data (Variable **R6** (**crust**):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	471.627	94.325	3227.867	< 0.0001
Error	12	0.351	0.029		
Corrected	17	471.978			
Computed against model Y=Mean(Y)					

Category	LS means	Groups			**R6-crust**
48	13.910	A			
24	0.747		B		
6	0.093			C	
3	0.073			C	
9	0.047			C	
0	0.000			C	

Analysis of variance, lactic acid for HPLC data (Variable **B30** (veins):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	476.948	95.390	716.198	< 0.0001
Error	12	1.598	0.133		
Corrected	17	478.546			
Computed against model Y=Mean(Y)					

Summary of all pairwise comparisons of veins isolates for time (Tukey (HSD):

Category	LS means	Groups			**B30-veins**
48	14.057	A			
24	4.363		B		
3	0.087			C	
9	0.080			C	
6	0.013			C	
0	0.000			C	

Analysis of variance, lactic acid for HPLC data (Variable **R25** (veins):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	555.412	111.082	590.323	< 0.0001
Error	12	2.258	0.188		
Corrected	17	557.670			
Computed against model Y=Mean(Y)					

Category	LS means	Groups			**R25-veins**
48	15.190	A			
24	1.843		B		
6	0.047			C	
9	0.043			C	
3	0.023			C	
0	0.000			C	

Analysis of variance, lactic acid for HPLC data (Variable **R40** (core):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	450.029	90.006	2419.871	< 0.0001
Error	12	0.446	0.037		
Corrected	17	450.475			
Computed against model Y=Mean(Y)					

Summary of all pairwise comparisons of core isolates for time (Tukey (HSD):

Category	LS means	Groups			**R40-core**
48	13.637	A			
24	1.143		B		
3	0.073			C	
9	0.063			C	
6	0.037			C	
0	0.000			C	

Analysis of variance, lactic acid for HPLC data (Variable W3**0** (core):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	1130.947	226.189	459.185	< 0.0001
Error	12	5.911	0.493		
Corrected	17	1136.858			
Computed against model Y=Mean(Y)					

Category	LS means	Groups			**W30-core**
48	21.400	A			
24	7.993		B		
3	0.070			C	
6	0.040			C	
0	0.000			C	
9	-0.020			C	

Analysis of variance, lactic acid for HPLC data (Variable *Ped. acidilactici*):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	510.457	102.091	418.818	< 0.0001
Error	12	2.925	0.244		
Corrected	17	513.382			
Computed against model Y=Mean(Y)					

Category	LS means	Groups			***Ped. acidilactici*** (control)
48	12.213	A			
24	10.303		B		
3	0.050			C	
9	0.050			C	
0	0.000			C	
6	-0.010			C	

Appendix 9 cont'd (d) Summary statistics showing the acetic acid production (HPLC) pair wise comparison of the data based on the Turkey test

Analysis of variance, acetic acid for HPLC data (Variable **R2** (**crust**):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	6.518	1.304	11.119	0.000
Error	12	1.407	0.117		
Corrected	17	7.925			
Computed against model Y=Mean(Y)					

Summary of all pairwise comparisons of crust isolates for time (Tukey (HSD):

Category	LS means	Groups			**R2-crust**
48	1.747	A			
24	0.287		B		
9	0.153		B		
3	0.150		B		
6	0.147		B		
0	0.000		B		

Analysis of variance, acetic acid for HPLC data (Variable **R6** (**crust**):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	3.568	0.714	8.506	0.001
Error	12	1.007	0.084		
Corrected	17	4.574			
Computed against model Y=Mean(Y)					

Category	LS means	Groups			**R6-crust**
48	1.373	A			
6	0.473		B		
3	0.300		B		
9	0.243		B		
24	0.183		B		
0	0.000		B		

Analysis of variance, acetic acid for HPLC data (Variable **B30** (veins):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	1.626	0.325	4.642	0.014
Error	12	0.841	0.070		
Corrected	17	2.467			
Computed against model Y=Mean(Y)					

Summary of all pairwise comparisons of veins isolates for time (Tukey (HSD):

Category	LS means	Groups		**B30-veins**
48	0.990	A		
9	0.373	A	B	
6	0.307	A	B	
24	0.307	A	B	
3	0.257		B	
0	0.000		B	

Analysis of variance, acetic acid for HPLC data (Variable **R25** (veins):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	2.036	0.407	9.939	0.001
Error	12	0.492	0.041		
Corrected	17	2.527			
Computed against model Y=Mean(Y)					

Category	LS means	Groups		**R25-veins**
48	1.063	A		
24	0.550	A	B	
3	0.347		B	
6	0.243		B	
9	0.230		B	
0	0.000		B	

Analysis of variance, acetic acid for HPLC data (Variable **R40** (core):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	0.847	0.169	3.673	0.030
Error	12	0.554	0.046		
Corrected	17	1.401			

Computed against model Y=Mean(Y)

Summary of all pairwise comparisons of core isolates for time (Tukey (HSD):

Category	LS means	Groups			**R40-core**
48	0.710	A			
3	0.327	A	B		
24	0.277	A	B		
9	0.260	A	B		
6	0.150	A	B		
0	0.000		B		

Analysis of variance, acetic acid for HPLC data (Variable W3**0** (core):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	3.645	0.729	21.451	< 0.0001
Error	12	0.408	0.034		
Corrected	17	4.053			

Computed against model Y=Mean(Y)

W30-core

Category	LS means	Groups		
48	1.337	A		
24	0.387		B	
3	0.270		B	
6	0.157		B	
9	0.073		B	
0	0.000		B	

Analysis of variance, acetic acid for HPLC data (Variable *Ped. acidilactici*):

Source	DF	m of squar	ean squar	F	Pr > F
Model	5	3.476	0.695	15.283	< 0.0001
Error	12	0.546	0.045		
Corrected	17	4.021			

Computed against model Y=Mean(Y)

Category	LS means	Groups			***Ped. acidilactici***
48	1.430	A			(control)
24	1.017	A	B		
9	0.660		B		
3	0.597		B		
6	0.547		B	C	
0	0.000			C	