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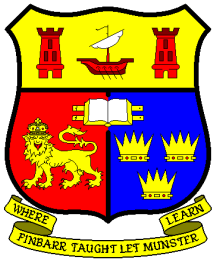

Evaluation of microbial adjuncts and their effect on the ripening of Cheddar cheese

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Table of contents

RISTAGNO, D., HANNON, J. A., BERESFORD, T. P. & MCSWEENEY, P. L. H. 2012. Effect of a bacteriocin-producing strain of *Lactobacillus paracasei* on the nonstarter microflora of Cheddar cheese. International Journal of Dairy Technology, 65, 523-530.

Abstract

A bacteriocin-producing strain of *Lactobacillus paracasei* DPC 4715 was used as an adjunct culture in Cheddar cheese in order to control the growth of "wild" non-starter lactic acid bacteria. No suppression of growth of the indicator strain was observed in the experimental cheese. The bacteriocin produced by *Lactobacillus paracasei* DPC 4715 was sensitive to chymosin and cathepsin D and it may have been cleaved by the rennet used for the cheese manufactured or by indigenous milk proteases.

A series of studies were performed using various microbial adjuncts to influence cheese ripening. *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 were added to the cheesemilk at level of 10^9 cfu/ml resulting in a final concentration of 10^8 cfu/g in Cheddar cheese. The strains significantly increased the level of pH 4.6-soluble nitrogen, total free amino acids after 60 and 180 d of ripening and some individual free amino acids after 180 d.

Yarrowia lipolytica DPC 6266, *Yarrowia lipolytica* DPC 6268 and *Candida intermedia* DPC 6271 were used to accelerate the ripening of Cheddar cheese. Strains were grown in YG broth to a final concentration of 10^7 cfu/ml, microfluidized, freeze-dried and added to the curd during salting at level of 2% w/w. The yeasts positively affected the primary, secondary proteolysis and lipolysis of cheeses and had aminopeptidase, dipeptidase, esterase and 5' phosphodiestere activities that contributed to accelerate the ripening and improve the flavor of cheese.

Hafia alvei was added to Cheddar cheesemilk at levels of 10^7 cfu/ml and 10^8 cfu/ml and its contribution during ripening was evaluated. The strain significantly increased the level of pH 4.6-soluble nitrogen, total free amino-acids, and some individual free amino-acids of Cheddar cheese, whereas no differences in the urea-polyacrylamide gel electrophoresis (urea-PAGE) electrophoretograms of the cheeses were detected. *Hafia alvei* also significantly increased the level of some biogenic amines.

A low-fat Cheddar cheese was made with *Bifidobacterium animalis* subsp. *lactis*, strain BB-12® at level of 10^8 cfu/ml, as a probiotic adjunct culture and Hi-Maize® 260 (resistant high amylose maize starch) at level of 2% and 4% w/v, as a prebiotic fiber which also played the role of fat replacer. *Bifidobacterium* BB-12 decreased by 1 log cycle after 60 d of ripening and remained steady at level of ~10^7 cfu/g during ripening. The Young's modulus also increased proportionally with increasing levels of Hi-maize. Hencky strain at fracture decreased over ripening and increased with increasing in fat replacer.

A cheese based medium (CBM) was developed with the purpose of mimicking the cheese environment at an early ripening stage. The strains grown in CBM showed aminopeptidase activity against Gly-, Arg-, Pro- and Phe-*p*-nitroanalide, whereas, when grown in MRS they were active against all the substrates tested. Both *Lb. danicus* strains grown in MRS and in CBM had aminotransferase activity towards aromatic amino acids (Phe and Trp) and also branched-chain amino acids (Leu and Val). Esterase activity was expressed against *p*-nitrophenyl-acetate (C2), *p*-nitrophenyl-butyrate (C4) and *p*-nitrophenyl-palmitate (C16) and was significantly higher in CBM than in MRS.

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1. Literature review: Acceleration of cheese ripening

1.1. Introduction to cheese microbiota

"Cheese is the generic name for a group of fermented milk-based food products produced throughout the world in a great diversity of flavours, textures, and forms" (Fox et al., 2000). The origin of cheesemaking dates back to about 8000 years ago and there are now more than 1000 cheese varieties worldwide (Fox et al., 2000). Cheese manufacture was originally adopted to extend the shelf life of milk and involves an acid or enzymatic coagulation of milk proteins followed by gel formation, whey expulsion, acid production and salt addition and period of ripening (Beresford et al., 2001). Two groups of bacteria are involved in cheese ripening: primary and secondary microbiota. Primary microbiota includes starter lactic acid bacteria which are responsible of acid formation during manufacture and cheese ripening. Secondary microbiota includes non-starter lactic acid bacteria (NSLAB) which are mainly facultatively heterofermentative lactobacilli (Chamba and Irlinger, 2004).

Lactobacilli are Gram-positive, non-motile and catalase negative bacteria, traditionally divided in three groups: (I) obligatory homofermentative, (II) facultatively heterofermentative, or (III) obligatory heterofermentative. NSLAB found in cheese are mainly *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum* and *Lactobacillus curvatus* (group II), although also *Pediococcus* spp. and obligate heterofermentative *Lactobacillus* spp. (group III) such as *Lactobacillus brevis* and *Lactobacillus fermentum* are also found occasionally (Fox et al., 2000; Beresford et al., 2001). NSLAB are present at very low numbers at the beginning of ripening and reach high levels (~10^8 cfu/g) throughout the ripening process. NSLAB can easily grow in cheese since they are facultative anaerobes and are salt and acid tolerant (Fox et al., 2000). Many strains of NSLAB grow in cheese by using as substrates compounds such as the products released after the autolysis of starter cells amino acids, organic and fatty acids, glycerol or carbohydrates released from the glycomacropeptide of κ-casein (galactose, *N*-acetylgalactosamine, *N*-acetylneuraminic acid), from glycoproteins and glycolipids in the milk-fat globule membrane (galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid) or from lysed cells of starter bacteria (ribose, deoxyribose, *N*-acetylglucosamine, *N*-acetylmuramic acid) (Adamberg et al., 2003).

NSLAB come generally from raw milk and from the factory environment. They may survive pasteurization or contaminate the curd after manufacture and grow in cheese during ripening. NSLAB do not contribute to acid production but play an important role during cheese ripening by production of proteolytic and lipolytic enzymes (Chamba and Irlinger, 2004). Some strains of NSLAB are used as adjunct cultures in cheese manufacture in order to accelerated the ripening process since they may contribute to increased production of free amino acids, accelerate and improve cheese flavour formation, reduce harshness and bitterness (Beresford and Williams, 2004). In spite of this, their role during ripening is still unclear and their effect on cheese sometimes can be also negative as some NSLAB strains can cause defects such as the formation of white spots of calcium lactate crystals on the cheese surface due to the racemization of L-lactate to D/L-lactate, off-flavours and slits formation caused by gas production (Banks and Williams, 2004).

1.2. Acceleration of Cheddar cheese ripening

Some varieties of hard and semi-hard cheeses need a long ripening time (e.g., up to 2 or more years for Parmigiano Reggiano); hence it may be necessary to accelerate the ripening process in order to reduce the costs of storage and maintenance, investments in buildings and machinery, costs of energy and labour (Walstra et al., 1999). Accelerating the ripening of cheese consists of accelerating proteolysis and/or lipolysis while maintaining a satisfactory texture. Proteolysis is perhaps the most important reaction during cheese ripening; it is responsible for changes in texture and flavour enhancement since it leads to the liberation of substrates (amino acids) for the generation of sapid compounds (amines, acids, thiols, and thioesters) (Fox et al., 2000). Lipolysis is that process that releases free fatty acids, glycerol, monoacylglycerides, or diacylglycerides from triacylglycerides and is essential for the development of the typical flavour of cheese (Broome et al., 2011).

As summarized in **Table 1.1**, techniques used in order to accelerate the ripening of cheese are as follows:

- increased ripening temperature
- use of enzyme preparations
- attenuated starters
- genetically modified starters
- enzyme-modified cheeses
- adjunct cultures

Table 1.1. Principal methods used to accelerate cheese ripening (Fox et al., 2000).

Method	*Advantages*	*Disadvantages*
Elevated temperature	No legal barriers; technically simple; no cost (perhaps saving)	Nonspecific action; increased risk of spoilage
Exogenous enzymes	Low cost; specific action, choice of flavour option	Limited choice of useful enzymes; possible legal barriers; difficult to ensure uniform incorporation of enzymes; risk of over ripening
Modified starter cells	Easy to incorporate; natural enzyme balance retained	Technically complex; rather expensive
Genetically engineered starters	Easy to incorporate; choice of options	Possible legal barriers; may experience consumer resistance
Cheese slurries and enzyme-modified cheese	Very rapid flavour development	High risk of microbial spoilage; final product requires processing

1.2.1. Increased ripening temperature

Increasing the ripening temperature is the simplest method for accelerating the maturation of cheeses. The disadvantages of this approach are a higher risk of microbial spoilage and non-specific reactions which could start and lead to the formation of undesirable off-flavours (Folkertsma et al., 1996). Proteolysis was accelerated in buffalo milk Cheddar cheese ripened at 12-13°C (Upadhyay et al., 1985), in Cheddar cheese ripened at 15, 17.5 and 20°C, but off-flavours developed after 32 weeks at 17.5°C and 16 weeks at 20°C (Aston et al., 1985).

In a study by Cromie et al. (1987), total bacteria, lactic acid bacteria, lactobacilli and 'undesirable lactobacilli' (producing off flavours and CO_2) and streptococcal counts were higher in Cheddar cheese stored at 15, 17.5 and 20°C than the control cheese ripened at 8°C whereas the total number of NSLAB was unchanged in cheese stored at elevated temperatures. No relationship was found between the presence of bacterial groups and off-flavours developed in cheeses stored at 17.5°C for 32 weeks and 20°C for 16 weeks.

In a study conducted by Folkertsma et al. (1996), Cheddar cheese was ripened at 8, 12 and 16°C. Cheeses ripened at 16°C showed a greater degradation of α_{s1}- and β-casein than the cheeses ripened at lower temperature and the level of water-soluble nitrogen was higher in cheeses ripened at 12 and 16°C. The highest mean flavour scores were received by the cheese ripened at 16°C after 3 months of ripening, but on extended ripening, the cheese developed lower quality and deteriorated in texture.

Sihufe et al. (2010) reported that in a traditional Argentinean hard cheese ripened at elevated temperature (18°C), the degradation of α_{s1}- and β-casein and the content of amino acids was higher than the cheese ripened at 12°C. Moreover, after 6 months of ripening significantly higher level of some fatty acids were observed in cheese ripened at 18°C.

Pachlova et al. (2012) showed that increasing the temperature up to 16°C could reduce the ripening time of Dutch type cheese to approximately one half compared to the period of maturation at standard temperature (10 °C); the cheese ripened at higher temperature had higher levels of free amino acids and greater hardness than the control cheese. However, it also developed higher levels of biogenic amines, such as tyramine, putrescine and cadaverine, which represents a risk for consumers.
These studies showed that raising the ripening temperature is an efficient method to accelerate the ripening of hard cheese, but disadvantages, such as development of an undesirable microbiota, formation of off-flavours and deterioration of texture must be taken into account. Cheddar cheese is normally ripened at 6-8 °C. When the ripening temperature is above 20°C, the cheese becomes soft and deforms easily because of fat exudation and moisture evaporation which occur especially in cheeses that are not film wrapped (Fox et al., 2000).

1.2.2. Use of enzyme preparations

Enzymes are directly responsible for most of the changes that occur during ripening. Therefore the addition of exogenous plasmin, chymosin, cell wall and/or intracellular proteinases and peptidases from LAB and NSLAB, fungal lipases and proteases to cheese during manufacture could accelerate the ripening process (Fox et al., 2000). The enzyme preparations can be added to the cheese milk resulting in a better distribution in the cheese, but most of the enzyme is usually lost in the whey; on the other hand, in Cheddar cheese enzymes can be added to the curd during salting or to the cheese milk after encapsulation in liposomes or fat globules (Walstra et al., 1999).

In a study by Ezzat (1990), intracellular cell-free enzyme extracts of *Lactobacillus delbrueckii* subsp *bulgaricus*, *Propionibacterium freudenreichii* and *Brevibacterium linens* were used to accelerate Ras cheese ripening in the presence of a commercial proteinase (Neutrase). The cheeses made with cell-free extracts had higher level of free fatty acids than the control cheese; enzyme extract from *Lb. delbrueckii* subsp *bulgaricus* showed the highest activity. No significant differences were found in the level of soluble nitrogen between experimental and control cheeses. After one month of ripening, cheeses treated with either Neutrase or cell-free extracts had significantly more intense and typical Ras cheese flavour than had the control cheese.

In another study by Hayashi et al. (1990a), the authors used a partially purified extracellular aminopeptidase from *B. linens* in combination with a commercial proteinase to accelerate Cheddar cheese ripening. According to the previous study, TCA-soluble and sulfosalicylic acid-soluble nitrogen were significantly higher and a stronger degradation of β-CN was detected in treated cheeses than control cheese. Furthermore, the treated cheese had a stronger flavour intensity than control cheese and no significant bitterness was detected. In a following study conducted by the same authors (Hayashi et al. 1990b), Cheddar cheese was made with partially purified extracellular proteinases produced by *B. linens*. After 2 months of ripening, the TCA-soluble nitrogen was higher and β-CN was degraded extensively in the treated cheeses than the control cheese.

Picon et al. (1995) showed that lipolysis in Manchego cheese increased in the presence of Palatase 200 from *Rhizomucor miehei* and Palatase 750L from *Aspergillus niger* whereas decreased in the presence of a fungal protease from *Aspergillus oryzae*. When the three enzymes were used in combination, soluble nitrogen and some amino acids increased and β-caseins showed a higher breakdown than α_s-caseins.

Kheadr et al. (2003) added liposome-entrapped Flavourzyme, neutral bacterial protease, acid fungal protease and lipase individually to cheese milk prior to renneting and reported that experimental cheeses treated with encapsulated enzymes had higher rates of proteolysis than the control cheese.

Kailasapathy and Lam (2005) encapsulated proteases enzymes (Flavourzyme) and added them in milk during manufacture of Cheddar cheese and evaluated their impact on ripening acceleration. The experimental cheeses treated with encapsulated

enzymes showed higher rates of proteolysis than the control cheese throughout the ripening period. Reduction of gumminess, hardness and chewiness was reported in experimental cheeses probably due to the proteolytic activity of the enzymes on casein. Similar results were also reported in other studies where different enzymes such as the esterase of *Micrococcus* sp. INIA 528 were added (Morales et al., 2005).

The effect of recombinant aminopeptidase (PepN) from *Lactobacillus rhamnosus* S93, in free or encapsulated form, on acceleration of Cheddar cheese ripening was investigated by Azarnia et al. (2010a). After 2 months of ripening, the experimental cheeses showed levels of phosphotungstic acid soluble nitrogen (PTA-N) and total free amino acids (FAA) similar to those found in control cheese after 6 months, suggesting that the addition of this recombinant aminopeptidase had a positive effect on acceleration of cheese ripening. Moreover, the experimental cheeses also received higher scores for sensory properties than the control cheese.

The use of enzyme preparations in cheese have shown to shorten the duration of cheese ripening but their use is not widespread because of the poor availability of approved commercial enzymes, difficulties in mixing homogenously the enzymes into the cheese matrix, waste of enzymes in the whey and whey contamination. The use of enzyme preparation might be a useful mean to overcome these problems; however, the cost of encapsulation is high and the efficiency of the process is still low (less than 50%) (Azarnia et al., 2006).

1.2.3. Attenuated starter bacteria

Starter bacteria are mainly responsible for the formation of small peptides, amino acids and for flavour development in cheese. Increasing the number of starter cells might represent a tool to accelerate the ripening of cheese, but in some cases (e.g. Cheddar cheese) high numbers of starter cells have been associated with bitterness (Fox et al., 2000). As an alternative, starter cells might be attenuated so that they lose their acid producing ability and their intracellular enzymes can be liberated and become involved in cheese ripening (Walstra et al., 1999; Fox et al., 2000).

There are different methods of attenuating cells: heating, freezing, spray-drying, freeze-drying, permeabilisation using lysozyme or solvents, or by the selection of lactose-negative mutants (Klein and Lortal, 1999). Attenuation by heating consists in growing up the culture in milk in order to achieve high cell densities and thus higher proteolytic and peptidase activities. The cells are then heat-treated directly in the milk or resuspended in hot milk at a selected temperature (60-70°C) for 10-15 s and then cooled. When cells are attenuated by freezing, they are usually freeze-shocked at ~ -20°C and then rapidly thawed at 40°C prior to addition to milk. Ice crystals which form inside the cells, cause damage to the cell membrane, therefore some enzymes might be lost to the suspending media. The use of spray-dried or freeze-dried cultures has a great impact on storage and shipping cost since they are much more practical and occupy less volume than frozen cultures.

Attenuation using lysozyme consists of treating a cell suspension with this enzyme. Lysozyme is a widely distributed enzyme that hydrolyzes the cell wall of certain bacteria. A limitation of this approach is the high cost of the enzyme. Other

substances used to attenuated cells are solvents such as *n*-butanol, which alters the lipid structure of the cell membrane making the cell unable to produce lactic acid, but the use of solvent-treated cells in cheesemaking may be difficult because of cost, health hazards, and legal barriers (Fox et al., 2000).

The use of lactose negative (Lac^-) starters might be a useful method to accelerate cheese ripening since they have lost their ability to grow in milk and therefore do not interfere with the rate of acid production. Lac^- cells occur naturally and are easily isolated since the Lac gene is encoded on a plasmid which is easily lost. Anyway, those cells still retain their enzymes and can therefore positively affect the ripening process (Klein and Lortal, 1999; Fox et al., 2000).

Attenuated starter cultures have been used to accelerate the ripening process in many cheese varieties. Johnson et al. (1995) made reduced fat Cheddar cheese with *Lb. helveticus* CNRZ-32 adjuncts attenuated by spray-drying at low (82°C) and high (120°C) outlet air temperature, freeze drying, or freezing. Ripening index (Phosphotungstic acid, PTA- and trichloroacetic acid, TCA-soluble N) were higher in cheeses made with lactobacilli spray-dried at low outlet air temperature, freeze-dried or frozen, whereas proteolysis in cheese made with lactobacilli spray-dried at high outlet air temperature was similar to the control cheese made without added lactobacilli. The flavour intensity of cheeses was enhanced in all cheeses containing the attenuated culture, but cheese made with spray-dried culture at high outlet air temperature had the least off-flavour intensity.

Kebary et al. (1996) studied the effect of freeze-shocked or heat-shocked *Lb. casei* NIH 334 or *Lb. helveticus* CNRZ 53 on the ripening of Ras cheese. Cheeses made with attenuated lactobacilli had higher ripening index (soluble nitrogen, soluble tyrosine, soluble tryptophan and total volatile fatty acids) and cheese scores than cheeses made without lactobacilli. Between the two methods of attenuation, the freeze-shocking seemed to be the best one, since cheeses made with either freeze-shocked *Lb. casei* or *Lb. helveticus* had a higher yield, ripening index and organoleptic scores than cheeses made with heat-shocked lactobacilli. Madkor et al. (2000) made Cheddar cheese with *Lb. helveticus* I or *Lb. casei* T that were freeze shocked, heat shocked or spray dried and reported only minor differences in the primary proteolysis between control cheeses and cheeses made with the attenuated cultures. However, cheeses made with freeze shocked or heat shocked *Lb. helveticus* adjuncts had significantly higher levels of free amino groups formation and both adjunct-treated cheeses had a higher level of free fatty acids. In a study by Di Cagno et al. (2012), *Lb. plantarum* CC3M8, *Lb. paracasei* CC3M35, and *Lb. casei* LC01, were used as viable adjunct cultures or adjunct cultures attenuated by sonication for the manufacture of Caciocavallo Pugliese cheese. The authors showed that cheese made with attenuated adjunct cultures had the highest concentration of free amino acids and aminopeptidase type N, iminopeptidase, endopeptidase type O, glutamate dehydrogenase and cystathionine lyase activities.

The use of attenuated cells to enhance the cheese ripening, however, is not widespread because the extent of attenuation is often strain dependent. Each attenuation method has advantages and limits; costs for attenuation are high. Moreover, further studies are needed to clarify the minimum cell concentration

needed to have a positive effect on cheese ripening and which is their impact on the rest of cheese microbiota (e.g. NSALB) (Klein and Lortal, 1999).

1.2.4. Genetically modified starters

Genetic engineering techniques have been used to control the activity of lactic acid bacteria, such as acid production, protein utilization, bacteriophage resistance, extracellular polysaccharide synthesis and flavour formation, in order to accelerate the ripening process of cheese (El Soda and Awad, 2011). These techniques can be divided into four categories: (i) food grade microorganisms genetically modified to overproduce recombinant enzymes such as peptidases, esterases, and amino acid catabolic enzymes, etc.; (ii) genetically engineered lactic starters producing selected peptidases, esterases and catabolic enzymes from *Lactococcus lactis* via a food grade cloning system; (iii) cloning of autolysin (hydrolase) into lactic starters; and (iv) metabolic engineering of lactic starters (Azarnia et al., 2006).

Lc. lactis is a good candidate for the intra- or extra-cellular production of numerous proteins of viral, bacterial or eukaryotic origins (Le Loir et al., 2005). Some authors have used genetic engineering techniques in order to express the natural proteinase produced by *Bacillus subtilis* in *Lc. lactis* (Vandeguchte et al., 1990; McGarry et al., 1994). Vandeguchte et al. (1990) cloned the *B. subtilis nprE* gene, encoding a neutral protease NprE, into *Lc. lactis* and showed that the strain was able to secrete the enzyme in an active form. The authors concluded that the modified strain of *Lc. lactis* could be used for the production of cheese since previous reports demonstrated that Npr protease can be helpful in the acceleration of cheese ripening (Law and Wigmore, 1983; Ardo and Pettersson, 1988).

McGarry et al. (1994) made Cheddar cheeses using a number of derivatives of *Lc. lactis* subsp. *lactis* UC317 in which the proteolytic system had been modified. Among this derivates, strains AM312 and AC322 harbored recombinant plasmid pMG36enpr, which contains the neutral proteinase gene from *B. subtilis*. Cheddar cheese made with these strains showed the highest level of water soluble nitrogen (WSN) and, after 6 months of ripening, the graders estimated the ages of cheeses to be more than one year, indicating that the ripening was accelerated by the proteinase activity. However, scores for body and texture were very low although the flavour was considered to be satisfactory.

Beside *B. subtilis* neutral protease, also *Lb. helveticus* peptidase has been expressed in *Lc. lactis* in order to develop novel starter strains with improved proteolytic properties (Christensen et al., 1995; Luoma et al., 2001; Joutsjoki et al., 2002; Courtin et al., 2002). In a study by Christensen et al. (1995), a recombinant *Lc. lactis* starter strain, producing the PepN of *Lb. helveticus* CNRZ32 increased the aminopeptidase activity in Cheddar cheese which resulted in a general increase in proteolysis (as determined by 10% trichloroacetic acid- and 5% phosphotungstic acid-soluble nitrogen) and free amino acids. Courtin et al. (2002) used *Lc. lactis* strains expressing two peptidases from *Lb. helveticus* (PepN and PepX) and four peptidases from *Lactobacillus delbrueckii* subsp. *lactis* (PepI, PepQ, PepG and PepW) to ripen a cheese model system. The author showed that the total amount of free amino acids increased up to 3-fold when the proline specific peptidases PepX

and PepQ or endopeptidase PepW of lactobacilli were produced in cheese. Joutsjoki et al. (2002), successfully transferred the genes encoding peptidases PepN, PepC, PepX and PepI of *Lb. helveticus* into *Lc. lactis.* When the peptidase activity was determined under cheesemaking conditions (pH 5.2, addition of 4% NaCl and 125 mmol/l $CaCl_2$ to the reaction mixture), a significant inhibition of the enzyme activity was detected compared to the activity obtained after the strain was grown under optimal conditions for each peptidase enzyme (purine-free medium or citrate-buffered milk). In particular, PepC activity was not detected, PepN and PepX activities were significantly inhibited, whereas PepI activity appeared to be stimulated by salt and was relatively high compared to PepN or PepX activities.

The use of genetically engineered starters has shown to be effective for the acceleration of cheese ripening. However, their use might raise concerns on safety and negatively affect the consumer and industry acceptance.

1.2.5. Enzyme-modified cheeses

Enzyme-modified cheeses (EMCs) may be defined as "concentrated cheese flavours produced enzymatically from cheeses of various ages and also from blends of casein, whey powder, skim milk powder and butterfat" (Wilkinson et al., 2011). EMCs are used as a cheese flavour ingredient in various food products, such as processed cheese, cheese analogues, cheese sauces, cheese dips, and products incorporating cheese, such as crackers and crisps (Fox et al., 2000; Wilkinson et al., 2011). The principle of producing EMCs is the addition of exogenous enzymes (proteinases, peptidases, lipases, and perhaps bacterial cultures) to cheese curd in order to generate intense cheese flavours rapidly. The advantages in using EMCs are a high degree of flavour intensity, wide range of flavours, reduced production costs, and extended shelf life (Wilkinson et al., 2011). The steps for the production of EMCs involve the homogenization of fresh curd or young cheese, followed by pasteurization (66-72°C, 4-6 min), cooling (e.g. 40°C) and addition of enzymes. The mixture is then left to incubate for a certain period of time, depending on the activity of the enzymes added, re-pasteurized to inactivate any residual microorganisms and enzymatic activity and then spray-dried or commercialized as a paste (Fox et al., 2000).

The advantages of EMCs are a high degree of flavour intensity, availability of a diverse range of flavours, reduced production costs, and extended shelf life (Wilkinson et al., 2011). Their flavour intensity is about 15- to 30-fold greater than that of natural cheese (Hulin-Bertaud et al., 2000; Azarnia et al., 2010b).

As reported by Hulin-Bertaud et al. (2000), the odor and flavour characteristics of some commercial EMCs were significantly different from those defined for natural Cheddar cheeses. EMCs were characterized by "vomit," "bitter," "astringent," "chemical," and "eggy/sulphur" flavours, whereas the natural Cheddar cheeses were characterized by "sweet," "caramel," "creamy," "nutty," and "buttery" flavours. Some of the negative attributes of EMCs were strongly correlated to a high fat content and low pH. Higher lipolysis was detected in EMCs than in natural Cheddar cheeses, in particular high levels of butyrate were found in some EMCs (Kilcawley et al., 2001).

Studies conducted on cheese have showed the positive impact that EMCs have on acceleration of ripening. In a study conducted by Hannon et al. (2006), fast-ripened Cheddar cheeses for ingredient purposes were produced by the addition of 0.25 and 1% w/w of a dried EMC at salting. The authors reported that cheese made with EMC had higher numbers of NSLAB and higher level of individual and free amino acids than the control cheese and a strong flavour developed up to 4 months of ripening. However, atypical Cheddar flavours developed when the cheeses were stored for a longer time.

Azarnia et al. (2010a) studied the effect of a natural crude enzyme and a recombinant aminopeptidase, both derived from *Lactobacillus rhamnosus* S93 in the presence of a commercial proteinase (Neutrase) on the proteolysis of enzyme-modified Cheddar cheese. Natural enzyme or recombinant aminopeptidase were added alone or in combination to the cheese slurries. No significant differences in the level of water-soluble nitrogen were observed between the samples, whereas higher levels of phosphotungstic acid-soluble nitrogen were detected in the cheeses treated with the enzymes from *Lb. rhamnosus* S93 than the control. Cheese with natural enzyme from *Lb. rhamnosus* S93 showed the highest levels of total free amino acids (FAA), the concentration of each FAA increased during maturation except for proline and arginine which during maturation period in all EMCs.

1.2.6. Adjunct cultures

Adjunct cultures may be defined as those cultures used for the manufacture of cheese in order to develop and control its flavour, colour, and texture (Rattray and Eppert, 2011). Adjunct cultures usually consist of a variety of species of bacteria (non-starter lactic acid bacteria, *Brevibacterium linens*, *Propionibacterium* spp.), yeasts (e.g., *Kluyveromyces*, *Debaryomyces*, *Candida* and *Saccharomyces*), moulds (e.g., *Geotrichum candidum*, *Penicillium camemberti*, *Penicillium roqueforti)*, and their contribution is indispensable for the development of the typical flavour of cheeses such as Roquefort, Emmental, Camembert, and Limburger (El Soda et al., 2000).

1.2.6.1. Non–Starter Lactic Acid Bacteria (NSLAB) adjuncts in Cheddar cheese

In some studies, lactobacilli such as *Lactobacillus casei*, *Lactobacillus paracasei, Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus curvatus, Lactobacillus rhamnosus* have been added to cheese in order to evaluate their impact on ripening and/or aroma development (Puchades et al., 1989; Drake et al., 1996; Lane and Fox, 1996; Thage et al., 2005; Phillips et al., 2006; Ong et al., 2007; Milesi et al., 2008). *Lactobacillus* adjunct cultures that grow in cheese during ripening produce desirable flavour attributes and suppress the growth of "wild" NSLAB (Broadbent et al., 2003). The properties and the effects of heterofermentative lactobacilli on cheese quality and sensory are strongly strain-dependant, for this reason, the selection of an appropriate adjunct culture is very important to improve the overall quality of cheese (El Soda et al., 2000; Katsiari et al., 2002). Generally, the selection of adjunct cultures should be based on their ability to produce flavour compounds, on their enzyme profiles and autolytic properties (El Soda et al., 2000).

Many studies have been conducted on the use of adjunct cultures in Cheddar cheese. An increase in the levels of free amino acids in Cheddar cheese made with added lactobacilli was also reported by Puchades et al. (1989) and Lynch et al. (1999). In a study by McSweeney et al. (1994), *Lb. casei* ssp. *pseudoplantarum*, *Lb. casei* ssp. *casei*, *Lb. plantarum* and *Lb. curvatus* were used as adjunct cultures in Cheddar cheese and their effect on cheese quality was evaluated. The authors showed that the cheeses made with adjunct lactobacilli received higher scores for flavour intensity and acceptability than the control cheese. No significant differences were found in the urea-polyacrylamide gel electrophoresis (urea-PAGE) electrophoretograms and levels of water-soluble nitrogen between cheeses, but the concentration of free amino acids was greater in experimental cheeses than in the control cheese, suggesting that lactobacilli contribute to proteolysis primarily by producing free amino acids.

Drake et al. (1996) reported that reduced-fat Cheddar cheese made with *Lb. helveticus* WSU19 as adjunct culture had higher level of proteolysis and exhibited greater oaky/nutty flavour and less bitterness than the reduced- and full-fat controls made without adjuncts. Reduced-fat Cheddar cheese made with *Lb. helveticus* WSU19 also received significantly higher consumer acceptance scores than reduced- and full-fat controls, after 6 months of aging.

In a study conducted by Lynch et al. (1996), the authors made Cheddar cheese under controlled microbiological conditions to study the effect of adjunct *Lb. casei* ssp. *casei*, *Lb. casei* ssp. *pseudoplantarum*, *Lb. curvatus* or *Lb. plantarum.* The use of added lactobacilli positively affected flavour acceptability of the cheese after ripening for 6 months at 7°C; in particular cheeses made with *Lb. plantarum* and *Lb. casei* ssp. *pseudoplantarum* adjuncts received the best grades, whereas slight bitterness was detected in the control cheeses. No differences in primary proteolysis were detected between levels of water-soluble nitrogen (WSN) of and urea-PAGE electrophoretograms of control and experimental cheeses during ripening. However, after 6 months, cheeses made with *Lb. plantarum* and *Lb. casei* ssp. *pseudoplantarum* and *Lb. curvatus* contained higher levels of free amino acid than control cheese and cheese made with *Lb. plantarum*.

In a further study, Drake et al. (1997) showed that primary proteolysis and consumer acceptance scores were higher in Cheddar cheese made with *Lb. helveticus* as adjunct than the control cheese. Similar results were also reported by Crow et al. (2001), who showed that strains of *Lb.paracasei* and *Lb.rhamnosus* significantly improved the flavour of Cheddar cheeses. Swearingen et al. (2001) studied the effect of two strains of *Lactobacillus* on Cheddar cheese ripening. The addition of these strains enhanced total free amino acids production in Cheddar cheese and the highest effect was found in cheese containing a blend of both lactobacilli strains. The use of *Lactobacillus* as adjunct culture also positively contribute to flavour development in Cheddar cheese.

Some lactobacilli are also recognized as probiotics and have been used for the production of cheeses. Ong et al. (2006) showed that after 4 months of ripening, Cheddar cheese made with probiotic cultures of *Lb. acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* spp. had higher level of free amino acids and hydrolysis of α_s-casein was significantly faster compared with the control cheese. In another study, the same authors showed that these probiotic cultures also increased

the level of water-, TCA- and PTA-soluble peptides in the probiotic Cheddar cheeses (Ong et al., 2007). Milesi et al. (2008) reported that *Lb. plantarum* I91 accelerated the ripening of Cheddar cheese modifying its peptide profile and increasing the level of some individual amino acids as well the total concentration of free amino acids.

1.2.6.2. Coryneform bacteria

Coryneform bacteria are asporogenous, aerobic Gram-positive, catalase-positive irregular rods or cocci, belonging to the class *Actinobacteria* and include organisms from the genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium* and *Microbacterium* (Denis and Irlinger, 2008). Coryneform bacteria of dairy interest are mainly found on the surface of smear-ripened cheeses such as Tilsit, Brick, Limburger, Romadur, Chaumes and Gubben (Brennan et al., 2004; Bockelmann et al., 2005).

These secondary cultures naturally grow on the surface of cheeses when they are exposed to air with a high relative humidity (> 95%) and contribute to the appearance and aroma development of the cheese. The sources of these bacteria are cheese milk, cheese brines, the air of ripening rooms, ripening shelves, and human skin (Bockelmann et al., 2005). Traditionally, the surface of old smear-ripened cheeses is washed with a brine solution which now contains part of the surface microbiota and is then used to inoculate the surface of young cheeses (Beresford et al., 2001). *Brevibacterum linens* is commonly used in cheese production since it contributes to proteolysis and lipolysis during ripening of cheese by producing protease, peptidase and lipolytic enzymes (Motta and Brandelli, 2008). *B. linens* is also a good producer of sulfur-containing compounds such as hydrogen sulfide, mercaptans, disulfides, hydrogen sulfide and methanethiol which enhance cheese flavour intensity (Rattray and Fox, 1999).

In a study by Oksuz et al. (2001), the inoculation of *B. linens* into the cheese milk or onto the surface of Kashar cheese significantly increased water soluble nitrogen and titratable acidity, thanks to its high proteolytic and lipolytic activity. Same results were also reported by Leclercq-Perlat et al. (2004) in a study conducted on Camembert-type made from pasteurized milk inoculated with *B. linens* at level of $8.1x10^3$ cfu/ml. The addition of *B. linens* to cheese has a very positive effect in reducing the ripening time thanks to its extracellular, cell-wall associated, intracellular proteinases as well as lipolytic and esterolytic enzymes. More research is needed to clarify the role of coryneform bacteria during ripening of cheeses. So far, the few studies conducted on *B. linens* have showed that this bacterium is able to accelerate the ripening of cheese by increasing the rate of proteolysis.

1.2.6.3. Enterobacteria

Enterobacteira belong to the family of *Enterobacteriaceae* and are Gram-negative, mostly catalase-positive straight rods able to grow in the presence and absence of oxygen and include organism from the genera *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Hafnia*, *Proteus*, *Salmonella* and *Yersinia.* Many Enterobacteriaceae are pathogenic for humans, causing a wide variety of diseases such as diarrhea, septicemia, respiratory disease; wound and burn infections, urinary tract infections and meningitis (Brenner and Farmer, 2005).

Enterobacteriaceae have been isolated from cheeses such as Valdeteja cheese (Alonso-Calleja et al., 2002), French cheeses (Coton et al., 2012), Spanish cheeses (Ortigosa et al., 2008), Turkish cheese (Aytac and Ozbas, 1992; Gonc et al., 2007). The contribution of Gram-negative bacteria in cheese ripening is still unclear. Usually, most Gram-negative bacteria in cheese are considered as spoilage flora that can be responsible for the formation of cheese texture and flavour defects (Delbes-Paus et al., 2012).

Morales et al. (2003) studied the effect of *Hafnia*, *Serratia*, *Enterobacter* and *Escherichia* on primary proteolysis of cheese. The strains showed to have a strong proteolytic activity on casein, in particular cheeses made with *Serratia* strains had a more extensive proteolysis. Some species such as *Proteus vulgaris* were found to affect positively the organoleptic properties of a model cheese by producing significant amounts of volatile compounds such as sulfur compounds (dimethyl disulphide), aldehydes (3-methylbutanal), alcohols (3-methylbutanol and 2-methylbutanol), esters (acetic acid 3-methyl butyl ester) and ketones (2-propanone) (Deetae et al., 2009a). In a further study by the same authors (Deetae et al., 2009b), *Proteus vulgaris* was used for the manufacture of Camembert-type cheese and showed a high proteolytic activity on casein and aminopeptidase activity on Met-, Leu- and Phe-*p*-nitroaniline (*p*NA) substrates. The strains also produced high amounts of volatile sulphur compounds, responsible for cooked cabbage and garlic notes, branched chain aldehydes and their corresponding alcohols which are associated with fruity taste. *Citrobacter freundii*, *Klebsiella oxytoca*, *Hafnia alvei*, *Pantoea agglomerans*, *Morganella morganii* and *Proteus vulgaris*, were used as adjunct culture in a study conducted by Delbès-Paus et al. (2012) on an uncooked pressed cheese. The strains did not produce significant amounts of volatile sulphur compounds and no effects on the colour or odour of the cheeses were detected; however, the strains slightly affected the texture since some assay cheeses was considered firmer than the control. Only cheese made with *K. oxytoca* had higher amounts of leucine and ornithine than control cheese, whereas no significant differences in the levels of free amino acids were observed in the other experimental cheeses.

In the study of Irlinger et al. (2012), *H. alvei* was inoculated on the surface of a soft smear cheese at level of 10^2 and 10^6 cfu/g and its impact on aroma compounds production was evaluated. Ketones, sulfur compounds, alcohols, aldehydes and esters were detected in experimental cheeses. Volatile sulfur compounds such as methanethiol, dimethyl disulfide and dimethyl trisulfide were the most abundant.

The role of Enterobacteria in cheese ripening is still unclear. However, in most cases their use as adjunct culture in cheese increase the level of volatile compounds and therefore improve the overall flavour of cheese. They can also contribute to acceleration of cheese ripening through their proteolytic activity and they can increase the level of some free amino acids.

1.2.6.4. Propionibacteria

Propionibacteria (PAB) are Gram-positive, anaerobic to aerotolerant and generally catalase positive non-spore forming, non-motile rods, belonging to the family *Propionibacteriacea*. They are originally used as adjunct cultures for the production of Swiss cheese varieties, where they are responsible for the characteristic flavour and eye formation, and certain fermented foods. The use of PAB as starter cultures require a co-inoculum with lactic acid bacteria (LAB) since they have poor growth ability in milk (Poonam et al., 2012). PAB are also used as adjunct cultures to improve flavour in some cheese where the eye formation is not required (Thierry et al., 2011). The dairy propionibacteria include *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium jensenii* and *Propionibacterium thoenii* which are industrially important as cultures in hard-cheese ripening and recently also as protective bio-preservatives and probiotics (Meile et al., 2008).

In a study of Fernandez-Espla and Fox (1998), *Propionibacterium freudenreichii* subsp. *shermanii* was used as adjunct culture in Cheddar cheeses at level of 10^5, 10^6, and 10^7 cfu/mL cheesemilk. No significant differences were found in urea-PAGE electrophoretograms, levels of water-soluble N, and peptide profiles by RP-HPLC, whereas major differences were found in amino acid level between control and experimental cheeses. The content of free amino acids (FAA) increased with increasing in PAB numbers; on the other hand, hydrophobic peptides decreased with increasing in PAB numbers. When *P. freudenreichii* subsp. *shermanii* was inoculated at low and medium numbers, the cheeses received the best scores for flavour development and body, whereas when inoculated at high level, the cheeses were characterized by an acute sweet and nutty flavour.

Chamba and Perreard (2002) showed the strong contribution of five strains of *P. freudenreichii* to lipolysis of Emmental cheese made from microfiltered milk. After 50 d and 90 d of ripening, the levels of FFA in experimental cheeses made with PAB were higher than the control cheese and the content of propionic acid was about seven times more than in cheese made without PAB. In a study of Thierry et al. (2005a), two mixtures of commercial strains of *P. freudenreichii* were used as adjunct cultures for the manufacture of Raclette cheese. The presence of PAB did not significantly affect the total amount of free amino acids (FAA) in cheeses, whereas the concentrations of all free fatty acids (FFA) were 2-5 fold higher in the experimental cheeses than in the control cheese; in particular, the highest level was reported for butanoic and hexanoic acids. Moreover, the inclusion of PAB in the experimental cheeses did not result in the development of eyes, but did increase the levels of volatile compounds such as esters and ketones and positively modified the odour and flavour.

In a further study (Thierry et al., 2005b), the same authors showed that Swiss cheeses made with three strains of *P. freudenreichii* had higher concentrations of all carboxylic acids and some of the neutral compounds, including 2-methylbutanol, 2-methylbutanal, methyl propionate, ethyl propionate and 5-methyl-2-heptanone. The production of volatile compounds by the three PAB strains tested was strain-dependent. The concentrations of all FFA were 2 to 8 times higher in the experimental cheeses made with *P. freudenreichii* than in the control cheese, in

particular, PAB strains released greater amounts of medium-chain and long-chain saturated FFA (C14:0, C15:0, C16:0 and C18:0).

Dherbecourt et al. (2010) showed that Swiss-type cheeses made with *P. freudenreichii* as ripening culture had higher levels of total FFA than the control cheese made without an adjunct. The concentration of FFA increased strongly comparing to the control cheese and was concomitant with the growth of *P. freudenreichii* showing that lipolysis could results from the activity of extracellular esterase(s) rather than intracellular esterases released after cell lysis.

In the study of Rehn et al. (2011), Grevé cheese was made by using *P. freudenreichii* ssp. *shermanii* SMR1137 in combination with different DL-starters. The strains were responsible for eye formation and increase of acetate, propionate, total content of FAA, 2-propanol, ethyl propionate and ethyl octanate. No differences were observed in the breakdown of casein. These studies showed that PAB can be used as adjunct culture not only for the production of Swiss type cheeses but also for other varieties. They improve the flavour and accelerate the ripening process trough their proteolytic and lipolytic activity. However, in some cheeses the production of eyes is not desirable and is considered a defect, therefore PAB should not be used at high level, also in order to prevent the formation of any off-flavour.

1.2.6.5. Yeasts and Moulds

In general, yeasts are mainly found in smear cheeses such as Tilsit, Beaufort, Münster, Brie, Camembert and Taleggio (Chamba and Irlinger, 2004) and mainly belong to the genera *Kluyveromyces*, *Debaryomyces*, and *Saccharomyces* (Rattray and Eppert, 2011). Yeasts are good producers of caseinolytic, aminopeptidase, carboxypeptidase and lipase enzymes (Chamba and Irlinger, 2004). In some cheese varieties they are undesirable since they contribute to the fruity, bitter, rancid and yeasty off-flavours and gassy, open texture (Fleet et al., 1990). However, in some cases they are used as adjunct cultures in order to improve flavour, texture and to accelerate proteolysis by the production of proteolytic and lipolytic enzymes (Beresford and Williams, 2004). Moulds such as *Pencillium roqueforti* are generally found in Roquefort, Gorgonzola, Stilton and Danish blue cheeses, whereas *Pencillium camemberti* is found in Camembert and Brie. Other varieties of cheeses are characterized by the presence of wild moulds such as *Mucor*, *Cladosporium*, *Geotrichum*, *Epicoccum* and *Sporotrichum. G. candidum* has properties of both yeasts and moulds and nowadays is considered to be a yeast (Beresford et al., 2001).

Wyder et al. (1999) investigated the contribution of *Galactomyces geotrichum*, *Pichia jadinii*, *Y. lipolytica* and *D. hanseni* to Raclette cheeses ripening. *Gal. geotrichum* negatively affected the concentrations of free short-chain acids and *D. hansenii* led to a decrease of both *n*-butyric and *n*-caproic acid. In contrast *Y. lipolytica* increased the level of *n*-butyric acid in cheese. Only *P. jadinii*, *D. hansenii* and *Gal. geotrichum* led to an increase in smaller peptides and free amino acids, whereas *Y. lipolytica* did not affect the breakdown of proteins and peptides. In a study by Martin et al. (1999), three strains of *G. candidum* and three species of yeast (*K. lactis*, *D. hansenii* and *Y. lipolytica*) were inoculated in a model lactic curd industrially used to make mold-ripened cheese.

Yeasts contributed to the formation of fruity odors whereas *G. candidum* was responsible for a Münster odour, sulfury and cheesy notes. In a study by Gaborit et al. (2001), strains of *Penicillium candidum, G. candidum, Rhodosporidium infirmominiatum* and *D. hansenii* were used as surface ripening cultures in the manufacture of Sainte Maure- and Camembert-type cheeses made with goat milk. The authors indicated that the association of these strains led in some cases to an improvement of the sensorial quality of cheeses, in particular Sainte Maure-type cheeses made with *G. candidum* and a combination of *G. candidum* + *D. hansenii* showed the highest scores for goat odour and flavour intensities, but lowest lipolysis levels. On the other hand, low goat odour, flavour intensities and high lipolysis levels were found in Sainte Maure-type cheeses made with *P. candidum* alone or associated with *D. hansenii* + *R. infirmominiatum.* In Camembert-type cheeses, high lipolysis levels were reached in the presence of *G. candidum* + *P. candidum* whereas the association *G. candidum* + *P. candidum* + *D. hansenii* led to a more intense goat flavour.

Ferreira and Viljoen (2003) reported that a fruity flavour developed after 2 months of ripening in Cheddar cheese made with *D. hansenii* but a bitter taste was detected after 6 months. Similar results were reported for Cheddar cheese made with *Y. lipolytica* which after 4 months developed a strong Cheddar flavour. In a study by Bintsis and Robinson (2004), *D. hansenii* and *Y. lipolytica* were inoculated together with *Lb. paracasei* subsp. *paracasei* in the brine used for the manufacture of Feta-type cheese. The adjunct of *Y. lipolytica* resulted in the production of undesirable aroma compounds in cheeses and in a softer texture, whereas cheeses made with *D. hansenii* had a higher concentration of aroma compounds than the other cheeses.
In a study by Das et al. (2005), *Yarrowia lipolytica* and *Geotrichum candidum* were used as adjunct cultures in the manufacture of dry-salted, washed-curd cheese and were found to increase the concentration of total free fatty acids. Free unsaturated long-chain fatty acids (oleic and linoleic acids) and free conjugated linoleic acid isomers were higher in the cheeses with added yeast than in the control cheese. Experimental cheeses also contained higher concentrations of esters of butanoic (C4), hexanoic (C6) and octanoic (C8) acids.

In a study by De Wit et al. (2005), *Y. lipolytica* and *D. hanseni* were inoculated into Cheddar cheese milk at level of 10^9 cfu/ml. The strains positively affected either lipolysis and proteolysis of cheeses. Cheeses made with *Y. lipolytica* and a combination of both *Y. lipolytica* and *D. hanseni* developed higher amounts of free fatty acids than control cheese and cheese only made with *D. hanseni* and a faster proteolysis was indicated by urea-PAGE. No differences in the level of WSN as % TN were observed in all cheeses. Leucine, phenylalanine, glutamine and asparagine were the most abundant FAAs in Cheddar cheeses made with both yeasts. In particular, Cheddar cheese made with *Y. lipolytica* contained higher levels of alanine, proline and tyramine than control cheese, while Cheddar cheese made a combination of *Y. lipolytica* and *D. hansenii* had higher levels of all amino acids than control cheese, except for His and Trp.

Four strains of *Y. lipolytica* were used as adjunct culture in a study by Lanciotti et al. (2005) and had a good impact on proteolysis and lipolysis of Caciotta-type cheese. The authors also reported that all strains preferentially released large amounts of branched chain saturated or unsaturated fatty acids.

Boutrou et al. (2006) studied the effect of *G. candidum* as a ripening agent on the surface of Camembert-type cheese. Results from urea-PAGE and reverse-phase HPLC showed that *G. candidum* was able to increase primary proteolysis at the cheese surface. At the end of ripening, the concentration of FAAs, glutamic acid, glutamine, proline, alanine, lysine, histidine and arginine in cheese surface was three times higher in cheese made with *G. candidum* than in control cheese made without and adjunct, showing that the yeasts was also able to increase secondary proteolysis.

Kesenkas and Akbulut (2008) used *Y. lipolytica*, *Debaryomyces hansenii*, and *Kluyveromyces marxianus* as adjunct cultures for the production of Turkish white brined cheese and reported that the strains had no effect on the concentration of free fatty acids, but increased the level of WSN/TN in cheeses, with *Y. lipolytica* showing the highest value (22.00%), followed by *K. marxianus* (21.02%) and *D. hansenii* (19.56%). Results of the sensory evaluation showed that cheeses containing *Y. lipolytica* were considered more acceptable than the other cheeses. De Freitas et al. (2008) showed that the use of *K. lactis* and *Pichia fermentans* as adjunct cultures in Cantalet cheese did not influence free amino acid or free fatty contents. Cheese made with an adjunct of *K. lactis* contained higher level of ethanol, ethyl hexanoate, four aldehydes, and two branched-chain acids than control cheese and cheese made with *P. fermentans*.

Similar results were reported by the same authors in a following study (De Freitas et al., 2009) where the ability of *Kluyveromyces lactis, Y. lipolytica*, and *P. fermentans* to promote flavour development in Cantalet cheese was investigated. The authors showed that the yeasts did not affect the content of total free amino acids, but enhanced the formation of some volatile aroma compound such as ethanol, ethyl esters and some branched-chain alcohols. Lipolysis was higher than the control cheese only in the cheese made with *Y. lipolytica*.

In a study conducted by Sørensen et al. (2011) on a cheese-surface model, *Y. lipolytica* produced sulphides, furans and short-chain ketones; *Saccharomyces cerevisiae* produced esters and *D. hansenii* produced branched-chain aldehydes and alcohols. Studies carried out so far suggest that adjunct yeasts contribute positively to proteolysis, lipolysis, flavour and texture development of cheese. They increase the concentration of free fatty acids, free amino acids and volatile aroma compounds although some authors reported off-flavours developed after 4-6 months of ripening. On the other hand, some studies showed that yeasts did not influence parameters such as free amino acids or free fatty acids content and *Y. lipolytica* caused the production of undesirable aroma compounds and the development of a soft texture. Little knowledge is available on the effect of adjunct moulds on cheese ripening, other than their traditional use in Blue and Camembert-type cheeses. Further studies are needed to elucidate their role in the acceleration of cheese ripening.

1.3. Conclusions

Cheese ripening can range from about 3 weeks for fresh cheeses like Mozzarella up to two or more years for Parmesan and extra mature Cheddar. In the latter case, ripening represents a time-consuming and expensive process which should be accelerated without affecting the flavour and textural properties of cheese. Increasing the ripening temperature is the simplest method to accelerate the maturation of cheese. Cheeses ripened at higher temperature than normal have faster proteolysis but, in some cases, formation of biogenic amines, off-flavours and deterioration of texture may occur. The addition of enzyme preparation to the cheese milk might be another useful mean to accelerate cheese ripening. However, most of the enzyme is usually lost in the whey, for this reason they are usually encapsulated in liposomes or fat globules, although the process is expensive and the efficiency still low. Attenuated starter cells have been proposed to accelerate the cheese ripening thanks to their ability to increase proteolysis and lipolysis without affecting the rate of lactic acid production. Enzyme modified cheeses are used as cheese ingredients in order to accelerate cheese ripening and to generate intense flavours rapidly, but in some cases, their high fat content and low pH caused high lipolysis and therefore negatively affected the flavour of cheese. Another approach to accelerate cheese ripening and improve the flavour is adding viable adjunct cultures such as bacteria, yeasts and moulds to the cheese milk. Secondary adjunct cultures enhanced lipolyisis, proteolysis and cheese flavour formation, but the choice of strain and the numbers to add should be considered carefully.

1.4. References

ADAMBERG, K., KASK, S., LAHT, T. M. & PAALME, T. 2003. The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. *International Journal of Food Microbiology,* 85, 171-183.

ALONSO-CALLEJA, C., CARBALLO, J., CAPITA, R., BERNARDO, A. & GARCIA-LOPEZ, M. L. 2002. Changes in the microflora of Valdeteja raw goat's milk cheese throughout manufacturing and ripening. *Lebensmittel-Wissenschaft Und-Technologie,* 35, 222-232.

ARDO, Y. & PETTERSSON, H. E. 1988. Accelerated cheese ripening with heat-treated cells of *Lactobacillus helveticus* and a commercial proteolytic-enzyme. *Journal of Dairy Research,* 55, 239-245.

ASTON, J. W., GILES, J. E., DURWARD, I. G. & DULLEY, J. R. 1985. Effect of elevated ripening temperatures on proteolysis and flavour development in Cheddar cheese. *Journal of Dairy Research,* 52, 565-572.

AYTAC, S. A. & OZBAS, Z. Y. 1992. Isolation of *Yersinia enterocolitica* from Turkish pickled white cheese. *Australian Journal of Dairy Technology,* 47, 60-&.

AZARNIA, S., LEE, B. H., ST-GELAIS, D., CHAMPAGNE, C. P. & KILCAWLEY, K. N. 2010a. Effect of free or encapsulated recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 on acceleration of Cheddar cheese ripening. *Food Biotechnology,* 24, 135-149.

AZARNIA, S., LEE, B. H., YAYLAYAN, V. & KILCAWLEY, K. N. 2010b. Proteolysis development in enzyme-modified Cheddar cheese using natural and recombinant enzymes of *Lactobacillus rhamnosus* S93. *Food Chemistry,* 120, 174-178.

AZARNIA, S., ROBERT, N. & LEE, B. 2006. Biotechnological methods to accelerate Cheddar cheese ripening. *Critical Reviews in Biotechnology,* 26, 121-143.

BANKS, J. M. & WILLIAMS, A. G. 2004. The role of the nonstarter lactic acid bacteria in Cheddar cheese ripening. *International Journal of Dairy Technology,* 57, 145-152.

BERESFORD, T. & WILLIAMS, A. 2004. The microbiology of cheese ripening. *Cheese: chemistry, physics and microbiology*, Vol. 1. Edited by P. F. Fox, P L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 287-317.

BERESFORD, T. P., FITZSIMONS, N. A., BRENNAN, N. L. & COGAN, T. M. 2001. Recent advances in cheese microbiology. *International Dairy Journal,* 11, 259-274.

BINTSIS, T. & ROBINSON, R. K. 2004. A study of the effects of adjunct cultures on the aroma compounds of Feta-type cheese. *Food Chemistry,* 88, 435-441.

BOCKELMANN, W., WILLEMS, K. P., NEVE, H. & HELLER, K. H. 2005. Cultures for the ripening of smear cheeses. *International Dairy Journal,* 15, 719-732.

BOUTROU, R., KERRIOU, L. & GASSI, J. Y. 2006. Contribution of *Geotrichum candidum* to the proteolysis of soft cheese. *International Dairy Journal,* 16, 775-783.

BRENNAN, N. M., COGAN, T. M., LOESSNER, M. & SCHERER, S. 2004. Bacterial surface-ripened cheeses. C*heese: chemistry, physics and*

microbiology, Vol. 2. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 199-225.

BRENNER D. J. & FARMER J.J. III, 2005. Order XIII. "Enterobacteriales", *Bergey's manual of systematic bacteriology*, Vol. 2: the Proteobacteria, part B: the Gammaproteobacteria. Edited by D. J. Brenner, N. R. Krieg, James T. Staley, G. M. Garrity, 587-850.

BROADBENT, J. R., HOUCK, K., JOHNSON, M. E. & 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.

BROOME, M. C., POWELL, I. B. & LIMSOWTIN, G. K. Y. 2011. Starter Cultures: Specific Properties. *Encyclopedia of dairy sciences*, Second edition. Edited by J. W. Fuquay, P. F. Fox & P. L. H. McSweeney, Academic Press, London, 559-566.

CHAMBA, J. F. & IRLINGER, F. 2004. Secondary and adjunct cultures. C*heese: chemistry, physics and microbiology*, Vol. 1. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 191-206.

CHAMBA, J. F. & PERREARD, E. 2002. Contribution of propionic acid bacteria to lipolysis of Emmental cheese. *Lait,* 82, 33-44.

CHRISTENSEN, J. E., JOHNSON, M. E. & STEELE, J. L. 1995. Production of Cheddar cheese using a *Lactococcus lactis* ssp. *cremoris* SK11 derivative with enhanced aminopeptidase activity. *International Dairy Journal,* 5, 367-379.

COTON, M., DELBES-PAUS, C., IRLINGER, F., DESMASURES, N., LE FLECHE, A., STAHL, V., MONTEL, M.-C. & COTON, E. 2012. Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiology,* 29, 88-98.

COURTIN, P., NARDI, M., WEGMANN, U., JOUTSJOKI, V., OGIER, J. C., GRIPON, J. C., PALVA, A., HENRICH, B. & MONNET, V. 2002. Accelerating cheese proteolysis by enriching *Lactococcus lactis* proteolytic system with lactobacilli peptidases. *International Dairy Journal,* 12, 447-454.

CROMIE, S. J., GILES, J. E. & DULLEY, J. R. 1987. effect of elevated ripening temperatures on the microflora of Cheddar cheese. *Journal of Dairy Research,* 54, 69-76.

CROW, V., CURRY, B. & 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.

DAS, S., HOLLAND, R., CROW, V. L., BENNETT, R. J. & MANDERSON, G. J. 2005. Effect of yeast and bacterial adjuncts on the CLA content and flavour of a washed-curd, dry-salted cheese. *International Dairy Journal,* 15, 807-815.

DE FREITAS, I., PINON, N., BERDAGUÉ, J. L., TOURNAYRE, P., LORTAL, S. & THIERRY, A. 2008. *Kluyveromyces lactis* but not *Pichia fermentans* used as adjunct culture modifies the olfactory profiles of Cantalet cheese. *Journal of Dairy Science,* 91, 531-543.

DE FREITAS, I., PINON, N., MAUBOIS, J.-L., LORTAL, S. & THIERRY, A. 2009. The addition of a cocktail of yeast species to Cantalet cheese changes bacterial survival and enhances aroma compound formation. *International Journal of Food Microbiology,* 129, 37-42.

DE WIT, M., OSTHOFF, G., VILJOEN, B. C. & HUGO, A. 2005. A comparative study of lipolysis and proteolysis in Cheddar cheese and yeast-inoculated Cheddar cheeses during ripening. *Enzyme and Microbial Technology,* 37, 606-616.

DEETAE, P., MOUNIER, J., BONNARME, P., SPINNLER, H. E., IRLINGER, F. & HELINCK, S. 2009a. Effects of *Proteus vulgaris* growth on the establishment of a cheese microbial community and on the production of volatile aroma compounds in a model cheese. *Journal of Applied Microbiology,* 107, 1404-1413.

DEETAE, P., SPINNLER, H.-E., BONNARME, P. & HELINCK, S. 2009b. 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.

DELBES-PAUS, C., POCHET, S., HELINCK, S., VEISSEIRE, P., BORD, C., LEBECQUE, A., COTON, M., DESMASURES, N., COTON, E., IRLINGER, F. & MONTEL, M.-C. 2012. Impact of Gram-negative bacteria in interaction with a complex microbial consortium on biogenic amine content and sensory characteristics of an uncooked pressed cheese. *Food Microbiology,* 30, 74-82.

DENIS, C. & IRLINGER, F. 2008. Safety assessment of dairy microorganisms: Aerobic coryneform bacteria isolated from the surface of smear-ripened cheeses. *International Journal of Food Microbiology,* 126, 311-315.

DHERBECOURT, J., BOURLIEU, C., MAILLARD, M. B., AUBERT-FROGERAIS, L., RICHOUX, R. & THIERRY, A. 2010. Time course and specificity of lipolysis in Swiss cheese. *Journal of Agricultural and Food Chemistry,* 58, 11732-11739.

DI CAGNO, R., DE PASQUALE, I., DE ANGELIS, M. & GOBBETTI, M. 2012. Accelerated ripening of Caciocavallo Pugliese cheese with attenuated adjuncts of selected nonstarter lactobacilli. *Journal of Dairy Science,* 95, 4784-4795.

DRAKE, M. A., BOYLSTON, T. D., SPENCE, K. D. & SWANSON, B. G. 1996. Chemical and sensory effects of a *Lactobacillus* adjunct in Cheddar cheese. *Food Research International,* 29, 381-387.

DRAKE, M. A., BOYLSTON, T. D., SPENCE, K. D. & SWANSON, B. G. 1997. Improvement of sensory quality of reduced fat Cheddar cheese by a *Lactobacillus* adjunct. *Food Research International*, 30, 35-40.

EL SODA, M. & AWAD, S. 2011. Cheese: accelerated cheese ripening. *Encyclopedia of dairy sciences*, Second edition. Edited by J. W. Fuquay, P. F. Fox & P. L. H. McSweeney, Academic Press, London, 795-798.

EL SODA, M., MADKOR, S. A. & TONG, P. S. 2000. Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science,* 83, 609-619.

ELSODA, M. A., HANTIRA, A. A., EZZAT, N. I. & ELSHAFEI, H. K. 1992. Accelerated ripening of Ras cheese using freeze-shocked mutant strains of *Lactobacillus casei*. *Food Chemistry,* 44, 179-184.

EZZAT, N. 1990. Accelerated ripening of Ras cheese with a commercial proteinase and intracellular enzymes from *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Propionibacterium freudenreichii* and *Brevibacterium linens*. *Lait,* 70, 459-466.

FERNANDEZ-ESPLA, M. D. & FOX, P. F. 1998. Effect of adding *Propionibacterium shermanii* NCDO 853 or *Lactobacillus casei* ssp. *casei* IFPL 731 on proteolysis and flavour development of Cheddar cheese. *Journal of Agricultural and Food Chemistry,* 46, 1228-1234.

FERREIRA, A. D. & VILJOEN, B. C. 2003. Yeasts as adjunct starters in matured Cheddar cheese. *International Journal of Food Microbiology,* 86, 131-140.

FLEET, G. H. 1990. Yeasts in dairy-products. *Journal of Applied Bacteriology,* 68**,** 199-211.

FOLKERTSMA, B., FOX, P. F. & McSWEENEY, P. L. H. 1996. Accelerated ripening of Cheddar cheese at elevated temperatures. *International Dairy Journal,* 6, 1117-1134.

FOX, P. F., GUINEE, T. P., COGAN, T. M. & McSWEENEY, P. L. H. (eds.) 2000. *Fundamentals of Cheese Science*, Aspen Publishers, Gaithersburg, MD.

GABORIT, P., MENARD, A. & MORGAN, F. 2001. Impact of ripening strains on the typical flavour of goat cheeses. *International Dairy Journal,* 11, 315-325.

GONC, S., KINIK, O., AKALIN, S. & KARAGOZLU, C. 2007. Isolation of *Yersinia enterocolitica* in Turkish cheese varieties. *Indian Veterinary Journal,* 84, 1062-1063.

HANNON, J. A., KILCAWLEY, K. N., WILKINSON, M. G., DELAHUNTY, C. M. & BERESFORD, T. P. 2006. Production of ingredient-type Cheddar cheese with accelerated flavour development by addition of enzyme-modified cheese powder. *Journal of Dairy Science,* 89, 3749-3762.

HAYASHI, K., REVELL, D. F. & LAW, B. A. 1990a. Accelerated ripening of Cheddar cheese with the aminopeptidase of *Brevibacterium linens* and a commercial neutral proteinase. *Journal of Dairy Research,* 57, 571-577.

HAYASHI, K., REVELL, D. F. & LAW, B. A. 1990b. Effect of partially purified extracellular serine proteinases produced by *Brevibacterium linens* on the accelerated ripening of Cheddar cheese. *Journal of Dairy Science,* 73, 579-583.

HULIN-BERTAUD, S., KILCAWLEY, K. N., WILKINSON, M. G. & DELAHUNTY, C. M. 2000. Sensory and compositional relationships between commercial Cheddar-flavoured enzyme-modified cheeses and natural cheddar. *Journal of Food Science,* 65, 1076-1082.

IRLINGER, F., IN YUNG, S. A. Y., SARTHOU, A.-S., DELBES-PAUS, C., MONTEL, M.-C., COTON, E., COTON, M. & HELINCK, S. 2012. Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food Microbiology,* 153, 332-8.

JOHNSON, J. A. C., ETZEL, M. R., CHEN, C. M. & JOHNSON, M. E. 1995. Accelerated ripening of reduced-fat Cheddar cheese using 4 attenuated *Lactobacillus-helveticus* CNRZ-32 adjuncts. *Journal of Dairy Science,* 78, 769-776.

JOUTSJOKI, V., LUOMA, S., TAMMINEN, M., KILPI, M., JOHANSEN, E. & PALVA, A. 2002. Recombinant *Lactococcus* starters as a potential source of additional peptidolytic activity in cheese ripening. *Journal of Applied Microbiology,* 92, 1159-1166.

KAILASAPATHY, K. & LAM, S. H. 2005. Application of encapsulated enzymes to accelerate cheese ripening. *International Dairy Journal,* 15, 929-939.

KATSIARI, M. C., VOUTSINAS, L. P. & KONDYLI, E. 2002. Improvement of sensory quality of low fat Kefalograviera-type cheese by using commercial special starter cultures. *Journal of Dairy Science,* 85, 2759-2767.

KEBARY, K. M. K., KHADER, A. E., ZEDAN, A. N. & MAHMOUD, S. F. 1996. Accelerated ripening of low fat Ras cheese by attenuated lactobacilli cells. *Food Research International,* 29, 705-713.

KEBARY, K. M. K., SALEM, O. M., HAMED, A. I. & EL-SISI, A. S. 1997. Flavour enhancement of direct acidified Kareish cheese using attenuated lactic acid bacteria. *Food Research International,* 30, 265-272.

KESENKAS, H. & AKBULUT, N. 2008. Yeasts as ripening adjunct cultures in Turkish white brined cheese production. *Turkish Journal of Veterinary & Animal Sciences,* 32, 327-333.

KHEADR, E. E., VUILLEMARD, J. C. & EL-DEEB, S. A. 2003. Impact of liposome-encapsulated enzyme cocktails on Cheddar cheese ripening. *Food Research International,* 36, 241-252.

KHEADR, E. E., VUILLEMARD, L. C. & EL-DEEB, S. A. 2002. Acceleration of Cheddar cheese lipolysis by using liposome-entrapped lipases. *Journal of Food Science,* 67, 485-492.

KILCAWLEY, K. N., WILKINSON, M. G. & FOX, P. F. 2001. A survey of lipolytic and glycolytic end-products in commercial Cheddar enzyme-modified cheese. *Journal of Dairy Science,* 84, 66-73.

KLEIN, N. & LORTAL, S. 1999. Attenuated starters: an efficient means to influence cheese ripening: a review. *International Dairy Journal,* 9, 751-762.

LANCIOTTI, R., VANNINI, L., LOPEZ, C. C., GOBBETTI, M. & GUERZONI, M. E. 2005. Evaluation of the ability of *Yarrowia lipolytica* to impart strain-dependent characteristics to cheese when used as a ripening adjunct. *International Journal of Dairy Technology,* 58, 89-99.

LANE, C. N. & FOX, P. F. 1996. Contribution of starter and adjunct lactobacilli to proteolysis in Cheddar cheese during ripening. *International Dairy Journal,* 6, 715-728.

LAW, B. A. & WIGMORE, A. S. 1983. Accelerated ripening of Cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. *Journal of Dairy Research,* 50, 519-525.

LE LOIR, Y., AZEVEDO, V., OLIVEIRA, S. C., FREITAS, D. A., MIYOSHI, A., BERMUDEZ-HUMARAN, L. G., NOUAILLE, S., RIBEIRO, L. A., LECLERCQ, S., GABRIEL, J. E., GUIMARAES, V. D., OLIVEIRA, M. N., CHARLIER, C., GAUTIER, M. & LANGELLA, P. 2005. Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microbial Cell Factories,* 4, Art. n. 2.

LECLERCQ-PERLAT, M. N., BUONO, F., LAMBERT, D., LATRILLE, E., SPINNLER, H. E. & CORRIEU, G. 2004. Controlled production of Camembert-type cheeses. Part I: Microbiological and physicochemical evolutions. *Journal of Dairy Research,* 71, 346-354.

LUOMA, S., PELTONIEMI, K., JOUTSJOKI, V., RANTANEN, T., TAMMINEN, M., HEIKKINEN, I. & PALVA, A. 2001. Expression of six peptidases from *Lactobacillus helveticus* in *Lactococcus lactis*. *Applied and Environmental Microbiology,* 67, 1232-1238.

LYNCH, C. M., McSWEENEY, P. L. H., FOX, P. F., COGAN, T. M. & DRINAN, F. D. 1996. Manufacture of Cheddar cheese with and without adjunct

lactobacilli under controlled microbiological conditions. *International Dairy Journal,* 6, 851-867.

LYNCH, C. M., MUIR, D. D., BANKS, J. M., McSWEENEY, P. L. H. & FOX, P. F. 1999. Influence of adjunct cultures of *Lactobacillus paracasei* ssp *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *Journal of Dairy Science,* 82, 1618-1628.

MADKOR, S. A., TONG, P. S. & EL SODA, M. 2000. Ripening of Cheddar cheese with added attenuated adjunct cultures of lactobacilli. *Journal of Dairy Science,* 83, 1684-1691.

MARTIN, N., SAVONITTO, S., MOLIMARD, P., BERGER, C., BROUSSE, M. & SPINNLER, H. E. 1999. Flavour generation in cheese curd by coculturing with selected yeast, mold, and bacteria. *Journal of Dairy Science,* 82, 1072-1080.

McGARRY, A., LAW, J., COFFEY, A., DALY, C., FOX, P. F. & FITZGERALD, G. F. 1994. Effect of genetically modifying the lactococcal proteolytic system on ripening and flavour development in Cheddar cheese. *Applied and Environmental Microbiology,* 60, 4226-4233.

McSWEENEY, P. L. H., WALSH, E. M., FOX, P. F., COGAN, T. M., DRINAN, F. D. & CASTELO GONZALEZ, M. 1994. A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. *Irish Journal of Agricultural and Food Research,* 33, 183-192.

MEILE, L., LE BLAY, G. & THIERRY, A. 2008. Safety assessment of dairy microorganisms: *Propionibacterium* and *Bifidobacterium*. *International Journal of Food Microbiology,* 126, 316-320.

MILESI, M. M., McSWEENEY, P. L. H. & HYNES, E. R. 2008. Viability and contribution to proteolysis of an adjunct culture of *Lactobacillus plantarum* in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology,* 105, 884-892.

MORALES, P., FERNANDEZ-GARCIA, E. & NUNEZ, M. 2003. Caseinolysis in cheese by *Enterobacteriaceae* strains of dairy origin. *Letters in Applied Microbiology,* 37, 410-414.

MORALES, P., MEDINA, M. & NUNEZ, M. 2005. Cysteine proteinase and tributyrin esterase production by *Micrococcus* sp. INIA 528 under cheese-making conditions. *Milchwissenschaft,* 60, 143-146.

MOTTA, A. S. & BRANDELLI, A. 2008. Properties and antimicrobial activity of the smear surface cheese coryneform bacterium *Brevibacterium linens*. *European Food Research and Technology,* 227, 1299-1306.

OKSUZ, O., KURULTAY, S. & SIMSEK, O. 2001. The effect of *Brevibacterium linens* on some physico-chemical properties and colour intensity of Kashar cheese. *Milchwissenschaft* 56, 82-85.

ONG, L., HENRIKSSON, A. & SHAH, N. P. 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium spp.* and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal,* 16, 446-456.

ONG, L., HENRIKSSON, A. & SHAH, N. P. 2007. Chemical analysis and sensory evaluation of Cheddar cheese produced with *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* or *Bifidobacterium* sp. *International Dairy Journal,* 17, 937-945.

ORTIGOSA, M., IRIGOYEN, A., URDIN, M., GARCIA, S., IBANEZ, F. C. & TORRE, P. 2008. Identification of enterococci and isolation of vancomycin-resistant strains in Spanish cheeses. *Milchwissenschaft,* 63, 164-167.

PACHLOVA, V., BUNKA, F., FLASAROVA, R., VALKOVA, P. & BUNKOVA, L. 2012. The effect of elevated temperature on ripening of Dutch type cheese. *Food Chemistry,* 132, 1846-1854.

PHILLIPS, M., KAILASAPATHY, K. & TRAN, L. 2006. Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium sp*., *L. casei*, *L. paracasei* and *L. rhamnosus*) in Cheddar cheese. *International Journal of Food Microbiology,* 108, 276-280.

PICON, A., GAYA, P., MEDINA, M. & NUNEZ, M. 1995. The effect of liposome-encapsulated *Bacillus subtilis* neutral proteinase on Manchego cheese ripening. *Journal of Dairy Science,* 78, 1238-1247.

POONAM, POPHALY, S. D., TOMAR, S. K., DE, S. & SINGH, R. 2012. Multifaceted attributes of dairy propionibacteria: a review. *World Journal of Microbiology & Biotechnology,* 28, 3081-95.

PUCHADES, R., LEMIEUX, L. & SIMARD, R. E. 1989. Evolution of free amino-acids during the ripening of Cheddar cheese containing added lactobacilli strains. *Journal of Food Science,* 54, 885-&.

RAKSAKULTHAI, R., ROSENBERG, M. & HAARD, N. F. 2002. Accelerated Cheddar cheese ripening with an aminopeptidase fraction from squid hepatopancreas. *Journal of Food Science,* 67, 923-929.

RATTRAY, F. P. & EPPERT, I. 2011. Cheese: Secondary Cultures. *Encyclopedia of dairy sciences*, Second edition. Edited by John W. Fuquay, Patrick F. Fox & McSweeney, P. L. H., Academic Press, London, 567-573.

RATTRAY, F. P. & FOX, P. F. 1999. Aspects of enzymology and biochemical properties of *Brevibacterium linens* relevant to cheese ripening: A Review. *Journal of Dairy Science,* 82, 891-909.

REHN, U., VOGENSEN, F. K., PERSSON, S. E., SAEDEN, K. H., NILSSON, B. F. & ARDO, Y. 2011. Influence of microflora on texture and contents of amino acids, organic acids, and volatiles in semi-hard cheese made with DL-starter and propionibacteria. *Journal of Dairy Science,* 94, 1098-1111.

SIHUFE, G. A., ZORRILLA, S. E., PEROTTI, M. C., WOLF, I. V., ZALAZAR, C. A., SABBAG, N. G., COSTA, S. C. & RUBIOLO, A. C. 2010. Acceleration of cheese ripening at elevated temperature. An estimation of the optimal ripening time of a traditional Argentinean hard cheese. *Food Chemistry,* 119, 101-107.

SØRENSEN, L. M., GORI, K., PETERSEN, M. A., JESPERSEN, L. & ARNEBORG, N. 2011. Flavour compound production by *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* in a cheese-surface model. *International Dairy Journal,* 21, 970-978.

SWEARINGEN, P. A., O'SULLIVAN, D. J. & WARTHESEN, J. J. 2001. Isolation, characterization, and influence of native, nonstarter lactic acid bacteria on Cheddar cheese quality. *Journal of Dairy Science,* 84, 50-59.

THAGE, B. V., BROE, M. L., PETERSEN, M. H., PETERSEN, M. A., BENNEDSEN, M. & ARDO, Y. 2005. Aroma development in semi-hard reduced-fat cheese inoculated with *Lactobacillus paracasei* strains with different aminotransferase profiles. *International Dairy Journal,* 15, 795-805.

THIERRY, A., FALENTIN, H., DEUTSCH, S. M. & JAN, G. 2011. Bacteria, Beneficial: *Propionibacterium* spp. *Encyclopedia of dairy sciences*, Second

edition. Edited by J. W. Fuquay, P. F. Fox & P. L. H. McSweeney, Academic Press, London, 403-411.

THIERRY, A., MAILLARD, M. B., BONNARME, P. & ROUSSEL, E. 2005a. The addition of *Propionibacterium freudenreichii* to Raclette cheese induces biochemical changes and enhances flavour development. *Journal of Agricultural and Food Chemistry,* 53, 4157-4165.

THIERRY, A., MAILLARD, M. B., RICHOUX, R., KERJEAN, J. R. & LORTAL, S. 2005b. *Propionibacterium freudenreichii* strains quantitatively affect production of volatile compounds in Swiss cheese. *Lait,* 85, 57-74.

UPADHYAY, K. G., VYAS, S. H., PANDYA, A. J., DESAI, H. K. & PATEL, J. N. 1985. Use of higher temperature for accelerating ripening of buffalo milk Cheddar cheese. *Asian Journal of Dairy Research,* 4, 209-216.

VANDEGUCHTE, M., KODDE, J., VANDERVOSSEN, J., KOK, J. & VENEMA, G. 1990. Heterologous gene-expression in *Lactococcus lactis* subsp *lactis* - synthesis, secretion, and processing of the *Bacillus subtilis* neutral protease. *Applied and Environmental Microbiology,* 56, 2606-2611.

WALSTRA, P., GEURTS, T. J., NOOMEN, A., JELLEMA, A. & VAN BOEKEL, M. A. J. S. 1999. *Dairy technology: Principles of milk properties and processes*. Edited by P. Walstra, T. J. Geurts, A. Noomen, A. Jelleman, M. A. J. S. van Boekel, Marcel. Dekker, Inc., New York, Basel, 601-638

WILKINSON, M. G., DOOLAN, I. A. & KILCAWLEY, K. N. 2011. Cheese: Enzyme-Modified Cheese. *Encyclopedia of dairy sciences*, Second edition. Edited by J. W. Fuquay, P. F. Fox & P. L. H. McSweeney, Academic Press, London, 799-804.

WYDER, M. T., BACHMANN, H. P. & PUHAN, Z. 1999. Role of selected yeasts in cheese ripening: An evaluation in foil wrapped Raclette cheese. *Lebensmittel-Wissenschaft & Technologie,* 32, 333-343.

YILMAZ, G., AYAR, A. & AKIN, N. 2005. The effect of microbial lipase on the lipolysis during the ripening of Tulum cheese. *Journal of Food Engineering,* 69, 269-274.

2. Control of the "wild" non-starter microbiota of Cheddar cheese using a bacteriocin-producing strain of *Lactobacillus paracasei*

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2.1. Abstract

Non-starter lactic acid bacteria contribute to cheese ripening as well as causing defects and spoilage. The use of bacteriocins may provide an approach for controlling the growth of this secondary microbiota. In this study, a bacteriocin-producing strain of *Lactobacillus paracasei* DPC 4715 was used as an adjunct culture in Cheddar cheese. Four control cheeses and one experimental cheese were made: the control cheese (S) was inoculated with the starter strain *Lactococcus lactis* ssp. *lactis* 303 (1.5%); the second control cheese (SC) with the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%) and a chloramphenicol-resistant indicator strain *Lactobacillus casei* DPC 2048^{CM} (0.25%); the control cheese SB with the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%) and the bacteriocin-producing strain *Lb. paracasei* DPC 4715 (0.25%), the cheese L with the starter strain *Lactococcus lactis* ssp. *lactis* DPC 3147 which produces the potent bacteriocin lacticin 3147 (1.5%); the experimental cheese SBC contained the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%), the bacteriocin-producing strain *Lb. paracasei* DPC 4715 (0.25%) and the indicator strain *Lb. casei* 2048^{CM} (0.25%) . The growth of the chloramphenicol-resistant indicator strain *Lb. casei* DPC 2048^{CM} was monitored on Rogosa agar containing chloramphenicol and to distinguish it from the "wild" and added NSLAB microbiota. No suppression of growth of the indicator strain was observed in the experimental cheese SBC during ripening and no bacteriocin production by *Lactobacillus paracasei* DPC 4715 was detected by the well diffusion method in cheese and cheese extracts. The bacteriocin produced by *Lactobacillus paracasei* DPC 4715 was sensitive to chymosin and cathepsin D and it may have been cleaved by the rennet used for the cheese manufacture or by indigenous milk proteases.

2.2. Introduction

Non-starter lactic acid bacteria (NSLAB) contribute to cheese ripening and flavour intensity, increasing aroma and reducing harshness and bitternes (Beresford and Williams, 2004). However, in some cases they are responsible for cheese defects such as the formation of white spots of calcium lactate crystals on the cheese surface due to the racemization of L-lactate to D/L-lactate, off-flavours and slit formation attributed to gas production (Banks and Williams, 2004). This microbiota can survive pasteurization of milk or can contaminate the curd during manufacture and grows in cheese during ripening (McSweeney, 2004).

Generally, low numbers of NSLAB positively affect cheese ripening, but high numbers are undesiderable (Fox et al., 1998). The overgrowth of NSLAB in cheese can be controlled by using microfiltered milk, aseptic conditions during manufacture, low ripening temperature, antibiotics or bacteriocin-producing starters (Shakeel Ur Rehman et al., 2000).

Many lactic acid bacteria (LAB) are able to produce bacteriocins which are anti-microbial proteinaceous molecules active against generally closely related species (Jack et al., 1995). Bacteriocins produced by LAB are generally recognized as safe (GRAS) and are used in food industry to improve food quality and microbial safety by inhibiting the growth of food-borne pathogens such as *Listeria monocytogenes, Staphylococcus aureus* and other spoilage or pathogenic bacteria (Sobrino-Lopez and Martin-Belloso, 2008).

Over the past years, much work has been focused on the use of bacteriocins from LAB as food biopreservatives to inhibit the growth of spoilage bacteria and pathogens (Cintas et al., 2001). This has led to the identification and characterization of many bacteriocins (e.g., Ferchichi et al., 2001; Maldonado et al., 2003; Bromberg et al., 2005; Todorov et al., 2005; Pangsomboon et al., 2006; Lozo et al., 2007; Todorov et al., 2008; Riazi et al., 2009), including some produced by *Lactobacillus paracasei*. Lozo et al. (2007) reported that the bacteriocin of *Lb. paracasei* BGSJ2-8 has a narrow antimicrobial spectrum since inhibits only the growth of closely related species. Bacteriocins produced by *Lb. paracasei* ST68, *Lb. paracasei* 171R2 (Tuma et al., 2008) and a strain of *Lb. paracasei* ssp. *paracasei* isolated from faeces of breast-fed newborn infants (Bendali et al., 2008) have anti-clostridial and anti-listerial effects. The bacteriocin produced by *Lb. paracasei* ST11BR is active against *Lb. casei*, *Lb. sakei* and Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Todorov et al., 2005) whereas, the bacteriocin of *Lb. paracasei* HL32 has activity against *Porphyromonas gingivalis* (Pangsomboon et al., 2006). Rodriguez et al. (2000) reported that the antimicrobial activity of five *Lactobacillus* strains isolated from raw milk was restricted to *Clostridium tyrobutyricum*, although, among them, two strain of *Lb. paracasei* ssp. *paracasei* showed to be effective also against *L. monocytogenes*.

The aim of this study was to investigate the ability of bacteriocin-producing *Lactobacillus paracasei* DPC 4715, isolated from Cheddar cheese, to suppress the growth of "wild" NSLAB when added as adjunct culture for Cheddar cheese manufacture. Since lactobacilli represent the large majority of NSLAB in Cheddar, *Lb. casei* 2048^{CM} (chloramphenicol resistant) was chosen as an indicator strain and its growth was monitored on Rogosa medium with added chloramphenicol in order to distinguish it from the adventitious NSLAB and added lactobacilli. *Lactococcus lactis* ssp. *lactis* DPC3147, was used as starter in the positive control cheese. This strain was isolated from an Irish kefir grain used for domestic "buttermilk" production and produces lacticin 3147, which is a broad-spectrum bacteriocin active against many Gram-positive food pathogens and spoilage microorganisms. *Lc. lactis* ssp. *lactis* DPC3147 is a strain suitable for Cheddar cheese manufacture as it reduces the pH within the desired time and produces sufficient levels of lacticin 3147 to reduce the levels of NSLAB during ripening (Ryan et al., 1996).

2.3. Materials and methods

2.3.1. Bacterial strains and cultivation conditions

The bacteriocin-producing strain *Lb. paracasei* DPC 4715 and the indicator strains *Lb. casei* DPC 2048^{CM}, *Lb. casei* DPC 2048, *Lb. casei* DPC 2061, *Lb. paracasei* DPC 3968, *Lb. paracasei* DPC 3990, *Lb. helveticus* CNRZ 32 were grown in MRS broth overnight at 30°C; the indicator strain *Lc. lactis* DPC 1638 the starter strain *Lc. lactis* ssp. *lactis* 303 and the lacticin 3147-producing *Lc. lactis* ssp. *lactis* DPC 3147, were grown in LM17 broth at 30°C overnight.

2.3.2. Bacteriocin sensitivity

The sensitivity of the indicator strains to the bacteriocins produced by *Lb. paracasei* DPC 4715 and *Lc. lactis* ssp. *lactis* DPC 3147 was determined using an agar well diffusion assay as follows: tempered molten (~45°C) agar was inoculated with 0.25% of an overnight culture of the appropriate indicator strain. The inoculated medium was dispensed in sterile Petri dishes and dried for 30 min under a laminar flow hood. Wells (5 mm) were cut in each plate using a sterile glass pipette. An overnight culture of the bacteriocin-producing strain *Lb. paracasei* DPC 4715 was centrifuged at 9,000 g for 10 min and the resulting supernatant was filter sterilized (pore size 0.2 µm; Pall Filters, Dublin, Ireland). Twenty-five µl of the bacteriocin solution was dispensed in each well, and plates were incubated overnight at 30°C. The sensitivity of each strain to the bacteriocin was observed by the formation of a clear inhibition zone around the wells.

2.3.3. Protease and catalase sensitivity

The sensitivity to chymosin (Chymax-180, Chr. Hansen, Hørshom, Denmark), plasmin (Sigma, 2.0 units/mg protein), cathepsin D (Sigma, 5.0 units/mg protein) and catalase (Sigma, 2,000-5,000 units/mg protein) was tested using the well diffusion assay as described above. Chymosin was diluted to the same concentration used for cheesemaking (0.03% v/v in milk), the other enzymes were diluited in distilled water to a final concentration of 50 mg/ml. The solutions were filter sterilized with disposable filters (pore size 0.2 µm; Pall Filters). Twenty-five microliters of the filtered overnight culture bacteriocin solution were dispensed into each well and 25 µl of each enzyme solution was spotted next to the well and allow to dry for 30 min. Plates were incubated overnight at 30°C and the protease sensitivity was shown by the formation of a half-moon-shaped zone of inhibition.

2.3.4. Cheesemaking

Four control cheeses and one experimental cheese were manufactured in 20 L scale in triplicate on different days. The cheesemaking was designed as follows. The control cheese S was inoculated with the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%); the control cheese SC with the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%) and the chloramphenicol-resistant indicator strain *Lb. casei* DPC 2048^{CM} (0.25%); the control cheese SB with the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%) and the bacteriocin-producing strain *Lb. paracasei* DPC 4715 (0.25%), the control cheese L with the starter strain *Lc. lactis* ssp. *lactis* DPC 3147 which produces lacticin 3147

(1.5%); the experimental cheese SBC contained the starter strain *Lc. lactis* ssp. *lactis* 303 (1.5%), the bacteriocin producing strain *Lb. paracasei* DPC 4715 (0.25%) and the indicator strain *Lb. casei* DPC 2048^{CM} (0.25%). The bulk culture were prepared the day before by inoculating 10% RSM with the appropriate cultures and growing them overnight at 30°C until the pH reached ~4.9. The cultures were then cooled using ice water and kept at 4 °C until use. Whole milk was standardized to casein: fat ratio of 0.7:1.0, pasteurised at 72 ºC for 15 s and cooled to 30 ºC for cheesemaking. Chymax-180 (Chr. Hansen, Hørshom, Denmark) was used as coagulant. Whey was drained at pH 6.2 and the curd cheddared, milled at pH 5.2 and salted to a level of 2.5 % (w/w). After pressing the curd at 150 kPa for 18 h, the cheeses were vacuum packed and ripened for 180 d at 8°C.

2.3.5. Analysis of cheese

Microbiological analyses were carried out in duplicate at regular intervals during ripening. Ten g of cheese were aseptically sampled and homogenized in 90 ml of sterile trisodium citrate (2% w/v) in a stomacher (Stomacher 400, Seward Limited, Worthing, West Sussex, UK) for 5 min. Starter cells were enumerated on LM17 agar after incubation at 30° for 3 d, NSLAB and added lactobacilli were enumerated on Rogosa medium after growth for 5 d at 30°C, the indicator strain *Lb. casei* DPC 2048^{CM} was enumerated on Rogosa medium containing cloramphenicol (10 µg/ml) after growth for 5 d at 30°C. Bacteriocin activity was determined using a well diffusion assay in cheese, by placing a piece of cheese into the wells and in cheese extracts as described by Ryan et al. (1996).

2.3.6. Compositional analysis

The cheeses were analysed for protein (Grappin, 1986), fat (Gerber method; IDF, 1986), moisture (oven drying at 102°C; IDF, 1983), salt (Fox, 1963). The pH was determined in cheese slurry (1:1 cheese:water) using a combined glass electrode (PHC3001-8, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standard pH Meter, Radiometer, Copenhagen, Denmark). The analyses were performed in triplicate after 14 d of ripening.

2.4. Results and discussion

Lactic acid bacteria play an important role during manufacture and preservation of many fermented foods by production of anti-microbial compounds such as organic acids, peroxide hydrogen, bacteriocins or antimicrobial peptides derived from the caseins (Toba et al., 1991; O'Sullivan et al., 2002; Bendali et al., 2008). The use of bacteriocin-producing LAB as adjunct culture in cheese manufacture may provide useful means for controlling the overgrowth of spoilage NSLAB. However, in some cases, it has limitation related to production of bacteriocins with a narrow spectrum of inhibition or sensitive to interactions with cheese ingredients (Tuma et al., 2008).

In this study, three Cheddar cheese trials were manufactured to investigate the ability of *Lb. paracasei* DPC 4715 to produce a bacteriocin in cheese and to inhibit the growth of the bacteriocin-sensitive indicator strain *Lb. casei* DPC 2048^{CM}.

2.4.1. Compositional analysis

The composition of each cheese at 14 d (**Table 2.1**) was typical of Cheddar cheese and the different starter culture systems and adjunct lactobacilli had no significant effect on composition. Similar results were reported by Madkor et al. (2000), Sallami et al. (2004), Ong et al. (2006) and Milesi et al. (2008).

Cheese	pH	% Salt	% Fat	%Moisture	% Protein
S	5.19±0,06^{a}	1.62±0.07^{a}	30.89±1.35^{a}	38.75±1.05^{a}	22.94±0.74^{a}
SB	5.11±0.09^{a}	1.68±0.16^{a}	30.67±0.57^{a}	38.83±0.73^{a}	22.86±1.28^{a}
SC	5.02±0.12^{a}	1.63±0.08^{a}	30.88±1.07^{a}	38.43±0.86^{a}	22.79±1.47^{a}
SBC	5.05±0.10^{a}	1.62±0.12^{a}	30.56±0.51^{a}	38.58±1.00^{a}	22.79±1.04^{a}
L	5.06±0.51^{a}	1.62±0.21^{a}	30.22±0.51^{a}	39.82±1.00^{a}	23.01±1.35^{a}

Table 2.1. pH and compositional analysis of Cheddar cheeses at 14 d of ripening. Cheese S, made with *Lc. lactis* ssp. *lactis* 303; cheese SB, made with *Lc. lactis* ssp. *lactis* 303 and the bacteriocin-producing strain *Lb. paracasei* DPC 4715; cheese SC, made with *Lc. lactis* ssp. *lactis* 303 and a chloramphenicol-resistant indicator strain *Lb. casei* DPC 2048^{CM}; cheese SBC, made with *Lc. lactis* ssp. *lactis* 303, the bacteriocin-producing strain *Lb. paracasei* DPC 4715 and the indicator strain *Lb. casei* 2048^{CM} ; cheese L, made with *Lc. lactis* ssp. *lactis* DPC 3147.
The results are shown as an average of three trials ± standard deviation.
[a]Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$)

2.4.2. Microbiological analysis

Starter lactic acid bacteria were inoculated at level of ~10^9 cfu/g and started decreasing around 30 d of ripening. Counts on LM17 showed numbers of ~10^7 cfu/g after 180 d in all trials (**Figure 2.1 A, B, C**), but by this time counts on LM17 would also include NSLAB. Similar results were reported by McSweeney et al. (1993), Midje et al. (2000), Awad et al. (2005), Hickey et al. (2006) and Shakeel Ur Rehman et al. (2008). The initial number of added lactobacilli in the cheeses SB, SC and SBC at d 1 were ~ 10^5 cfu/g in all trials and increased to ~10^8 cfu/g at the end of ripening (**Figure 2.2 A, B, C**). No NSLAB were detected in control cheese L made with lacticin 3147-producing starter strain *Lc. lactis* ssp. *lactis* DPC 3147 until 150 d of ripening when the adventitious non-starter lactobacilli reached levels of 10^6 and 10^7 cfu/g in Trials A and B, respectively, and 10^6 cfu/g in Trial C after 180 d of ripening (**Figure 2.2 A, B, C**). Ryan et al. (1996) reported that the NSLAB were absent up to six months of ripening in Cheddar cheese made with a mix of lacticin 3147-producing strains, which also contained *Lc. lactis* DPC 3147. NSLAB in the control cheese S reached numbers of ~ 10^6 cfu/g after 60 d in Trial B; after 90 d in Trials A and C, numbers had increased to ~ 10^6-10^7 cfu/g. It is known that the non-starter microbiota of Cheddar cheese increases from a low number (10^2-10^3 cfu/g) at the early stage of ripening to become the dominant viable microbiota (10^7-10^8 cfu/g) after 3-4 months (Fox et al., 1998).

Bacteriocin production in cheese was monitored by comparing the growth rate of the sensitive indicator strain *Lb. casei* 2048^{CM} in the experimental cheese SBC (made with the bacteriocin-producing strain) and the control cheese SC (made without bacteriocin-producing strain). Since the indicator strain *Lb. casei* 2048^{CM} was chloramphenicol resistant, its growth was easily monitored and it could be discriminated from "wild" and other added lactobacilli when grown on Rogosa medium with added chloramphenicol. The indicator strain showed the same growth trend in cheese SBC and SC in all trials, suggesting that its growth was not inhibited in cheese made with the bacteriocin-producing strain (**Figure 2.3 A, B, C**). However, in Trial A, the numbers of *Lb. casei* 2048^{CM} reduced by 1 log cycle at 90 d. This might be due to the bacteriocin activity, but its production was not detected by the well-diffusion assay (results not shown). It is possible that the bacteriocin was produced at very low concentration which was not detectable by the well diffusion assay. This method is very sensitive to the medium composition, diffusion ability of the bacteriocin, strain used and inoculum concentration (Yaakoubi et al., 2009).

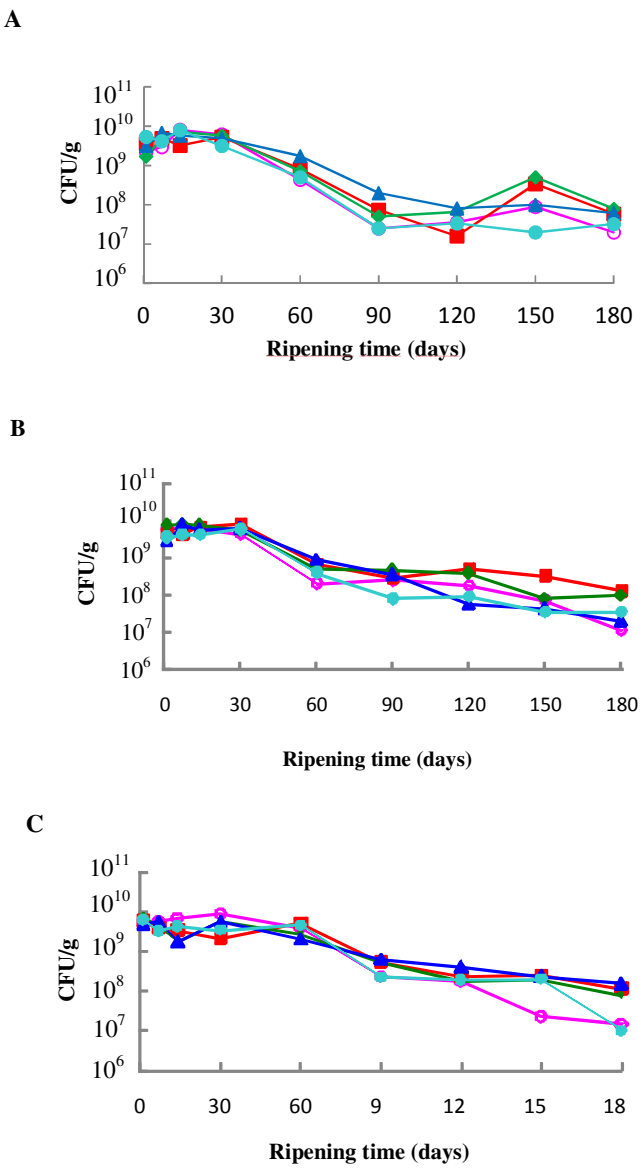

Figure 2.1. Growth of starter lactic acid bacteria (SLAB) on LM17 agar incubated 30°C for 3 days during ripening of Cheddar cheeses in Trials A, B, C. Cheese S (o), made with *Lc. lactis* ssp. *lactis* 303; cheese SB (■), made with *Lc. lactis* ssp. *lactis* 303 and the bacteriocin-producing strain *Lb. paracasei* DPC 4715; SC (♦), made with *Lc. lactis* ssp. *lactis* 303 and a chloramphenicol-resistant indicator strain *Lb. casei* DPC 2048^{CM}; cheese SBC (▲), made with *Lc. lactis* ssp. *lactis* 303, the bacteriocin-producing strain *Lb. paracasei* DPC 4715 and the indicator strain *Lb. casei* 2048^{CM} ; cheese L (●), made with *Lc. lactis* ssp. *lactis* DPC 3147.

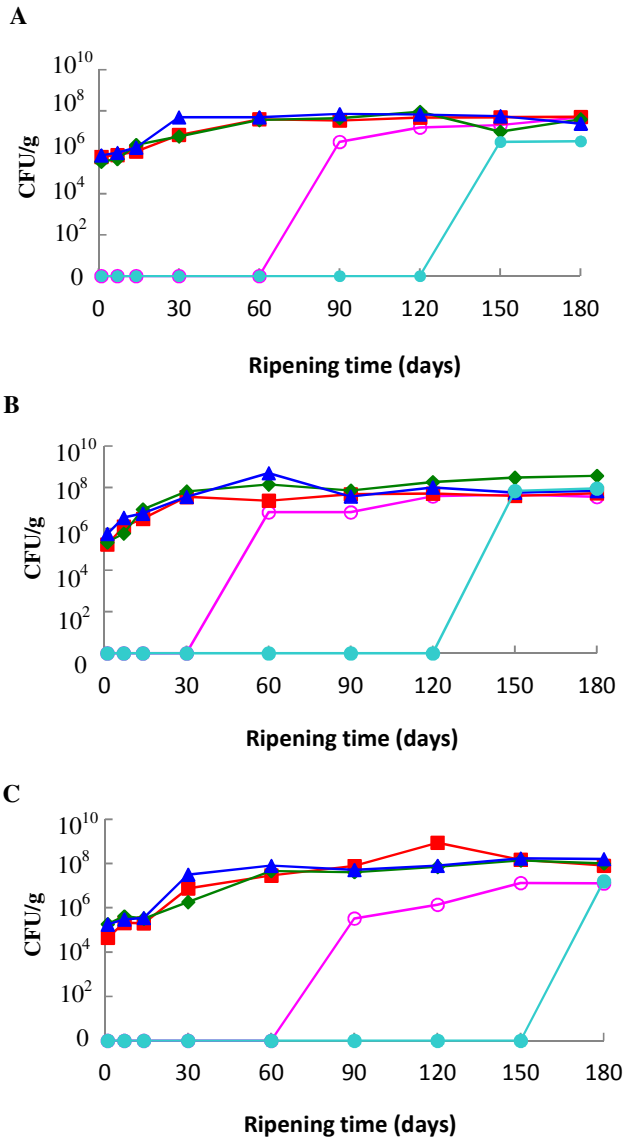

Figure 2.2. Growth of non-starter lactic acid bacteria (NSLAB) enumerated on Rogosa agar incubated at 30°C for 5 days during ripening of Cheddar cheeses in Trial A, B, C. Cheese S (o), made with *Lc. lactis* ssp. *lactis* 303; cheese SB (■), made with *Lc. lactis* ssp. *lactis* 303 and the bacteriocin-producing strain *Lb. paracasei* DPC 4715; cheese SC (♦), made with *Lc. lactis* ssp. *lactis* 303 and a chloramphenicol-resistant indicator strain *Lb. casei* DPC 2048^{CM}; cheese SBC (▲), made with *Lc. lactis* ssp. *lactis* 303, the bacteriocin-producing strain *Lb. paracasei* DPC 4715 and the indicator strain *Lb. casei* 2048^{CM} ; cheese L (●), made with *Lc. lactis* ssp. *lactis* DPC 3147.

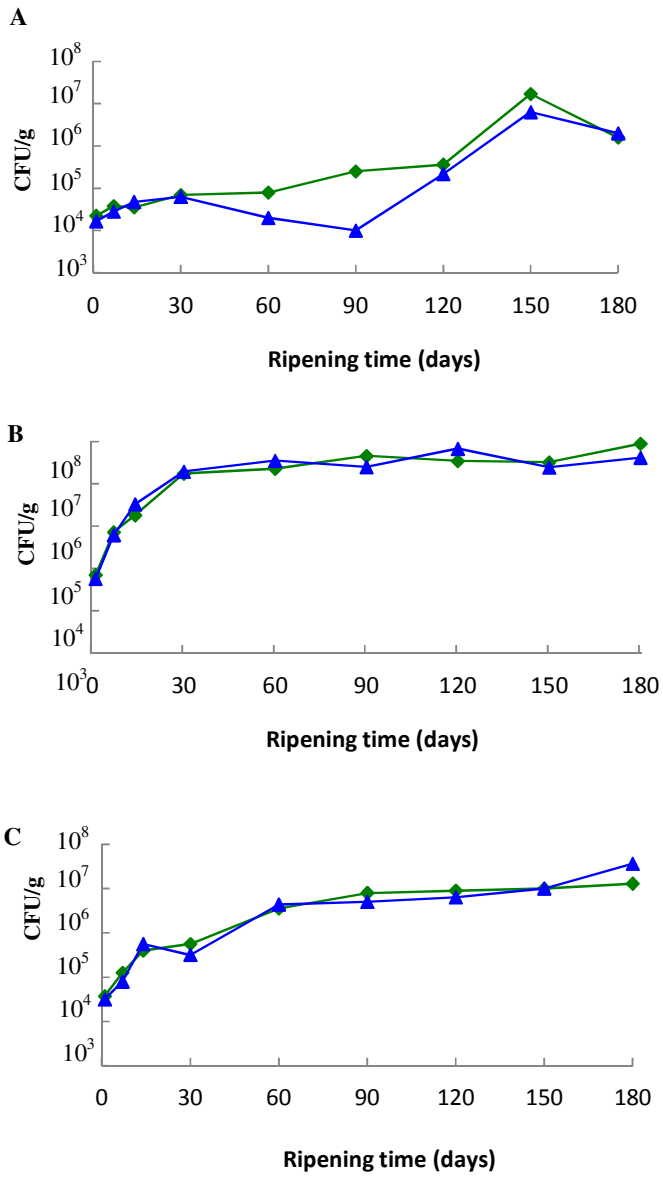

Figure 2.3. Growth of the indicator strain *Lc. lactis* 2048^{CM} enumerated on Rogosa medium with added cloramphenicol after growth for 5 d at 30° during ripening of Cheddar cheeses in Trial A, B, C. Cheese SC (♦), contained the starter strain *Lc. lactis* ssp. *lactis* 303 and a chloramphenicol-resistant indicator strain *Lb. casei* DPC 2048CM; cheese SBC (▲), contained *Lc. lactis* ssp. *lactis* 303, the bacteriocin-producing strain *Lb. paracasei* DPC 4715 and the indicator strain *Lb. casei* 2048CM , in Trials A, B and C.

2.4.3. Well-diffusion assay

The production of bacteriocin by *Lb. paracasei* DPC 4715 was tested *in vitro* by well-diffusion assay (**Table 2.2**). The bacteriocin inhibited the growth of *Lb. casei* DPC 2048^{CM}, *Lb. casei* DPC 2048, *Lb. casei* DPC 2061, *Lb. paracasei* DPC 3968 and *Lb. paracasei* DPC 3990, whereas it did not show antimicrobial activity against *Lb. helveticus* CNRZ 32 and *Lc. lactis* DPC1638 (*Lc. lactis* HP), suggesting that the bacteriocin has a narrow spectrum of inhibition.

Table 2.2. Inhibition spectrum of the bacteriocin of *Lb. paracasei* DPC 4715.

Indicator strains	Code	Medium	Sensitivity[a]	Source
***Lactobacillus casei*^{CM}**	DPC 2048	MRS	++	DPC
Lactobacillus casei	DPC 2048	MRS	++	DPC
Lactobacillus casei	DPC 2061	MRS	++	DPC
Lactobacillus helveticus	CNRZ 32	MRS[b]	–	CNRZ
Lactobacillus paracasei	DPC 3968	MRS	++	DPC
Lactobacillus paracasei	DPC 3990	MRS	+	DPC
Lactococcus lactis (HP)	DPC1638	LM17	–	DPC

[a] -, no zone of inhibition; +, 1-3 mm; ++ 3-5 mm.
[b] Incubated at 37°C. All the others medium were incubated at 30°C.

Bacteriocin production in cheese and cheese extracts was monitored during ripening by a well-diffusion assay. No inhibition activity was detected in the cheese extracts and cheese SB and SBC against the sensitive indicator strains in all trials (results not shown), indicating that the bacteriocin was not produced in cheeses during ripening or was produced at a very low concentration which was not detectable by the assay. In the control cheese L made with *Lc. lactis* DPC 3147, the inhibition activity of lacticin during ripening was detected only against *Lb. helveticus* CNRZ 32 and *Lc. lactis* DPC1638 (Figure 2.4 A, B).

Figure 2.4. Bacteriocin production by *Lc. lactis* DPC 3147 in cheese (A) and cheese extract (B) after 180 d of ripening in Trial A.
Indicator strain: *Lb. helveticus* CNRZ32 (A). Bacteriocin production by *Lc. lactis* DPC 3147 in cheese (c) and cheese extract (d) after 180 d of ripening in Trial A. Indicator strain: *Lc. lactis* DPC 1638 (B).

Bacteriocin production depends on pH, nutrient source and incubation temperature. High level of bacteriocin production may be obtained at different conditions from those required by the producer strain and in some cases it is not related to cell mass or growth rate (Todorov et al., 2005). Data regarding bacteriocin production by LAB and microbial growth at different temperature and pH were reported by Hugas et al. (1998) who showed that *Lactobacillus sakei* CTC494 produced a higher amount of bacteriocin when incubated at lower temperature (4°C and 10°C) than at its optimal (30°C). Bromberg et al. (2005) showed that *Lactococcus lactis* ssp. *cremoris* CTC204 is able to produce a bacteriocin when incubated at 4°C. On the other hand, Bendali et al. (2008) reported that *Lb. paracasei* ssp. *paracasei* produced an anti-listerial bacteriocin when incubated at 37°C, but it was not able to grow and produce bacteriocin when incubated at 6°C.

In order to exclude the possibility that the antimicrobial activity was due to the production of hydrogen peroxide, well diffusion test was performed by spotting catalase next to the well containing the bacteriocin. No half-moon-shaped halo was detected where catalase was spotted, showing that hydrogen peroxide was not produced by *Lb. paracasei* DPC 4715 and that the inhibition activity was due to bacteriocin production (results not shown).

In order to discover if the ripening temperature, salt-in-moisture level and pH of cheese affected the production of bacteriocin, *Lb. paracasei* DPC 4715 was inoculated in MRS broth containing 0% and 5% NaCl, the pH values was adjusted to 5.0 and 5.5, and incubated at 8°C. The results were compared to those obtained after incubation at 30°C. The well diffusion assay confirmed that the strain was able to produce the bacteriocin under these conditions (**Figure 2.5 A, B**). However, a narrow halo of inhibition (~3 mm) was detected in the cultures incubated at 8°C indicating that the bacteriocin was produced at lower concentration and a much longer incubation period (14 d) was required for growth (**Figure 2.5 A**). A narrow zone of inhibition was also detected in the culture incubated at 30°C in MRS adjusted to pH 5 indicating that the production of bacteriocin is also affected by low pH (**Figure 2.5 B**).

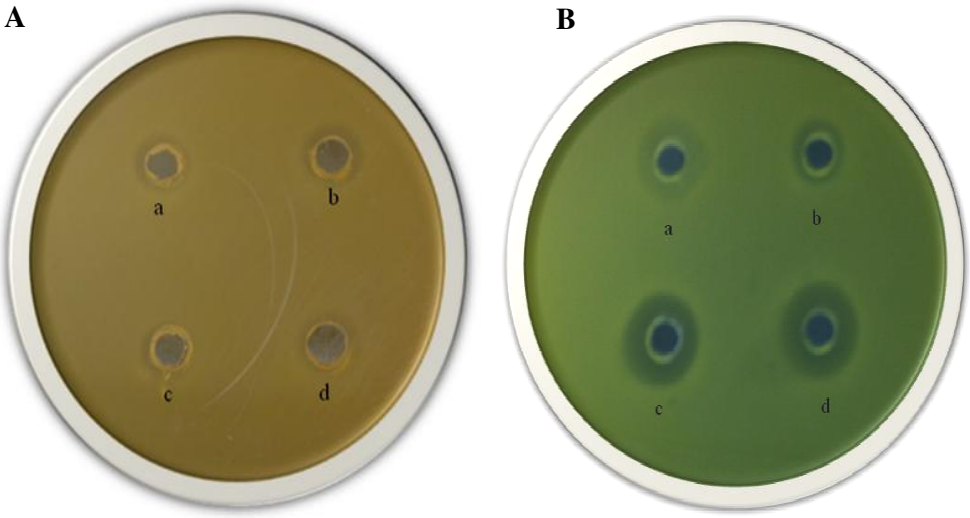

Figure 2.5. *Lb. paracasei* DPC 4715 bacteriocin production in MRS broth containing 0% and 5% NaCl, adjusted to pH 5 and 5.5 and incubated at 8°C. Wells: a, pH 5, 0% NaCl; b, pH 5, 5% NaCl; c, pH 5.5, 0% NaCl; d, pH 5.5, 5% NaCl. The bacteriocin production is shown by the formation of an inhibition zone around the wells. Indicator strain: *Lb. paracasei* DPC 2048 (A). *Lb. paracasei* DPC 4715 bacteriocin production in MRS broth containing 0% and 5% NaCl, adjusted to pH 5 and 5.5 and incubated at 30°C. Wells: a, pH 5, 0% NaCl; b, pH 5. 5% NaCl; c, pH 5.5, 0% NaCl; d, pH 5,5, 5% NaCl. The bacteriocin production is shown by the formation of an inhibition zone around the wells. Indicator strain: *Lb. paracasei* DPC 2048 (B).

These results showed that pH lower than 5.5 and the temperature used for ripening affected the production of bacteriocin; however, its absence from cheese might also be due to an interaction between the bacteriocin and other cheese ingredients. Hence, the bacteriocin was tested for its sensitivity to some proteases known to be present in milk by the well diffusion test and it was shown to be resistant to the action of plasmin, but sensitive to chymosin and cathepsin D (**Figure 2.6**).

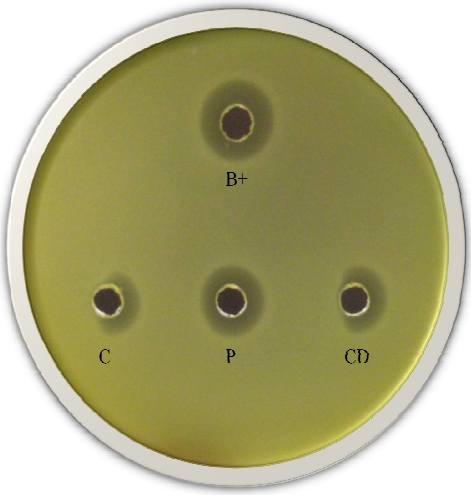

Figure 2.6. **Sensitivity of the bacteriocin to proteolytic enzymes.**
Wells: B+, positive control (*Lb. paracasei* DPC 4715 overnight culture supernatant); C, chymosin; P, plasmin; CD, cathepsin D. Sensitivity of the bacteriocin to the proteases is characterized by a half-moon-shaped zone of inhibition around the well. Indicator strain: *Lb. paracasei* DPC 2048.

Similar results were obtained by Sarantinopoulos et al. (2002) who showed that *Enterococcus faecium* FAIR-E 198 was able to produce a bacteriocin when incubated in skimmed milk, but no enterocin activity was observed when fermentations were performed in skimmed milk in the presence of rennet and $CaCl_2$ and when the strain was used as adjunct starter in Feta cheese making. Nascimento et al. (2008) showed that no antimicrobial effect was observed against *L. monocytogenes* Scott A and *S. aureus* ATCC 27154 in Minas Frescal cheese manufactured with the addition of bacteriocin-producing adjunct cultures *Lactococcus lactis* ssp. *lactis* ATCC 11454, *Lactobacillus plantarum* ALC 01 and *Enterococcus faecium* FAIR-E 198. However, Moreno et al. (2003) showed that the bacteriocin-producing strains *E. faecium* RZS C5 and *E. faecium* DPC 1146 were able to produce an enterocin with anti-listerial activity in Cheddar cheese.

2.5. Conclusion

In some cases, *in vitro* bacteriocin production does not guarantee *in situ* efficacy. Bacteriocin activity in cheese may be affected by adsorption to the caseins, by action of proteases, rennet, or by adsorption to the fat molecules or low diffusion capacity in the cheese matrix (Nascimento et al., 2008). This work showed that the bacteriocin-producing strain *Lb. paracasei* DPC 4715 is not suitable as an adjunct culture in Cheddar cheese for controlling the growth of NSLAB. No suppression growth of the sensitive indicator strain *Lb. casei* DPC 2048^{CM} occurred and no bacteriocin activity was detected by well diffusion assay in the experimental cheese during ripening. The reason could be due to an effect of low pH and ripening temperature on the production of bacteriocin in cheese, or to an interaction between the bacteriocion and some proteases known to be present in milk such as cathepsin D and/or an interaction with chymosin used for cheesemaking which in turn led to the inactivation of the bacteriocin.

2.6. References

AWAD, S., HASSAN, A. & HALAWEISH, F. 2005. Application of exopolysaccharide-producing cultures in making reduced fat Cheddar cheese. Composition and proteolysis. *Journal of Animal Science*, 83, 269-270.

BANKS, J. M. & WILLIAMS, A. G. 2004. The role of the nonstarter lactic acid bacteria in Cheddar cheese ripening. *International Journal of Dairy Technology*, 57, 145-152.

BENDALI, F., GAILLARD-MARTINIE, B., HEBRAUD, M. & SADOUN, D. 2008. Kinetic of production and mode of action of the *Lactobacillus paracasei* subsp *paracasei* anti-listerial bacteriocin, an Algerian isolate. *Lwt-Food Science and Technology*, 41, 1784-1792.

BERESFORD, T. & WILLIAMS, A. 2004. The microbiology of cheese ripening. *Cheese: chemistry, physics and microbiology*, Vol. 1. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 287-317.

BROMBERG, R., MORENO, I., DELBONI, R. R., CINTRA, H. C. & OLIVEIRA, P. T. V. 2005. Characteristics of the bacteriocin produced by *Lactococcus lactis* subsp *cremoris* CTC 204 and the effect of this compound on the mesophilic bacteria associated with raw beef. *World Journal of Microbiology & Biotechnology*, 21, 351-358.

CINTAS, L. M., CASAUS, M. P., HERRANZ, C., NES, I. F. & HERNANDEZ, P. E. 2001. Review: Bacteriocins of lactic acid bacteria. *Food Science and Technology Internationa*l, 7, 281-305.

FERCHICHI, M., FRERE, J., MABROUK, K. & MANAI, M. 2001. Lactococcin MMFII, a novel class IIa bacteriocin produced by *Lactococcus lactis* MMFII, isolated from a Tunisian dairy product. *FEMS Microbiology Letters*, 205, 49-55.

FOX, P. F. 1963. Potentiometric determination of salt in cheese. *Journal of Dairy Science*, 46, 744-745.

FOX, P. F., McSWEENEY, P. L. H. & LYNCH, C. M. 1998. Significance of non-starter lactic acid bacteria in cheddar cheese. *Australian Journal of Dairy Technology*, 53, 83-89.

GRAPPIN, R. 1986. Milk determination of nitrogen content Kjeldahl method and calculation of crude protein content. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 20A, 1-3.

HICKEY, D. K., KILCAWLEY, K. N., BERESFORD, T. P. & WILKINSON, M. G. 2006. Starter bacteria are the prime agents of lipolysis in Cheddar cheese. *Journal of Agricultural and Food Chemistry*, 54, 8229-8235.

HUGAS, M., PAGÉS, F., GARRIGA, M. & MONFORT, J. M. 1998. Application of the bacteriocinogenic *Lactobacillus sakei* CTC494 to prevent growth of Listeria in fresh and cooked meat products packed with different atmospheres. *Food Microbiology*, 15, 639-650.

IDF 1983. Milk, cream and evaporated milk - determination of total solids content (reference method)-provisional international IDF standard 21A-1982. *Milchwissenschaft-Milk Science International*, 38, 21-22.

IDF 1986. Cheese and processed cheese products determination of fat content gravimetric method reference method. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 1-5.

JACK, R. W., TAGG, J. R. & RAY, B. 1995. Bacteriocins of Gram-positive bacteria. *Microbiological Reviews*, 59, 171-200.

LOZO, J., JOVCIC, B., KOJIC, M., DALGALARRONDO, M., CHOBERT, J. M., HAERTLE, T. & TOPISIROVIC, L. 2007. Molecular characterization of a novel bacteriocin and an unusually large aggregation factor of *Lactobacillus paracasei* subsp *paracasei* BGSJ2-8, a natural isolate from homemade cheese. *Current Microbiology*, 55, 266-271.

MADKOR, S. A., TONG, P. S. & EL SODA, M. 2000. Ripening of Cheddar cheese with added attenuated adjunct cultures of lactobacilli. *Journal of Dairy Science*, 83, 1684-1691.

MALDONADO, A., RUIZ-BARBA, J. L. & JIMENEZ-DIAZ, R. 2003. Purification and genetic characterization of plantaricin NC8, a novel coculture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. *Applied and Environmental Microbiology*, 69, 383-389.

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

McSWEENEY, P. L. H., FOX, P. F., LUCEY, J. A., JORDAN, K. N. & COGAN, T. M. 1993. Contribution of the indigenous microflora to the maturation of Cheddar cheese. *International Dairy Journal*, 3, 613-634.

MIDJE, D. L., BASTIAN, E. D., MORRIS, H. A., MARTIN, F. B., BRIDGEMAN, T. & VICKERS, Z. M. 2000. Flavor enhancement of reduced fat Cheddar cheese using an integrated culturing system. *Journal of Agricultural and Food Chemistry*, 48, 1630-1636.

MILESI, M. M., McSWEENEY, P. L. H. & HYNES, E. R. 2008. Viability and contribution to proteolysis of an adjunct culture of *Lactobacillus plantarum* in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology*, 105, 884-892.

MORENO, M. R. F., REA, M. C., COGAN, T. M. & DE VUYST, L. 2003. Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. *International Journal of Food Microbiology*, 81, 73-84.

NASCIMENTO, M. S., MORENO, I. & KUAYE, A. Y. 2008. Applicability of bacteriocin-producing *Lactobacillus plantarum*, *Enterococcus faecium* and *Lactococcus lactis* ssp. *lactis* as adjunct starter in Minas Frescal cheesemaking. *International Journal of Dairy Technology*, 61, 352-357.

ONG, L., HENRIKSSON, A. & SHAH, N. P. 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb casei*, *Lb paracasei* and *Bifidobacterium* ssp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal*, 16, 446-456.

O'SULLIVAN, L., ROSS, R. P. & HILL, C. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, 84, 593-604.

PANGSOMBOON, K., KAEWNOPPARAT, S., PITAKPORNPREECHA, T. & SRICHANA, T. 2006. Antibacterial activity of a bacteriocin from *Lactobacillus paracasei* HL32 against *Porphyromonas gingivalis*. *Archives of Oral Biology*, 51, 784-793.

RIAZI, S., WIRAWAN, R. E., BADMAEV, V. & CHIKINDAS, M. L. 2009. Characterization of lactosporin, a novel antimicrobial protein produced by

Bacillus coagulans ATCC 7050. *Journal of Applied Microbiology*, 106, 1370-1377.

RODRIGUEZ, E., GONZALEZ, B., GAYA, P., NUNEZ, M. & MEDINA, M. 2000. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal*, 10, 7-15.

RYAN, M. P., REA, M. C., HILL, C. & ROSS, R. P. 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Applied and Environmental Microbiology*, 62, 612-619.

SALLAMI, L., KHEADR, E. E., FLISS, I. & VUILLEMARD, J. C. 2004. Impact of autolytic, proteolytic, and nisin-producing adjunct cultures on biochemical and textural properties of Cheddar cheese. *Journal of Dairy Science*, 87, 1585-1594.

SARANTINOPOULOS, P., LEROY, F., LEONTOPOULOU, E., GEORGALAKI, M. D., KALANTZOPOULOS, G., TSAKALIDOU, E. & VUYST, L. D. 2002. Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. *International Journal of Food Microbiology*, 72, 125-136.

SHAKEEL-UR-REHMAN, DRAKE, M. A. & FARKYE, N. Y. 2008. Differences between Cheddar cheese manufactured by the milled-curd and stirred-curd methods using different commercial starters. *Journal of Dairy Science*, 91, 76-84.

SHAKEEL-UR-REHMAN, FOX, P. F. & McSWEENEY, P. L. H. 2000. Methods used to study non-starter microorganisms in cheese: a review. *International Journal of Dairy Technology*, 53, 113-119.

SOBRINO-LOPEZ, A. & MARTIN-BELLOSO, O. 2008. Use of nisin and other bacteriocins for preservation of dairy products. *International Dairy Journal*, 18, 329-343.

TOBA, T., YOSHIOKA, E. & ITOH, T. 1991. Lacticin, a bacteriocin produced by *Lactobacillus-delbrueckii* subsp *lactis*. *Letters in Applied Microbiology*, 12, 43-45.

TODOROV, S. D. 2008. Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of the adsorption of bacteriocin AMA-K to Listeria sp. *Brazilian Journal of Microbiology*, 39, 178-187.

TODOROV, S. D., VAN REENEN, C. A. & DICKS, L. M. T. 2005. Optimized production of bacteriocin ST11BR, generated by *Lactobacillus paracasei* subsp. *paracasei* ST11BR isolated from traditional South African beer. *South African Journal of Science*, 101, 182-186.

TUMA, S., KUCEROVA, K. & PLOCKOVA, M. 2008. Isolation of anticlostridially active lactobacilli from semi-hard cheese. *Czech Journal of Food Sciences*, 26, 324-332.

YAAKOUBI, K., BENKERROUM, N., WIOROWSKI, F., SANSON, F., HAYDERSAH, J. & CHEVALLIER, I. 2009. Development of a multiwell antagonistic activity assay for the detection of bacteriocin production by lactic acid bacteria. *Journal of Rapid Methods and Automation in Microbiology*, 17, 32-45.

3. Role of coryneform bacteria in the ripening of Cheddar cheese

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3.1 Abstract

Coryneform bacteria are generally found on the surface of smear-ripened cheeses and contribute to cheese ripening by the production of proteolytic and lipolytic enzymes and pigments which are responsible for the formation of a glistening, viscous and red-orange-yellow layer at the surface. In this study, *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305, originally isolated from a smear-ripened cheese, were used as adjunct cultures in Cheddar cheese and their role during ripening was evaluated. The strains were grown to a final concentration of 10^9 cfu/ml and the cultivated broth was added to the cheesemilk at level of 1% v/v. Control cheese (C1) was made with 1% of sterile broth, cheese 2 (C2) contained 1% of a culture of *Microbacterium casei* DPC 5281, cheese 3 (C3) contained 1% of *Corynebacterium casei* DPC 5281, cheese 4 (C4) contained 1% of *Corynebacterium variabile* DPC 5305. Numbers of coryneform bacteria in experimental cheeses were around ~10^8 cfu/g in all trials and decreased by ~2 log cycles during the first week of ripening but the numbers remained almost constant thereafter. The composition of each cheese at 14 days was typical for Cheddar cheese indicating that the use of coryneform bacteria as adjunct cultures did not have significant effect on composition. No differences between the urea-PAGE electrophoretograms of experimental and control cheeses were observed. Levels of pH 4.6-soluble nitrogen after 60 and 180 days of ripening observed in experimental cheeses were significantly higher than control cheese in Trial A, whereas in Trials B and C, only cheeses C2 and C3 were significantly higher than control cheese. After 60 and 180 d of ripening all the experimental cheeses showed a significantly higher level of total free amino acids than the control cheese and higher levels of some individual free amino acids at 180 d. Cheese C4 made with *Corynebacterium variabile* DPC 5305 had the highest level of total free fatty acids in all trials whereas no significant difference was observed in levels of free fatty acids between the control and experimental cheeses. These results show that *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 helped to accelerate the ripening of Cheddar cheese by producing proteolytic enzymes; moreover the lattermost strain also produced lipases as increased levels of total fatty acids were observed in cheese C4.

3.2. Introduction

Coryneform bacteria are asporogenous, aerobic Gram-positive irregular rods or cocci, belonging to the class *Actinobacteria* and include organisms from the genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium* and *Microbacterium*. They are generally environmental residents and/or commensal flora of humans, although some species have been found in fermented dairy products (Denis and Irlinger, 2008). Coryneform bacteria of dairy interest grow typically on the surface of cheeses such as Tilsit, Brick, Limburger and Gubben. These varieties are called smear-ripened cheeses because of the presence of a glistening, viscous and red-orange-yellow layer on the cheese surface (Brennan et al., 2004).

Traditionally, the surface of old smear-ripened cheeses is washed with a brine solution which was then used to wash, and so inoculate, the surface of young cheeses. Therefore, all the microorganisms are transferred from the old to the young cheese. This method has the disadvantage of transferring also pathogenic microorganisms, like *Listeria* spp. and other contaminants such as enterobacteria, enterococci and moulds eventually present on the surface of the old cheeses. For this reason, the use of commercial smear preparation containing different strains such as *Brevibacterium linens*, *Debaryomyces hansenii* and/or *Geotrichum candidum* is preferable in order to improve hygiene (Beresford et al., 2001). Coryneform bacteria come generally from the cheese milk, cheese brine, cheesemaking utensils, cheese factory environment, human skin and start developing on the surface of cheeses after yeasts, which grow during the first days of ripening, oxidize the lactate to CO_2 and H_2O and deaminate amino acids with the consequent production of NH_3 and increase in pH from 5.0 to > 6.

The rise in pH, the production of stimulatory growth substances, such as vitamins, by yeast and the ripening conditions (high temperature of 10 to 14 °C and high relatively humidity of >95%) allows the growth of a secondary salt-tolerant microbiota composed mainly of staphylococci, micrococci and coryneforms (Valdes Stauber et al., 1997; Corsetti et al., 2001; Chamba and Irlinger, 2004; Bockelmann et al., 2005). It is known that coryneform bacteria play an important role in the ripening of smear-cheeses by producing enzymes which contribute to lipolysis, proteolysis and to flavour, colour, aroma and texture development (Brennan et al., 2001; Gobbetti et al., 2001; Curtin et al., 2002).

Curtin (2001) reported that *C. ammoniagenes* 8, *Corynebacterium* spp. B, E and V have cystathionine lyase activity, but they did not show any L-methionine aminotransferase activity. The same author showed that *Corynebacterium* sp. sub-group *flavescens* CA8 had decarboxylase activity on phenylalanine and tyrosine, *M. gubbeenense* DPC5288 had decarboxylase activity only on phenylalanine, whereas, both strains had aminotransferase activity on phenylalanine, tyrosine and tryptophan. Initially, Brennan et al. (2001) isolated a bacterial strain from the surface of a smear-ripened cheese which was named *C. mooreparkense*. Following a further study, Gelsomino et al. (2005) showed that there are no genomic difference between isolates of *C. mooreparkense* and *C. variabile* and concluded that the two species are heterotypic synonym. Gobbetti et al. (2001) showed that *C. variabile* NCDO 2101 produces an extracellular PepI active on Pro-containing dipeptides and tripeptides which may contribute to proteolysis and flavour formation of smear-cheeses. Curtin

et al. (2002) reported that brevibacteria, corynebacteria, staphylococci and brachybacteria strains isolated from the surface of Tilsit and Gubbeen cheeses have esterase activity, tripeptidase and dipeptidase activity especially on dipeptides containing Met residue. Moreover, all the smear strains have cystathionine lyase activity, whereas only one strain showed L-methionine aminotransferase activity. These enzymes are involved in the catabolism of sulphur amino acids, which on degradation, lead to the liberation of sulphur compounds such as methanethiol, *S*-methylthioesthers, dimethyldisulphide, responsible for the "cheesy", "cabbage" and "garlic" flavor in cheese such as Cheddar, Limburger and Camembert (Bonnarme et al., 2001; Curtin et al., 2002). *C. casei, C. mooreparkense* and *M. gubbeenense* produce methanthiol from methionine, which is one of the major flavour compounds in smear-ripened cheeses (Brennan et al., 2001; Yvon and Rijnen, 2001).

Coryneform bacteria are also able to produce *S*-methylthioesthers from carboxylic acids such as acetic, propionic, isobutyric or isovaleric acids (Yvon and Rijnen, 2001). In this study, *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 were used as adjunct cultures in Cheddar cheese and their role during ripening was evaluated.

3.3. Materials and methods

3.3.1. Strains

The bacteria strains used for cheesemaking were *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 from the collection of the Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. The strains were grown for 2 d at 30°C under agitation (150 rpm) in 3 L flask containing 500 ml of broth consisting of casein hydrolysate (10 g/l), peptone (5 g/l), yeast extract (2.5 g/l), glucose (1 g/l), skim milk powder (1 g/l) and NaCl (30 g/l) (Curtin et al., 2002).

3.3.2. Cheesemaking

The strains were grown to a final cell count of 10^9 cfu/ml and the cultivated broth was added to the cheese milk at level of 1% (v/v). Control cheese (C1) was made with 1% of sterile broth, cheese 2 (C2) contained 1% of a culture of *Microbacterium casei* DPC 5281, cheese 3 (C3) contained 1% of *Corynebacterium casei* DPC 5281, cheese 4 (C4) contained 1% of *Corynebacterium variabile* DPC 5305. Cheesemaking was carried out in triplicate on different days. Whole milk was standardized to casein: fat ratio of 0.7:1.0, pasteurised at 72ºC for 15 s and cooled to 30ºC. DVS starter culture (R604 Chr-Hansen, Hørsholm, Denmark) was added at a rate of level 0.03% w/v to cheesemilk. Chymax-180 (Chr. Hansen, Hørshom, Denmark) was used as coagulant. Whey was drained at pH 6.2 and the curd cheddared, milled at pH 5.2 and salted at a level of 2.5 % (w/w). After pressing the curd at 150 kPa for 18 h, the cheeses were vacuum packed in a vacuum bag and ripened for 180 days at 8°C.

3.3.3. Microbiological analysis

Samples were taken under aseptic conditions using a cheese trier at day 1, 7, 14 and every 30 days thereafter for 6 months. Ten g of cheese were homogenized in 90 ml of sterile trisodium citrate (2% w/v) in a stomacher bag (Stomacher 400, Seward Limited, Worthing, West Sussex, UK) for 5 min. Starter cells were enumerated on LM17 agar after incubation at 30° for 3 days, NSLAB were enumerated on Rogosa medium incubated anaerobically at 30°C for 5 days, coryneform bacteria were enumerated on Plate Count Agar (PCA) with 6% of NaCl after incubation at 30° for 3 days.

3.3.4. Compositional analysis

Protein (Grappin, 1986), fat (Gerber method; IDF 1986), moisture (oven drying at 102°C; IDF 1983), salt (Fox, 1963) and pH were determined in triplicate after 14 days of ripening. The pH was measured in cheese slurry (1:1 cheese:water) using a combined glass electrode (PHC3001-8, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Copenhagen, Denmark).

3.3.5. Analysis of cheese

The pH 4.6 soluble and insoluble fractions of the cheeses were prepared in according to the method of Kuchroo and Fox (1982). The extraction was carried out in triplicate for each cheese. The N content of the pH 4.6-soluble fraction of the cheeses was measured by the macro-Kjeldahl method (Grappin, 1986) and expressed as a % of the total N content of the cheeses. Urea-polyacrylamide gel electrophoresis (PAGE) was carried out on the samples after 60 d and 180 d of ripening, using the method of Andrews (1983) as modified by Shalabi and Fox (1987). Gels are stained directly by the method of Blakesely and Boezi (1977). Peptide profiles of the pH 4.6-soluble fractions were determined by reverse-phase HPLC using a Varian HPLC system (Varian Associates Inc., Walnut Creek, CA, USA) according to the method described by Sousa and McSweeney (2001). Total free amino acids (FAA) were determined for the 14 d, 60 d and 180 d old cheeses by the trinitrobenzenesulphonic (TNBS) acid method (Polychroniadou, 1988). Individual free amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Welwyn Garden City, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column. Levels of free fatty acids at 180 d were measured by gas chromatograpy (GC) as described by De Jong and Badings (1990). Extraction of free individual fatty acids (FFA) for GC analysis was carried out by using a solid-phase extraction technique; 500 mg Varian Bond Elut-NH_2 cartridges were used. FFAs were quantified using a gas chromatograph (Varian Star 3400 CX with Varian 8200 CX autosampler and flame ionization detector (300ºC) interfaced with Star Chromatography Workstation 5.0 software for data acquisition; Varian Analytical Instruments, Harbor City, CA, USA). A wall-coated open tubular fused silica capillary column (25 m length x 0.32 mm internal diameter) coated with FFAP-CB was used. FFAs were separated and identified by reference to known standards and quantified by peak area. A standard calibration mix of 17 fatty acid (including internal standards, valeric acid C5:0; pelargonic acid C9:0; margaric acid C17:0) was prepared at concentrations 1000, 500, 300, 200, 100 and 50 ppm. One-way analysis of variance of data from of the composition of the cheeses was performed using SPSS Version 18.0 for Windows XP (SPSS Inc., Chicago, IL, USA). Significance was declared at $P \leq 0.05$. The HPLC peak height data were pre-processed according to the method of Piraino et al. (2004) and principal component analysis (PCA) was performed using SPSS Version 18.0 for Windows XP.

3.3.6. Biogenic amines

Lyophilized 4.6-soluble fractions samples (obtained from 1 ml of extract) were diluted using perchloric acid (0.6 $mol.l^{-1}$) and 0.5 mL of obtained mixtures (vigorously vortexted) were subjected to derivatisation with dansylchloride according to Dadáková et al. (2009); 1,7-heptanediamine was used as an internal standard. The derivatised samples were filtered (porosity 0.22 μm) and applied on a column (Zorbax Eclipse XDB-C18, 150 x 4.6 mm, 3.5μm, Agilent Technologies, Santa Clara, CA, USA). The concentration of eight biogenic amines (histamine, tyramine, phenylethylamine, tryptamine, putrescine, cadaverine, spermidine, spermine) was monitored by an high performance liquid chromatography system equipped with a binary pump; an autosampler (LabAlliance, State College, MD, USA); a column thermostat; a UV/VIS DAD detector (λ = 254 nm) and a degasser (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA). The conditions for separation of the

monitored BA are described by Smělá et al. (2004). Each of the lyophilized samples was derivatised three times. Standards, reagents and eluents were obtained from Sigma Aldrich (St. Louis, MO, USA).

3.4. Results and discussion

Coryneform bacteria are important for colour and aroma development in smear-ripened cheeses. Many contribute to cheese ripening by producing proteinases, peptidases, lipases and liberating amino acids and fatty acids which are precursors of various flavour compounds. In this study, *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 were used separately as adjunct cultures in Cheddar cheese and their role during ripening was evaluated.

3.4.1. Compositional analysis

The composition of each cheese at 14 days was typical for Cheddar cheese indicating that the use of coryneform bacteria as adjunct cultures did not have a significant effect ($P \leq 0.05$) on any compositional parameter (**Table 3.1**).

Similar results were also shown by other authors who used different adjunct cultures in Cheddar cheese, such as *Enterococcus faecium Lactobacillus paracasei* ssp *paracasei*, *Lactobacillus plantarum* and attenuated lactobacilli (Gardiner et al., 1999; Lynch et al., 1999; Madkor et al., 2000; Milesi et al., 2008; Burns et al., 2012).

Table 3.1. pH and composition of Cheddar cheeses at 14 days of ripening.
C1, Control cheese; C2, cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

	Cheese	pH	% Salt	% Fat	%Moisture	% Nitrogen	% Protein
Trial A	**C1**	4.89±0.1[a]	1.40±0.01[a]	30.00±0.00[a]	40.27±0.32[a]	3.56±0.10[a]	22.72±0.62[a]
	C2	4.93±0.02[a]	1.30±0.01[a]	30.33±0.00[a]	40.17±0.33[a]	3.51±0.08[a]	22.36±0.52[a]
	C3	4.88±0.02[a]	1.37±0.00[a]	29.67±0.58[a]	40.34±0.31[a]	3.42 ±0.02[a]	21.81±0.13[a]
	C4	4.96±0.03[a]	1.36±0.15[a]	30.00±0.00[a]	39.71±0.15[a]	3.53 ±0.03[a]	22.55±0.20[a]
Trial B	**C1**	4.92±0.07[a]	1.23±0.00[a]	31.00±0.00[a]	40.10±0.81[a]	3.41±0.15[a]	21.78±0.93[a]
	C2	4.92±0.03[a]	1.25±0.01[a]	31.33±0.58[a]	40.04±0.23[a]	3.35±0.09[a]	21.39±0.59[a]
	C3	4.94±0.04[a]	1.21±0.03[a]	31.67±0.58[a]	39.58±0.07[a]	3.40±0.03[a]	21.68±0.22[a]
	C4	4.97±0.02[a]	1.22±0.11[a]	31.33±0.58[a]	39.36±0.26[a]	3.60±0.11[a]	22.95±0.72[a]
Trial C	**C1**	4.97±0.04[a]	1.28±0.03[a]	30.67±0.58[a]	40.82±0.15[a]	3.60±0.07[a]	23.00±0.43[a]
	C2	4.94±0.06[a]	1.34±0.02[a]	30.33±0.58[a]	40.97±0.56[a]	3.62±0.06[a]	23.10±0.39[a]
	C3	4.91±0.03[a]	1.36±0.02[a]	31.33±0.58[a]	40.56±0.58[a]	3.70±0.03[a]	23.60±0.21[a]
	C4	4.96±0.09[a]	1.29± 0.00[a]	31.00±0.00[a]	40.18±0.25[a]	3.71±0.08[a]	23.68±0.50[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviations.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

3.4.2 Microbiological analysis

Starter lactic acid bacteria (SLAB) were present at level of ~10^9 cfu/g after manufacture and their evolution during ripening in Trials A, B and C is shown in **Figure 3.1 A, B, C**. In Trial A, their numbers decreased by~ 1 log cycle after 60 days in cheeses C3, C4 and by ~ 2 log cycles after 90 days in C1 and C2. At the end ripening they were present at level of 10^5 cfu/g in cheeses C1, C4 and 10^6 cfu/g in cheeses C2 and C3.

In Trial B, they dropped from 10^9 cfu/g to 10^8 cfu/g in all cheeses after 90 days and reached levels of 10^7 cfu/g in C1, C2, C3 and 10^6 cfu/g in C4 after 150 days. In Trial C the number of starter lactic acid bacteria decreased by ~ 1 log cycle in cheeses C1, C2 after 60 days, and by this amount in cheeses C3, C4 after 90 days. They reached a level of 10^6 cfu/g in C1 and 10^7 cfu/g in C2, C3 and C4 after 120 days of ripening. Similar results were reported by Shakeel-ur-Rehman et al. (2000), McSweeney et al. (1993a) and Hickey et al. (2006), for changes in starter numbers during the ripening of Cheddar cheese.

Numbers of non-starter lactic acid bacteria (NSLAB) increased slightly during the first 30 days in all cheeses in Trials A and C and reached levels of 10^4 cfu/g and 10^3 cfu/g, respectively. In Trial B, numbers reached 10^4 cfu/g in C1, C2, C3 and 10^5 cfu/g in C4 after 60 days of ripening (**Figure 3.2 A, B, C**). Similar results were also reported by Lane et al. (1997), Swearingen et al. (2001) and Broadbent et al. (2003) who studied the growth of non-starter lactic acid bacteria in cheese. It is known that the non-starter microbiota of Cheddar cheese increases from a low number (10^2-10^3 cfu/g) at the early stage of ripening to become the dominant viable microbiota (10^7-10^8 cfu/g) after 3-4 months (Fox et al., 1998).

The initial number of coryneform bacteria in the cheeses C2, C3 and C4 at day 1 was ~10^8 cfu/g in all trials (**Figure 3.3 A, B, C**). Generally, numbers started decreasing by ~2 log cycles during the first week and the level remained almost constant thereafter during ripening. In particular, after 7 days of ripening, numbers of viable *M. casei* DPC 5281 decreased from 10^8 to ~10^6 cfu/g in cheese C2 in all trials; the numbers of *C. casei* DPC 5293 reduced from 10^8 ~10^6 cfu/g in Trials A, B and from 10^8 to ~10^5 cfu/g in Trial C; numbers of *C. variabile* DPC 5305 decreased from 10^8 to ~10^6 cfu/g in Trials A and B and from 10^8 to ~10^5 cfu/g in Trial C. At the end of ripening, coryneforms were present at 10^5 cfu/g in all cheeses in Trials A and B; 10^4 cfu/g in C2, C3 and 10^5 cfu/g in C4 in Trial C.

Coryneform bacteria are facultatively anaerobic and generally grow on the surface of smear cheeses. In this study they were used as adjunct cultures and inoculated into the milk, therefore, their growth in cheese was probably affected by the absence of oxygen. In fact, coryneform population did not grow during ripening but started dying off during the first week and perhaps released intracellular enzymes which could contribute to ripening. It might be possible that the coryneform strains used were acid-sensitive and the co-inoculum into the milk with starter lactic acid bacteria could have affected their growth (Goerges et al., 2008). NSLAB colonies were not detected on PCA medium containing 6% of NaCl used to count coryneform bacteria. Colonies were checked microscopically and showed a typical irregular rod shape of coryneforms.

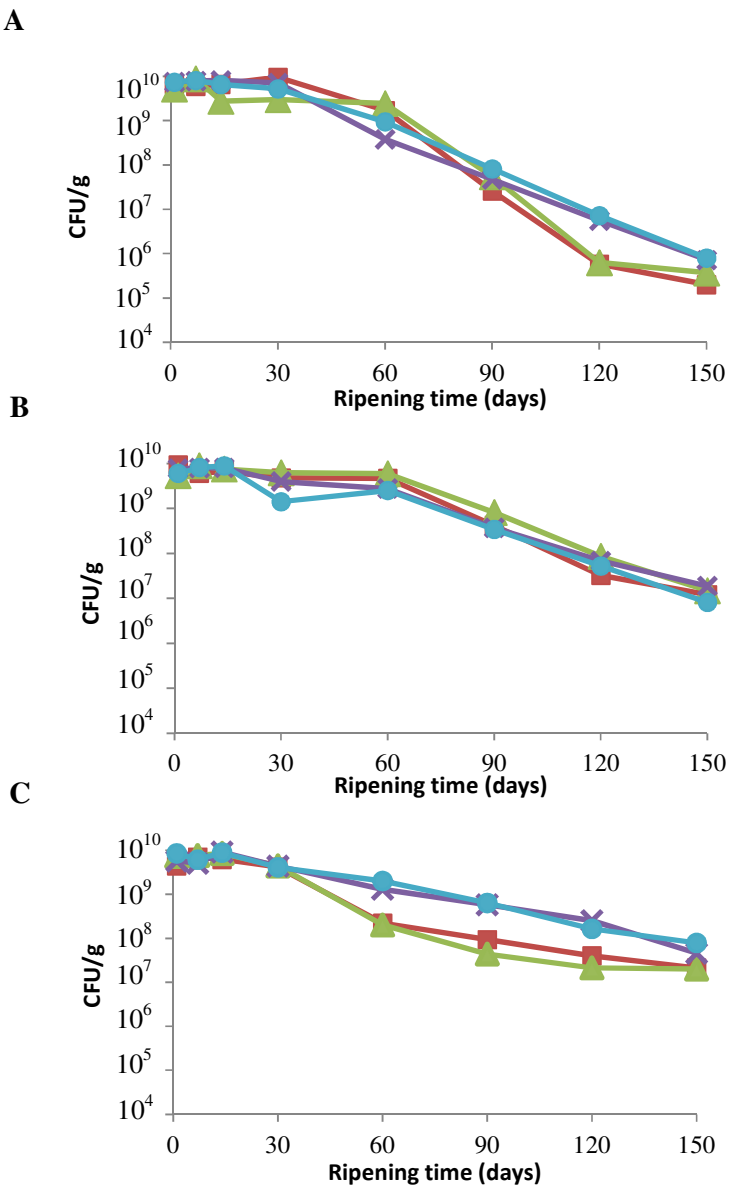

Figure 3.1. Growth of starter lactic acid bacteria (SLAB) on LM17 agar incubated 30°C for 3 days during ripening of Cheddar cheeses in Trials A, B, C. C1 (■), Control cheese; C2 (▲), Cheese made with *Microbacterium casei* DPC 5281; C3(×), cheese made with *Corynebacterium casei* DPC 5293; C4 (●), cheese made with *Corynebacterium variabile* DPC 5305.

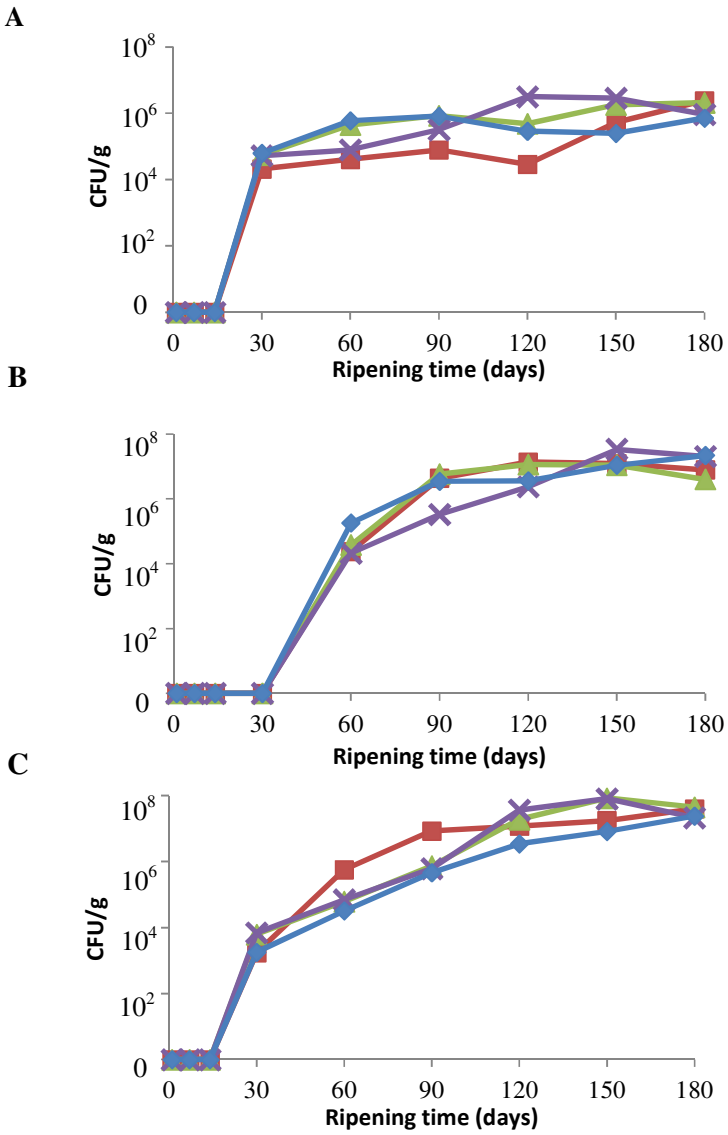

Figure 3.2. Growth of non-starter lactic acid bacteria (NSLAB) enumerated on Rogosa agar incubated at 30°C for 5 days during ripening of Cheddar cheeses in Trial A, B, C. C1 (■), Control cheese; C2 (▲), Cheese made with *Microbacterium casei* DPC 5281; C3(×), cheese made with *Corynebacterium casei* DPC 5293; C4 (●), cheese made with *Corynebacterium variabile* DPC 5305.

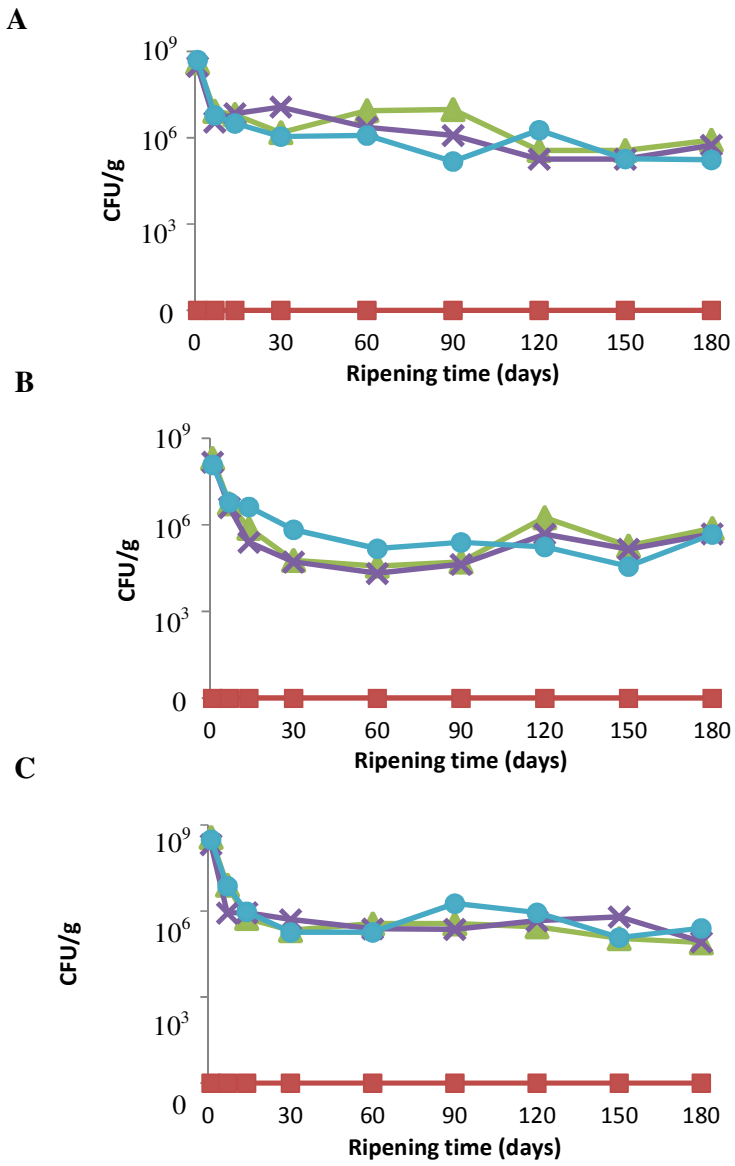

Figure 3.3. Numbers of coryneform bacteria enumerated on PCA containing 6% salt incubated at 30° for 3 days during ripening of Cheddar cheeses in Trial A, B, C. C1 (■), Control cheese; C2 (▲), Cheese made with *Microbacterium casei* DPC 5281; C3(×), cheese made with *Corynebacterium casei* DPC 5293; C4 (●), cheese made with *Corynebacterium variabile* DPC 5305.

3.4.3. Assessment of proteolysis

3.4.3.1. Urea-PAGE

Electrophoretograms of the cheeses after 60 and 180 days of ripening are shown in **Figures 3.4** and **3.5**. Proteolysis was typical for Cheddar cheese; both α_{s1}-casein and β-casein were degraded gradually during ripening by the action of chymosin and plasmin, respectively, while γ-caseins accumulated in all cheeses. No differences between the experimental and control cheeses were observed. These results confirm that proteolysis in Cheddar cheese is mainly due to the action of chymosin and plasmin (McSweeney et al., 1993b; Shakeel-Ur Rehman et al., 2000; Sousa et al., 2001; Upadhyay et al., 2004). Moreover, Ghosh et al. (2009) showed that *C. casei* 95332 and *C. variabile* 95324 have no proteolytic activity against casein during growth in modified milk medium, irrespective of whether extracellular or intracellular enzymes fraction were assayed. In agreement with the results of this study were also Lynch et al. (1999), Hynes et al. (2003), Di Cagno et al. (2006) and Milesi et al. (2008), who used a range of different adjunct cultures and showed that thay had no effect on proteolysis.

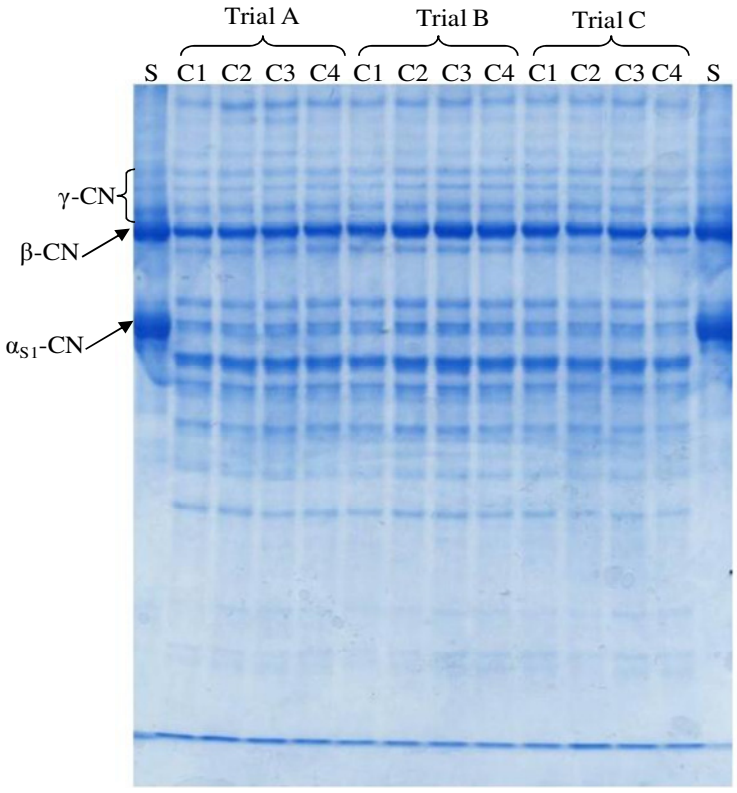

Figure 3.4. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses at 60 d of ripening in Trial A, B, C.
C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

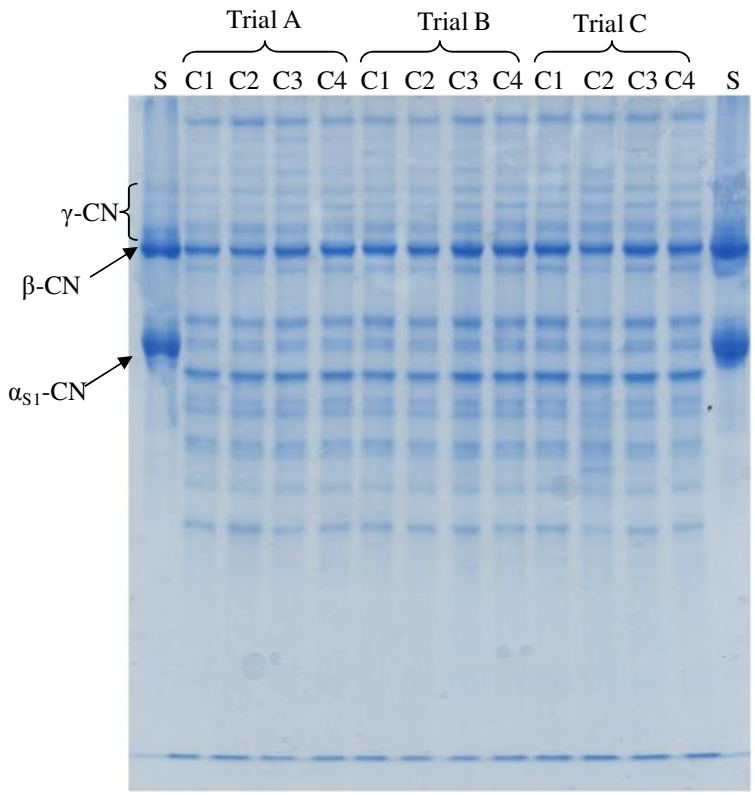

Figure 3.5. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses at 180 d of ripening in Trial A, B, C.
C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

3.4.3.2 Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed on the pH 4.6-soluble fraction of the 60 and 180 days old cheeses. Only results from Trial A are shown in **Figure 3.6** and **3.7**.

At 60 days of cheese ripening chromatograms were similar for all the cheeses; however, minor variations in the height of certain peaks were seen. The peaks at ~ 55 min were present at higher levels in the cheese made with *M. casei* DPC 5281 than in the control cheese. A similar occurrence was seen with the same peaks eluting at ~ 55 min at 180 days of ripening: the peak in cheese made with *C. variabile* DPC 5305 and the peak in control cheese had a higher height than the peaks in the other experimental cheeses. In general, no major differences were observed between the control and the coryneform bacteria inoculated cheese in terms of their peptide profiles determined by RP-HPLC.

Figure 3.8 A, B and **3.9 A, B** shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for of the processed peak height data from RP-HPLC of the pH 4.6 soluble extract at 60 and 180 days of ripening in Trial A, B and C. At 60 days, cheeses C1 and C4 are well grouped on the first principal component. After 180 days of ripening all cheese are well grouped together on the first principal component apart from cheese C2 from Trial A and C and cheese C3 from trial A.

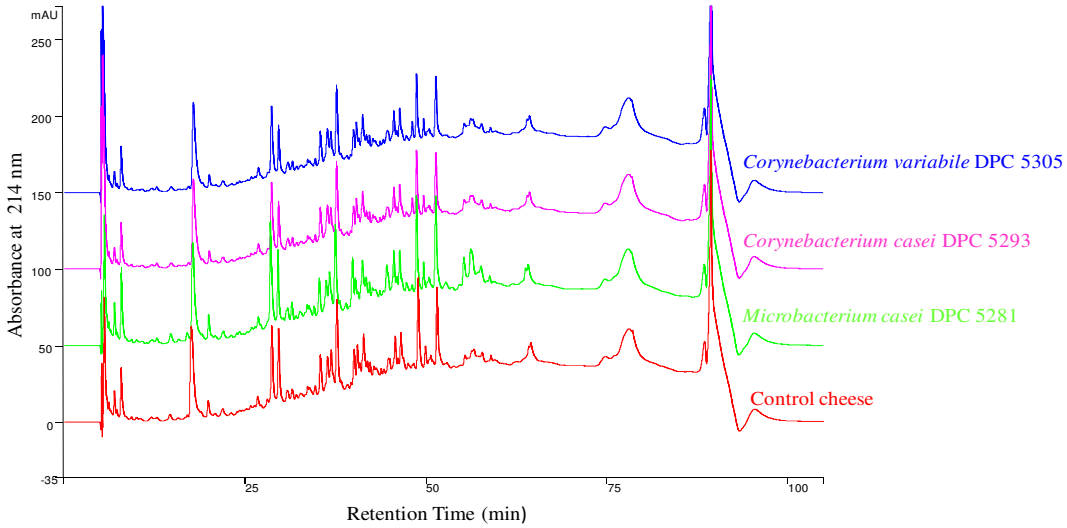

Figure 3.6. Reversed-phase HPLC chromatograms of Control and experimental Cheddar cheeses made with coryneform adjuncts at 60 days of ripening in Trial A.

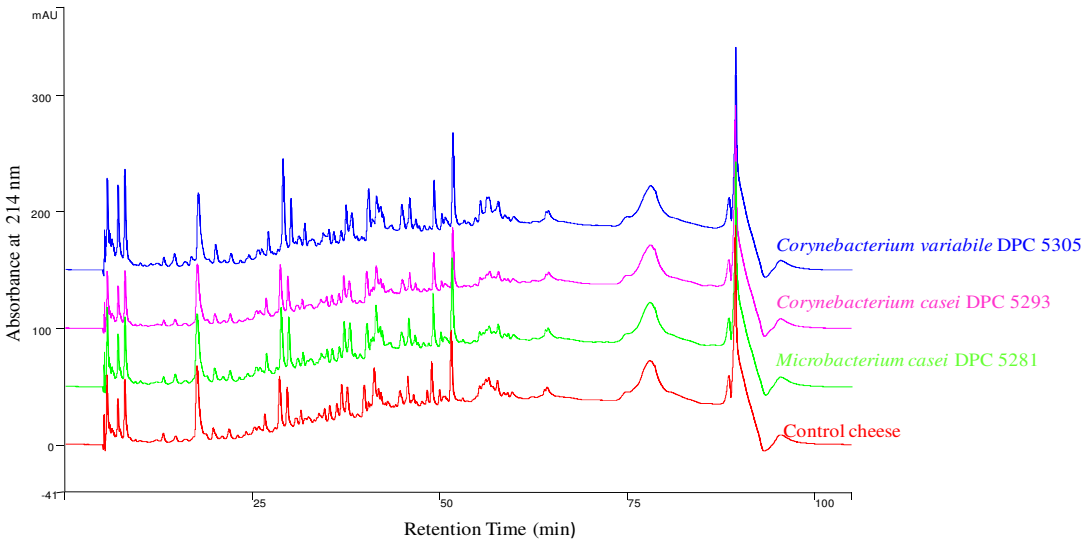

Figure 3.7. Reversed-phase HPLC chromatograms of Control and experimental Cheddar cheeses made with coryneform adjuncts at 180 days of ripening in Trial A.

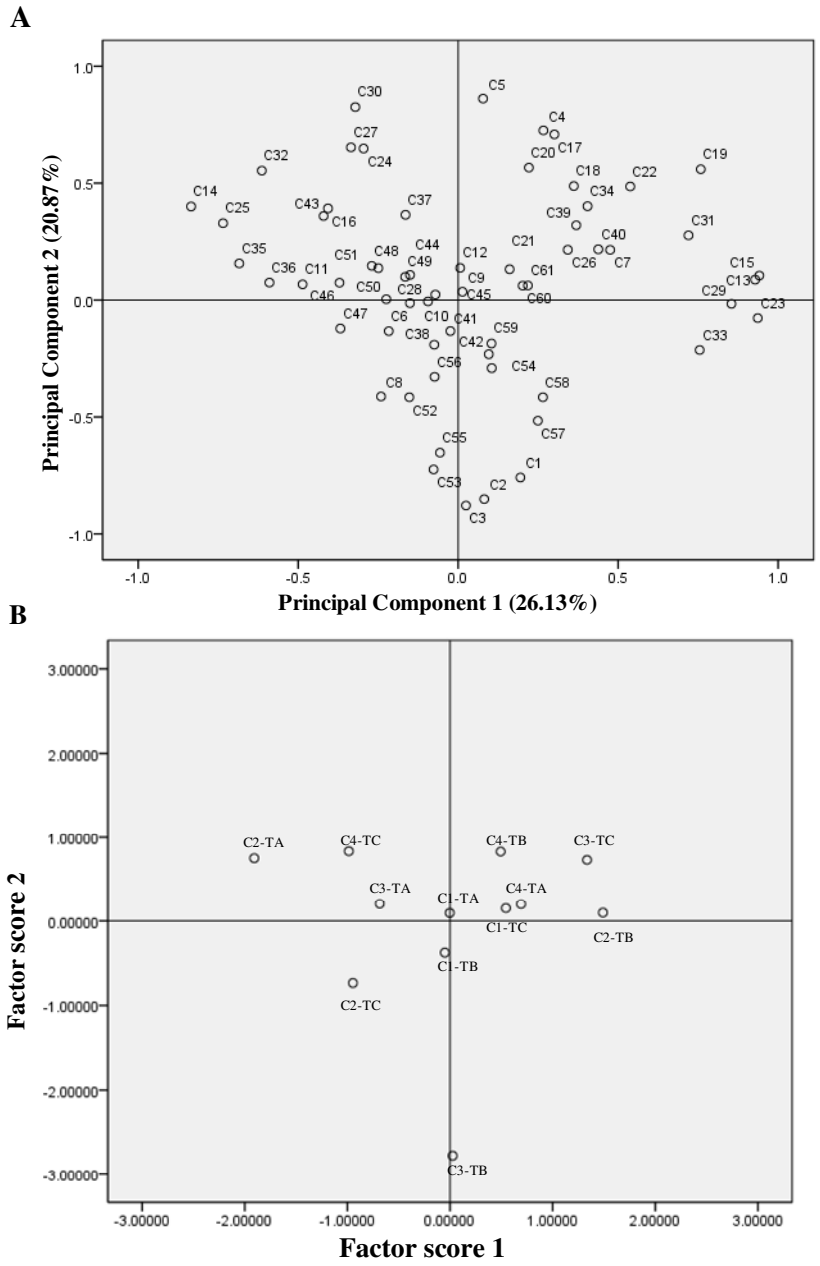

Figure 3.8. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheeses at 60 d of ripening in Trials A, B and C. C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

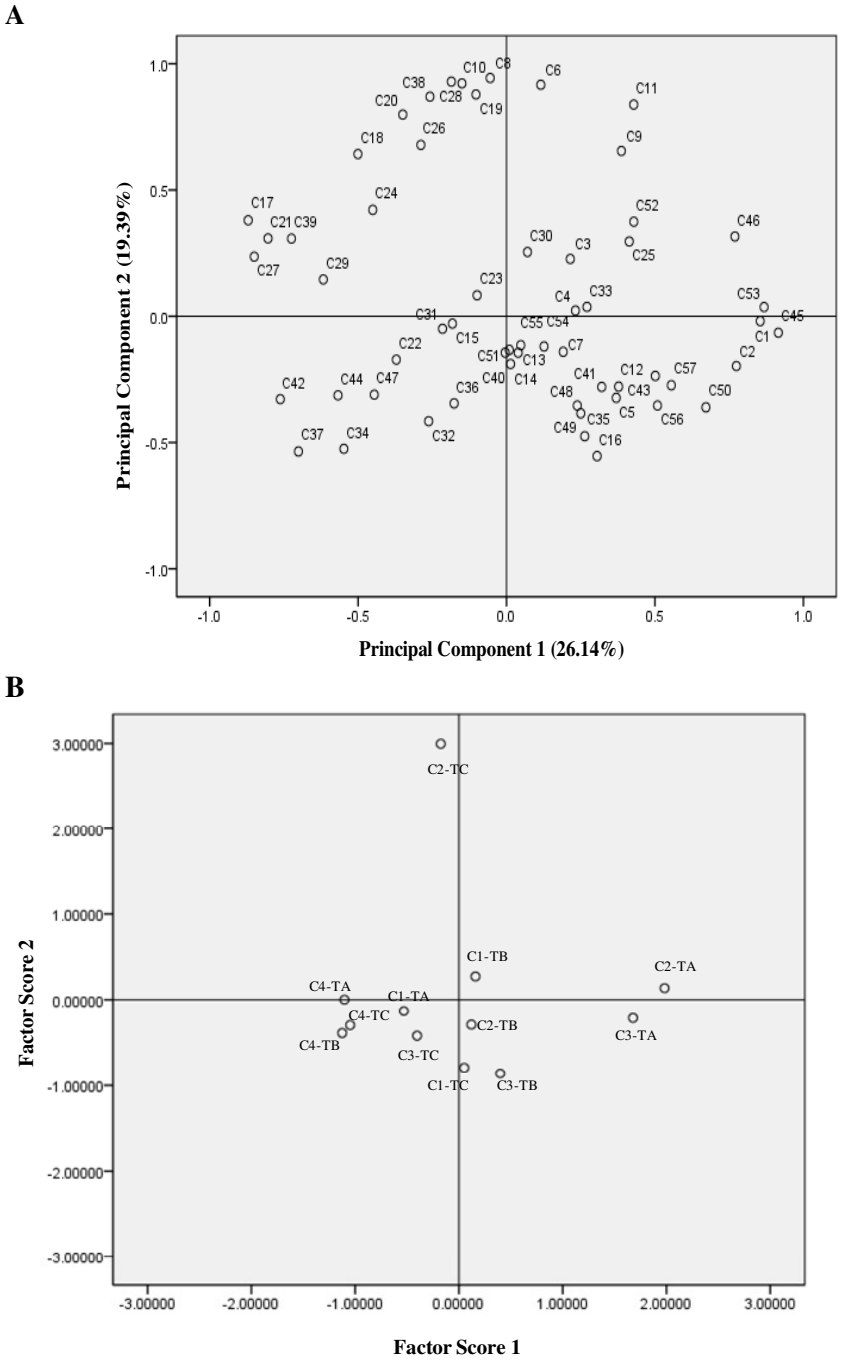

Figure 3.9. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheeses at 180 d of ripening in Trials A, B and C. C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

3.4.3.3. pH 4.6-Soluble nitrogen

Mean levels of pH 4.6-soluble nitrogen as percentage of total nitrogen at 14 d were found not to be significantly different ($P \leq 0.05$) in all cheeses in Trials A, B, C. (**Table 3.2**). Levels of pH 4.6-soluble nitrogen after 60 and 180 days of ripening observed in experimental cheeses C2, C3, C4 were significantly higher ($P \leq 0.05$) than in control cheese C1 in Trial A, whereas in Trial B and C, only levels in cheeses C2 and C3 were significantly higher ($P \leq 0.05$) than control cheese C1. Although it is known that the liberation of pH 4.6-soluble nitrogen in Cheddar cheese is mainly due to chymosin and plasmin, coryneform strains contributed to increase the level of pH 4.6-soluble nitrogen in the cheeses C2, C3, C4 in Trial A and in cheese C2, C3 in Trial B and C. Similar results are reported by authors who used different adjunct cultures, such as lactobacilli (Lynch et al., 1996; Kenny et al., 2006), probiotic lactobacilli and bifidobacteria (Ong et al., 2006). No differences in the level of pH 4.6-soluble nitrogen were detected in cheeses made with adjunct cultures and the control cheese.

Table 3.2. Mean levels of pH 4.6-soluble nitrogen as percentage of total nitrogen in Cheddar cheeses at 14, 60 and 180 days of ripening.
C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

%TN	Cheese	Ripening time		
		14d	60d	180d
Trial A	C1	11.74±0.19[a]	16.64±0.33[a]	24.70±0.63[a]
	C2	12.30±0.29[a]	19.75±0.57[b]	28.03±0.48[c]
	C3	12.47±0.52[a]	18.85±0.83[b]	27.46±0.85[bc]
	C4	11.70±0.45[a]	18.65±0.76[b]	26.44±0.07[b]
Trial B	C1	10.61±0.57[a]	17.60±0.57[a]	25.66±0.88[a]
	C2	11.01±0.22[a]	19.87±0.53[b]	28.21±0.43[b]
	C3	11.13±0.11[a]	19.43±0.45[b]	28.14±0.41[b]
	C4	10.63±0.02[a]	17.90±0.47[a]	26.00±0.32[a]
Trial C	C1	10.45±0.33[a]	16.05±0.80[a]	24.15±0.21[a]
	C2	10.37±0.76[a]	19.32±0.73[b]	27.78±0.13[b]
	C3	10.57±0.26[a]	18.46±0.15[b]	26.88±0.95[b]
	C4	10.24±0.24[a]	16.77±0.26[a]	24.54±0.12[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviations.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

3.4.3.4. Free and individual amino acids

The levels of total free amino acids at 14, 60 and 180 days were determined in cheeses by TNBS assay and were expressed as mg leucine/mg cheese (**Table 3.3**). Statistically significant differences ($P \leq 0.05$) between control cheese and experimental cheeses were found at 14 days in Trial A, where C2 and C3 contained a higher level of total amino acids than control cheese C1. In particular, cheese C3, made with *C. casei* DPC 5293 contained the highest level. In contrast, no significant differences existed between the mean level of total free amino acids in all cheeses in Trials B and C at 14 days of ripening. After 60 days, levels of total amino acids were significantly higher in all the experimental cheeses than the control cheeses in all trials. In particular, C2 and C3 showed the highest level in Trial A, whereas C2 had the highest level in Trial C. At the end of ripening, all experimental cheeses showed a significantly higher level of free amino acids than the control cheese in all trials, confirming the ability of coryneform bacteria to produce peptidases which lead to the liberation of free amino acids.

Table 3.3. Level of total free amino acids in Cheddar cheeses at 14, 60 and 180 days of ripening. C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

FAA (mgLeu/g Cheese)	**Cheese**	**Ripening time**		
		14d	60d	180d
Trial A	C1	5.07±0.28[a]	10.87±0.52[a]	23.34±0.98[a]
	C2	7.43±0.59[b]	23.39±0.35[c]	28.02±0.71[c]
	C3	10.02±0.36[c]	22.35±0.20[c]	26.32±0.78[bc]
	C4	6.00±0.29[a]	13.90±0.90[b]	25.39±0.74[b]
Trial B	C1	4.73 ±0.08[a]	12.40±0.30[a]	20.05±0.21[a]
	C2	5.34 ±0.78[a]	14.37±0.35[b]	21.67±0.96[b]
	C3	5.48±0.72[a]	14.29±0.58[b]	21.84±0.49[b]
	C4	5.72±0.72[a]	14.43±0.49[b]	22.11±0.31[b]
Trial C	C1	5.27±0.70[a]	10.42±0.84[a]	26.95±0.46[a]
	C2	4.80±0.22[a]	13.45±0.38[c]	29.75±0.46[b]
	C3	4.73±0.39[a]	12.07±0.18[b]	28.49±0.79[b]
	C4	5.86±0.58[a]	11.85±0.14[b]	29.01±0.38[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviations.

[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

The levels of individual free amino acids is showed in **Table 3.4** and are expressed as mg/100mg cheese. Generally, higher levels of some free amino acids were found in cheese C3 and C4 in Trial A and B, and in all experimental cheeses in Trial C than the control cheese C1, confirming that coryneform strains contributed to proteolysis producing peptidases which liberate free amino acids. In particular, in Trial A, cheese C3 made with *C. casei* DPC 5293 contained higher levels of all amino acids than the control cheese, except for Tyr which was statistically similar to the control. Cheese C4, made with *C. variabile* DPC 5305, contained significantly higher levels of Glu Met, Gaba and Lys. Ile was present only in C2 and C3; the latter cheese showed the highest level. Similarly, in Trial B, higher levels of most amino acids were found in cheese C3 and C4 than the control cheese C1. The levels of Asp, Thr, Val, Leu and Lys in cheeses C3 and C4 were significantly higher than cheese C2 and control cheese C1, whereas only C4 contained a higher concentration of Glu, Met, Gaba, His and Arg than the control cheese. Ile was detected only in cheese C3 and C4. In Trial C, all the experimental cheeses (C2, C3 and C4) contained a significantly higher concentration of Asp, Met and Leu than the control cheese. C2 and C4 both contained a higher level of Thr and Ser than C1. Levels of Gly, Ala and His were higher in C2 than the control cheese. Levels of Gaba were significantly higher in C2 and C3, Lys and Arg in C3 and C4. Ile was present at the highest concentration in C2 whereas it was not detected in control cheese C1. In Trial B, cheese C2, made with *M. casei* DPC 5281 had a significantly lower level of Thr, Met, Leu, Gaba, Lys and Arg than the control cheese. Proteolysis is a major factor in the development of the texture and organoleptic qualities of smear cheeses which involves chemical, physical, and microbiological changes. It is affected by several factors, including the secondary microbiota (Leclercq-Perlat et al., 2000). During ripening, some amino acids may be catabolised by the action of aminotransferases or amino acid lyases leading to the formation of flavour compound in cheese (Curtin and McSweeney, 2003). Many cheese-related bacteria, including coryneforms, are able to produce flavour compounds from amino acids (Yvon and Rijnen, 2001). Some authors showed that strains of coryneform bacteria have aminotransferase and decarboxylase activity on some amino acids (Leuschner et al., 1998; Curtin et al., 2002; Curtin and McSweeney 2003). Volatile aromatic sulphur compounds originating from methionine and cysteine are probably key components of smear cheese flavour and contribute to the garlic note (Wilhelm, 2002).

Figure 3.10 A, B shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for individual free amino acids at 6 months of ripening in Trial A, B and C. Results suggest that there is no trial effect between all control cheeses C1 in all trials, whereas there is a big trial effect in samples C2 from Trial C, C3 from Trial A and C4 from Trial B. In particular, samples C3 from Trial A, had the highest concentration of all the amino acids except for Tyr.

Table 3.4. Levels of individual free amino acids in Cheddar cheeses at 180 days of ripening.

C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

IFA	***Trial A***			
mg/100gCheese	C1	C2	C3	C4
Asp	5.24±0.16^{a}	5.24±0.11^{a}	13.61±0.64^{b}	5.82±0.38^{a}
Thr	2.01±0.08^{a}	2.43±0.07^{a}	5.34±0.45^{b}	3.20±1.33^{a}
Ser	4.44±0.09^{a}	4.76±0.21^{a}	10.11±1.38^{b}	5.06±0.25^{a}
Glu	10.45±0.38^{a}	10.39±0.39^{a}	16.65±0.62^{c}	11.67±0.24^{b}
Gly	3.21±0.26^{a}	3.45±0.11^{a}	6.20±0.80^{b}	3.66±0.13^{a}
Ala	6.82±0.12^{a}	6.69±0.18^{a}	11.03±0.61^{b}	7.30±0.24^{a}
Val	7.89±0.85^{a}	9.95±1.14^{a}	13.55±0.70^{b}	9.28±0.93^{a}
Met	9.64±0.10^{a}	9.38±0.42^{a}	17.65±0.31^{c}	10.95±0.64^{b}
Ile	-	0.90±0.80^{a}	4.24±0.36^{b}	-
Leu	43.69±0.62^{a}	47.53±1.10^{a}	80.09±2.45^{b}	47.67±1.55^{a}
Tyr	26.88±0.32^{a}	14.36±12.64^{a}	16.43±7.91^{a}	24.15±0.61^{a}
Phe	48.68±0.87^{a}	51.76±1.02^{a}	60.08±1.08^{b}	52.36±2.26^{a}
Gaba	22.15±0.17^{ab}	20.60±1.26^{a}	37.64±0.97^{c}	23.42±0.63^{b}
His	15.81±0.86^{a}	21.69±4.20^{a}	33.54±1.21^{b}	21.27±6.60^{a}
Lys	9.59±0.14^{a}	10.48±0.19^{ab}	15.27±0.77^{c}	11.27±0.33^{b}
Arg	2.70±0.44^{a}	2.35±0.25^{a}	3.76±0.29^{b}	2.31±0.30^{a}
Pro	-	3.48±1.65^{ab}	6.41±1.62^{b}	1.91±1.95^{a}

IFA	***Trial B***			
mg/100gCheese	C1	C2	C3	C4
Asp	5.03±0.21^{a}	4.69±0.08^{a}	5.60±0.65^{b}	6.27±0.16^{c}
Thr	1.82±0.12^{b}	1.48±0.10^{a}	2.18±0.45^{c}	2.88±0.05^{d}
Ser	3.92±0.11^{a}	3.55±0.07^{a}	4.45±1.38^{a}	4.77±0.05^{a}
Glu	10.01±0.17^{ab}	5.46±4.74^{a}	11.32±0.62^{ab}	11.77±0.27^{b}
Gly	2.77±0.02^{ab}	2.44±0.03^{a}	2.85±0.80^{ab}	3.24±0.07^{b}
Ala	5.93±0.05^{ab}	5.44±0.19^{a}	5.82±0.61^{a}	6.85±0.32^{b}
Val	7.21±0.83^{a}	5.60±0.04^{a}	10.69±0.70^{b}	12.83±1.07^{c}
Met	9.05±0.27^{b}	7.89±0.25^{a}	8.88±0.31^{b}	10.17±0.42^{c}
Ile	-	-	1.39±0.36^{a}	1.72±1.50^{a}
Leu	41.48±0.34^{b}	37.07±0.57^{a}	44.97±2.45^{c}	53.57±1.03^{d}
Tyr	21.75±2.43^{a}	11.88±10.40^{a}	20.48±7.91^{a}	22.71±1.17^{a}
Phe	45.64±1.59^{ab}	34.90±11.69^{a}	48.95±1.08^{ab}	53.18±1.70^{b}
Gaba	20.15±0.51^{b}	17.73±0.76^{a}	20.81±0.97^{b}	23.26±0.16^{c}
His	15.09±2.36^{a}	15.04±2.06^{a}	17.32±1.21^{ab}	23.34±4.05^{b}
Lys	8.60±0.19^{b}	8.07±0.23^{a}	9.54±0.77^{c}	12.23±0.12^{d}
Arg	3.15±0.15^{b}	2.11±0.30^{a}	2.83±0.29^{b}	4.89±0.15^{c}
Pro	2.40±2.09^{a}	2.49±2.40^{a}	2.03±1.62^{a}	4.60±1.68^{a}

IFA	***Trial C***			
mg/100gCheese	C1	C2	C3	C4
Asp	4.47±0.16[a]	6.55±0.38[c]	5.43±0.33[b]	5.76±0.15[b]
Thr	2.04±0.15[a]	4.33±0.32[c]	2.84±0.22[ab]	3.26±0.65[b]
Ser	3.63±0.12[a]	8.99±0.49[c]	4.51±0.17[ab]	5.16±1.01[b]
Glu	6.20±5.38[a]	12.41±0.47[a]	12.43±0.74[a]	11.87±0.26[a]
Gly	2.87±0.11[a]	4.78±0.48[b]	3.35±0.34[a]	3.33±0.22[a]
Ala	5.86±0.10[a]	8.23±1.17[b]	6.85±0.31[ab]	6.88±0.23[ab]
Val	9.61±0.28[a]	10.66±2.19[a]	11.60±0.67[a]	12.75±1.29[a]
Met	8.17±0.25[a]	11.32±0.46[c]	9.93±0.37[b]	9.94±0.37[b]
Ile	-	5.47±0.18[b]	0.95±0.83[a]	1.53±1.35[a]
Leu	41.70±0.89[a]	66.11±3.67[c]	48.23±2.02[b]	50.81±1.93[b]
Tyr	18.55±0.80[a]	17.25±2.37[a]	13.79±11.95[a]	13.54±8.59[a]
Phe	44.89±0.78[a]	45.37±2.46[a]	45.64±15.73[a]	46.43±14.83[a]
Gaba	19.01±0.52[a]	25.09±1.27[b]	21.38±2.00[b]	21.82±1.00 [ab]
His	16.34±3.64[a]	33.86±1.89[b]	17.79±4.64[a]	21.79±5.85[a]
Lys	8.65±0.20[a]	8.17±0.37[a]	10.89±0.52[b]	11.68±0.35[b]
Arg	1.98±0.17[a]	2.39±0.07[ab]	2.52±0.22[b]	2.84±0.20[c]
Pro	3.67±3.29[a]	3.72±0.13[a]	4.41±1.73[a]	2.72±1.72[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

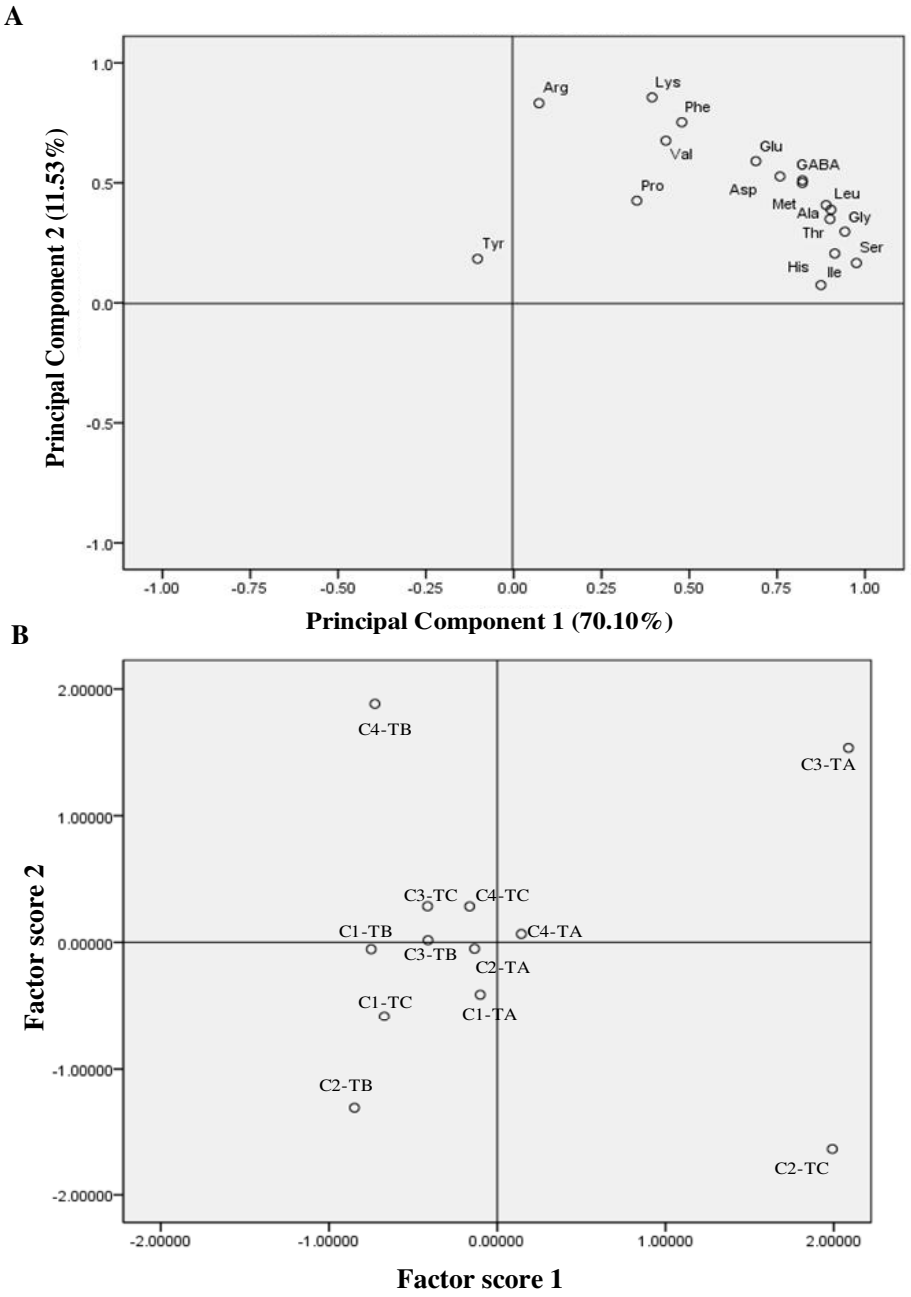

Figure 3.10. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for individual free amino acids at 6 months of ripening in Trials A, B and C. C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

3.4.4. Assesment of lipolysis

3.4.4.1 Free fatty acid profile

Lipolysis in cheese during ripening is catalysed by the action of lipases and esterases naturally present in milk or produced by microorganisms and leads to the liberation of free fatty acids which contribute to cheese flavor. In particular, short- and medium-chain fatty acids are precursors of flavour and aroma compound such as methyl ketones, lactones, esters, and secondary alcohols (Collins et al., 2003). Levels of total and each free carboxylic acids (from butyric C4:0, to arachidic, C20:0) of cheeses at 180 days are shown in **Table 3.5** and are expressed as mg/kg of cheese. In general, cheese C4 made with *C. variabile* DPC 5305 had the highest level of total free fatty acids in Trials A, B and C. In Trial A, cheese C2 made with *M. casei* DPC 5281 had a significantly lower level of total free fatty acids than the control cheese C1, whereas non-significant differences were observed between the control cheese C1 and the cheese C3 made with *C. casei* DPC 5293. In Trials B and C no significant differences were observed between the control cheese C1 and the experimental cheeses C2 and C3 made with *M. casei* DPC 5281 and *C. casei* DPC 5293, respectively. The strain *C. variabile* DPC 5305 showed the highest lipolytic activity in cheese C4 compared to the other cheeses.

Brennan et al. (2001) showed that this strain is able to produce esterase, esterase/lipase and lipase activity whereas *C. casei* produces only esterase and esterase/lipase. Curtin et al. (2002) showed that *C. casei* B, *Corynebacterium* spp. I and *C. ammoniagenes* CA8 have esterase activity on β-naphthyl butyrate, β-naphthyl caprate, β-naphthyl palmitate. No data concerning the lipolytic activity of *M. casei* DPC 5281 are available in the literature. Palmitic acid was the most abundant free fatty acid in all cheeses, even though the levels of miristic, oleic and linoleic acids were also high (**Table 3.5**).

In Trial A, significantly higher concentration of butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), myristic acid (C14:0) were found in cheese C4 than in the control cheese and the other experimental cheeses. Levels of capric acid (C10:0), lauric acid (C12:0), oleic acid (C18:1) in both cheeses C3 and C4 were higher than the others, while, the concentration of linolenic acid (C18:3) in the cheese C3 was found to be the highest. Butyric (C4:0), lauric (C12:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1) and arachidic (C20:0) acids were significantly higher in cheese C4 than control cheese C1, while, the level of linolenic acid (C18:3) was higher in cheese C3 and C4 than the other cheeses.

Finally, in Trial C, the concentrations of butyric acid (C4:0) and caproic acid (C6:0) were significantly higher in C3 and C4 than the other cheese. Levels of caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linolenic acid (C18:3) and arachidic acid (C20:0) was higher in the cheese C4 than all the other cheeses. These results showed that in cheese C4 *C. variabile* DPC 5305 affected qualitative and quantitative lipolysis in all trials more than the other strains, and confirmed that this strain is a good producer of lipase and/or esterase (Brennan et al., 2001).

Figure 3.11 A, B shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for free fatty acids at 6 months of ripening in Trial A, B and C. Results showed that there is a trial effect in samples C3 and C4 from Trial C, whereas no big variation is found between the samples from the other trials, especially in samples C1. Highest concentration of C18:2 are found in samples C1 in all trials, whereas samples C4 had the highest concentration of all the fatty acids except of C18:2.

Table 3.5. Levels of fatty acids in Cheddar cheeses at 180 days of ripening in trial A, B and C. C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

FFA	***Trial A***			
mg/kg cheese	C1	C2	C3	C4
C4:0	39.11±1.15^{a}	47.28±1.44^{a}	51.01±5.13^{a}	71.04±10.80^{b}
C6:0	12.98±0.99^{a}	13.51±0.22^{a}	19.36±0.38^{ab}	25.09±5.42^{b}
C8:0	9.69±0.37^{a}	12.28±3.15^{ab}	22.64±4.51^{ab}	28.34±12.54^{c}
C10:0	86.47±6.32^{a}	92.65±0.47^{a}	111.14±0.61^{b}	121.40±7.93^{b}
C12:0	116.41±6.65^{a}	123.85±5.01^{ab}	150.81±8.74^{bc}	176.87±17.96^{c}
C14:0	286.10±24.84^{a}	307.43±13.24^{a}	378.30±53.89^{ab}	429.51±50.75^{b}
C16:0	1002.82±36.22^{b}	764.45±10.65^{a}	845.27±19.75^{a}	981.37±62.18^{b}
C18:0	397.51±24.74^{b}	318.81±11.26^{a}	325.86±10.41^{a}	374.23±22.27^{b}
C18:1	362.18±12.08^{a}	361.94±7.83^{a}	445.31±4.56^{b}	481.63±19.15^{c}
C18:2	38.80±14.19^{b}	15.76±6.77^{a}	14.80±5.41^{a}	12.87±2.04^{a}
C18:3	17.82±11.62^{ab}	9.96±4.17^{a}	41.14±1.60^{c}	28.13±8.26^{b}
C20:0	48.75±1.37^{b}	40.41±1.01^{a}	51.00±2.57^{b}	52.28±0.65^{b}
Total	2568.54±27.75^{b}	2308.36±27.93^{a}	2629.95±36.48^{b}	3016.47±169.90^{c}

FFA mg/Kg cheese	***Trial B***			
	C1	C2	C3	C4
C4:0	56.20±10.29^{a}	52.14±3.53^{a}	53.74±1.84^{a}	70.80±1.56^{b}
C6:0	15.05±1.28^{a}	13.95±0.81^{a}	14.76±0.25^{a}	22.76±0.68^{a}
C8:0	34.90±37.16^{a}	16.58±1.15^{a}	15.44±1.92^{a}	19.78±1.52^{a}
C10:0	105.01±17.01^{a}	95.18±8.57^{a}	98.55±2.33^{a}	119.40±1.56^{a}
C12:0	141.07±23.85^{ab}	127.48±11.51^{a}	134.20±4.12^{ab}	166.43±5.59^{b}
C14:0	372.51±77.11^{a}	333.60±31.92^{a}	359.84±10.72^{a}	424.39±7.09^{a}
C16:0	774.81±14.27^{a}	767.36±65.68^{a}	828.97±30.87^{a}	996.01±35.17^{b}
C18:0	355.85±26.96^{a}	326.00±17.54^{a}	345.84±17.17^{a}	428.13±9.29^{b}
C18:1	401.91±23.44^{a}	405.53±31.66^{a}	436.39±10.33^{a}	505.25±18.50^{b}
C18:2	30.15±22.64^{a}	14.16±1.18^{a}	15.78±1.68^{a}	28.00±5.65^{a}
C18:3	16.12±1.62^{a}	24.24±7.04^{ab}	34.88±3.78^{bc}	40.00±3.33^{c}
C20:0	47.54±1.82^{a}	44.39±3.89^{a}	51.22±2.49^{ab}	58.37±3.83^{b}
Total	2625.67±221.09^{a}	2593.04±157.59^{a}	2597.22±68.37^{a}	3135.35±68.93^{b}

FFA	***Trial C***			
mg/kg cheese	C1	C2	C3	C4
C4:0	22.17±2.08[a]	43.61±0.30[a]	54.76±1.9[b]	87.90±1.27[c]
C6:0	16.62±0.70[a]	15.69±0.42[a]	23.14±0.5[b]	35.58±0.33[c]
C8:0	21.80±1.43[a]	18.36±1.03[a]	29.27±4.3[a]	43.83±6.76[b]
C10:0	116.84±2.62[a]	114.06±2.59[a]	118.34±2.30[a]	150.73±3.68[b]
C12:0	154.26±5.24[a]	158.02±10.1[a]	157.17±265[a]	225.95±12.10[b]
C14:0	381.70±15.36[a]	393.31±15.0[a]	397.22±9.66[a]	554.56±41.87[b]
C16:0	890.29±66.78[ab]	830.06±5.88[a]	969.28±51.08[b]	1096.86±33.33[c]
C18:0	343.19±6.04[a]	327.12±17.1[a]	336.95±9.48[a]	386.46±1.00[b]
C18:1	362.91±16.86[a]	365.93±17.99[a]	375.32±18.11[a]	434.37±13.45[b]
C18:2	19.70±2.52[a]	13.90±3.69[a]	11.87±0.94[a]	16.62±4.91[a]
C18:3	20.29±0.35[ab]	29.43±2.57[c]	16.54±0.75[a]	20.84±0.44[b]
C20:0	41.98±0.52[ab]	45.52±2.43[b]	38.08±0.61[a]	56.13±3.84[c]
Total	2559.93±104.10[a]	2481.04±24.36[a]	2705.43±71.80[a]	3351.65±107.47[b]

The results are shown as an average of three replicates for each trial (A, B, C)c± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

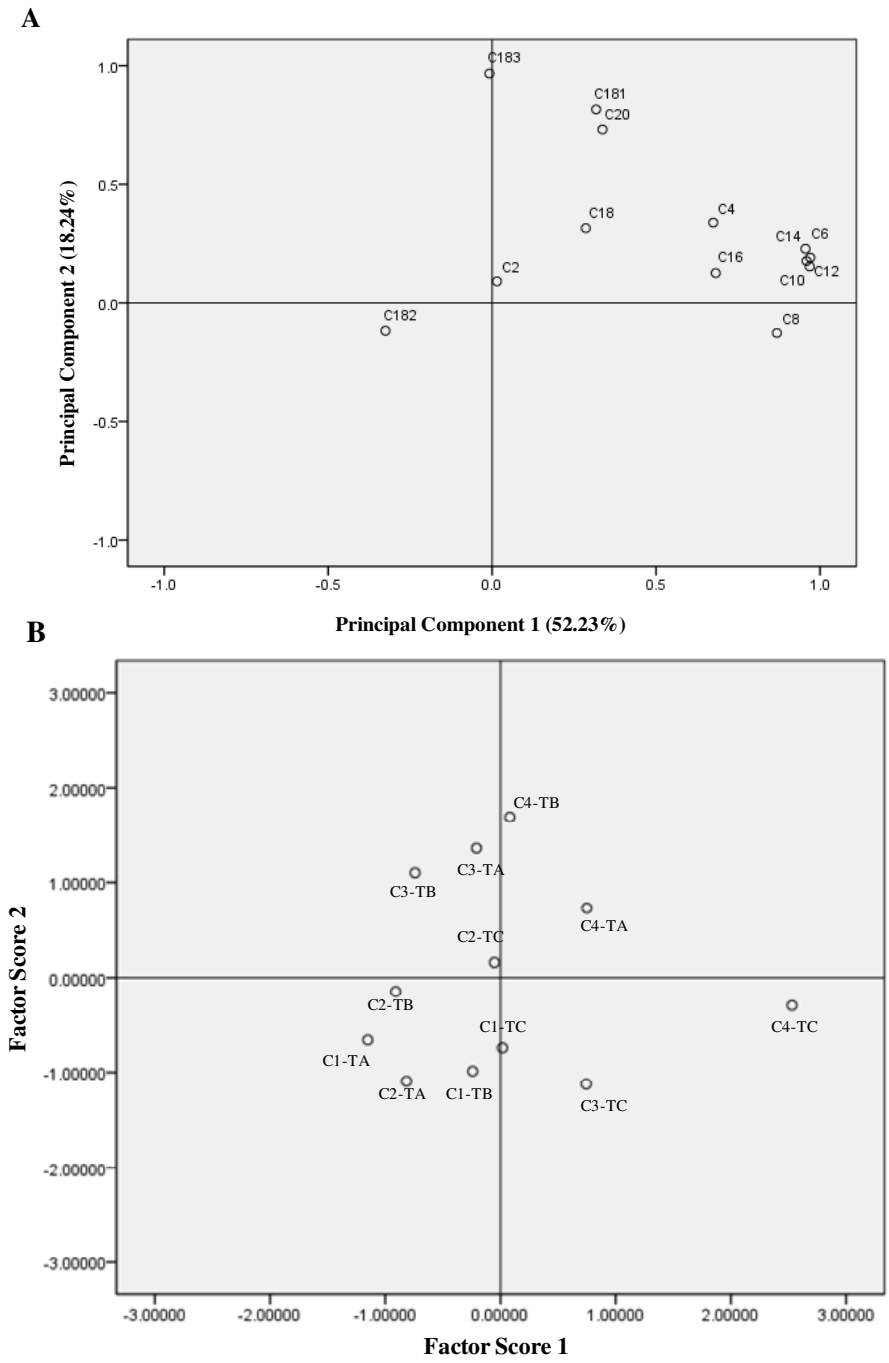

Figure 3.11. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for free fatty acids at 6 months of ripening in Trials A, B and C. C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

3.4.5. Biogenic amines

Levels of biogenic amines (BA) are shown in **Table 3.6** and expressed as µg of BA in 1 g of cheese. In Trial A, significantly higher ($P \leq 0.05$) levels of phenylethylamine and tyramine were found in all experimental cheeses than the control cheese C1. Only cheese C2 and C3 had a significantly higher ($P \leq 0.05$) level of putrescine and spermine than control cheese C1, respectively. Both experimental cheeses C2 and C3 had significant higher ($P \leq 0.05$) levels of spermidine than control cheese C1. In Trial B, only experimental cheese C4 showed significantly higher ($P \leq 0.05$) levels of phenylethylamine and spermidine than control cheese C1; whereas in Trial C, experimental cheeses C3 and C4 had significantly higher ($P \leq 0.05$) levels of putrescine and spermidine than control cheese C1, respectively. Results from Trial B and C showed that the coryneform strains used in this work are not good producers of biogenic amines apart from *C. casei* DPC 5293 and *C. variabile* DPC 5305 who significanty increased only the level of phenylethylamine, putrescine and spermidine.

These results are in contrast with those found in Trial A, where almost all strains showed to be good producers of phenylethylamine, putrescine, tyramine, spermidine and spermine. Biogenic amines in foods are mainly formed by amino acid decarboxylases of bacteria (Leuschner et al., 1998). Curtin and McSweeney (2003) reported that three strains of *Brevibacterium linens* ATCC 9174, *Corynebacterium* sp. subgroup *flavescens* CA8 and *Microbacterium gubbeenense* DPC5288, isolated from the surface of smear cheese, had decarboxylase activity when incubated at pH 9 and 30°C in a medium containing phenylalanine, tyrosine and tryptophan. *Brevibacterium linens* ATCC 9174, was active against all three substrates; *Corynebacterium* sp. subgroup *flavescens* CA8 had decarboxylase activity only against phenylalanine and tyrosine, whereas *Microbacterium gubbeenense* DPC5288 was active only against phenylalanine and tryptophan. After incubation under cheese like conditions, only *Corynebacterium* sp. subgroup *flavescens* CA8 showed a decrease in tyrosine and a corresponding increase in tyramine concentrations. No data for biogenic amine production by coryneform bacteria are available in the literature, apart from a study conducted by Straub et al. (1995) where the authors indicated *Micrococcus* and *Kocuria*, isolated from fermented sausages, as producers of biogenic amines. On the other hand, Leuschner et al. (1998) showed that some strains of *Arthrobacter* sp., *Micrococcus* sp., *Brevibacterium linens* and *Geotrichum candidum* are capable of degrading histamine and tyramine.

Table 3.6. Levels of biogenic amines in Cheddar cheeses at 180 days of ripening.C1, Control cheese; C2, Cheese made with *Microbacterium casei* DPC 5281; C3, cheese made with *Corynebacterium casei* DPC 5293; C4, cheese made with *Corynebacterium variabile* DPC 5305.

BA	***Trial A***			
(μg/gr of cheese)	C1	C2	C3	C4
tryptamine	n.d.	n.d.	n.d.	n.d.
phenylethylamine	4.19±0.27[a]	12.36±0.43[c]	10.75±0.11[b]	13.09±0.75[c]
putrescine	12.86±1.64[a]	242.53±20.83[b]	28.46±11.12[a]	16.88±0.92[a]
cadaverine	n.d.	n.d.	27.32±1.60	n.d.
histamine	n.d.	n.d.	n.d.	n.d.
tyramine	67.62±8.99[a]	109.08±5.13[c]	119.33±5.14[c]	92.65±2.83[b]
spermidine	55.20±7.15[a]	113.60±27.64[b]	141.54±2.56[b]	72.93±3.65[a]
spermine	18.37±1.40[a]	18.90±3.87[a]	33.00±7.83[b]	20.37±4.62[ab]

BA	***Trial B***			
(μg/gr of cheese)	C1	C2	C3	C4
tryptamine	n.d.	n.d.	n.d.	n.d.
phenylethylamine	6.49±1.86[a]	6.21±1.25[a]	6.14±0.81[a]	12.42±1.80[b]
putrescine	22.32±7.91[a]	23.37±8.16[a]	15.75±0.91[a]	15.44±1.09[a]
cadaverine	n.d.	n.d.	n.d.	n.d.
histamine	n.d.	n.d.	n.d.	n.d.
tyramine	72.02±9.27[a]	93.16±6.88[a]	76.91±7.13[a]	89.24±8.79[a]
spermidine	71.02±13.65[a]	86.83±10.52[a]	61.56±8.09[a]	125.72±5.72[b]
spermine	21.57±4.38[a]	20.49±1.46[a]	15.73±1.76[a]	22.82±7.48[a]

BA	***Trial C***			
(μg/gr of cheese)	C1	C2	C3	C4
tryptamine	n.d.	n.d.	n.d.	n.d.
phenylethylamine	15.61±1.96[b]	12.15±0.33[a]	12.42±0.94[a]	10.13±1.32[a]
putrescine	20.52±5.44[a]	19.98±3.09[a]	55.09±10.54[b]	18.45±1.27[a]
cadaverine	n.d.	n.d.	n.d.	n.d.
histamine	n.d.	n.d.	n.d.	n.d.
tyramine	105.45±6.33[b]	95.23±12.61[ab]	82.53±1.65[a]	87.81±4.66[ab]
spermidine	94.82±10.44[a]	100.28±22.42[a]	89.41±7.42[a]	146.19±4.66[b]
spermine	16.22±1.53[a]	25.29±4.55[a]	25.04±8.82[a]	25.43±8.06[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

n.d. not detected

3.5. Conclusions

The results of this study showed that the use of *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 as adjunct cultures in Cheddar cheese contributed to the ripening process through their proteolytic and lipolytic activity. This effect may be due to the release of lipolytic and proteolytic enzymes which some coryneform are able to produce (Brennan et al., 2004, Chamba and Irlinger, 2004). The coryneform population died off during the early stage of ripening and then remained almost constant over 180 days. It is hard to know whether coryneform bacteria cells lysed, were damaged or remained intact during ripening and therefore whether the extracellular enzymes were secreted during ripening or liberated after lysis together with intracellular enzymes. At the end of ripening, significantly higher level of pH 4.6-soluble nitrogen were found in all experimental cheeses than the control cheese in Trial A and only in cheese C2, C3 in Trial B. Higher level of total free amino acids were reported in all the experimental cheeses and qualitative difference in individual free amino acids were found between experimental and control cheeses. Total lipolysis was found to be significantly higher in C4 made with *Corynebacterium variabile* DPC 5305. *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 were not good producers of biogenic amines in Trial B and C, since they increased only the level of phenylethylamine, putrescine and spermidine, while in Trial A, almost all strains appeared to be good producers of phenylethylamine, putrescine, tyramine, spermidine and spermine.

3.6. References

ANDREWS, A. T. 1983. Proteinases in normal bovine-milk and their action on caseins. *Journal of Dairy Research*, 50, 45-55.

BERESFORD, T. P., FITZSIMONS, N. A., BRENNAN, N. L. & COGAN, T. M. 2001. Recent advances in cheese microbiology. *International Dairy Journal,* 11, 259-274.

BLAKESLEY, R. W. & BOEZI, J. A. 1977. New staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry,* 82, 580-582.

BOCKELMANN, W., WILLEMS, K. P., NEVE, H. & HELLER, K. H. 2005. Cultures for the ripening of smear cheeses. *International Dairy Journal,* 15, 719-732.

BONNARME, P., LAPADATESCU, C., YVON, M. & SPINNLER, H. E. 2001. L-Methionine degradation potentialities of cheese-ripening microorganisms. *Journal of Dairy Research,* 68, 663-674.

BRENNAN, N. M., BROWN, R., GOODFELLOW, M., WARD, A. C., BERESFORD, T. P., SIMPSON, P. J., FOX, P. F. & COGAN, T. M. 2001. *Corynebacterium mooreparkense* sp nov and *Corynebacterium casei* sp nov., isolated from the surface of a smear-ripened cheese. *International Journal of Systematic and Evolutionary Microbiology,* 51, 843-852.

BRENNAN, N. M., COGAN, T. M., LOESSNER, M. & SCHERER, S. 2004. Bacterial surface-ripened cheeses. *Cheese: chemistry, physics and microbiology*, Vol. 2. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 199-225.

BROADBENT, J. R., HOUCK, K., JOHNSON, M. E. & 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.

BURNS, P., CUFFIA, F., MILESI, M., VINDEROLA, G., MEINARDI, C., SABBAG, N. & HYNES, E. 2012. Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheeses. *Food Microbiology,* 30, 45-50.

CHAMBA, J. F. & IRLINGER, F. 2004. Secondary and adjunct cultures. *Cheese: chemistry, physics and microbiology*, Vol. 1. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 191-206.

COLLINS, Y. F., McSWEENEY, P. L. H. & WILKINSON, M. G. 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal,* 13, 841-866.

CORSETTI, A., ROSSI, J. & GOBBETTI, M. 2001. Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *International Journal of Food Microbiology,* 69, 1-10.

CURTIN, A. C. & McSWEENEY, P. L. H. 2003. Catabolism of aromatic amino acids in cheese-related bacteria: aminotransferase and decarboxylase activities. *Journal of Dairy Research,* 70, 249-252.

CURTIN, A. C., DE ANGELIS, M., CIPRIANI, M., CORBO, M. R., McSWEENEY, P. L. H. & GOBBETTI, M. 2001. Amino acid catabolism in cheese-related bacteria: selection and study of the effects of pH, temperature and NaCl by quadratic response surface methodology. *Journal of Applied Microbiology,* 91, 312-321.

CURTIN, A. C., GOBBETTI, M. & McSWEENEY, P. L. H. 2002. Peptidolytic, esterolytic and amino acid catabolic activities of selected bacterial strains from the surface of smear cheese. *International Journal of Food Microbiology,* 76, 231-240.

DADAKOVA, E., KRIZEK, M. & PELIKANOVA, T. 2009. Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC). *Food Chemistry,* 116, 365-370.

DE JONG, C. & BADINGS, H. T. 1990. Determination of free fatty acids in milk and cheese procedures for extraction clean up and capillary gas chromatographic analysis. *Journal of High Resolution Chromatography,* 13, 94-98.

DENIS, C. & IRLINGER, F. 2008. Safety assessment of dairy microorganisms: Aerobic coryneform bacteria isolated from the surface of smear-ripened cheeses. *International Journal of Food Microbiology,* 126, 311-315.

DI CAGNO, R., QUINTO, M., CORSETTI, A., MINERVINI, F. & GOBBETTI, M. 2006. Assessing the proteolytic and lipolytic activities of single strains of mesophilic lactobacilli as adjunct cultures using a Caciotta cheese model system. *International Dairy Journal,* 16, 119-130.

FOX, P. F. 1963. Potentiometric determination of salt in cheese. *Journal of Dairy Science,* 46, 744-745.

FOX, P. F., McSWEENEY, P. L. H. & LYNCH, C. M. 1998. Significance of non-starter lactic acid bacteria in cheddar cheese. *Australian Journal of Dairy Technology,* 53, 83-89.

GARDINER, G. E., ROSS, R. P., WALLACE, J. M., SCANLAN, F. P., JAGERS, P., FITZGERALD, G. F., COLLINS, J. K. & STANTON, C. 1999. Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of Cheddar cheese. *Journal of Agricultural and Food Chemistry,* 47, 4907-4916.

GELSOMINO, R., VANCANNEYT, M., SNAUWAERT, C., VANDEMEULEBROECKE, K., HOSTE, B., COGAN, T. M. & SWINGS, J. 2005. *Corynebacterium mooreparkense*, a later heterotypic synonym of *Corynebacterium variabile*. *International Journal of Systematic and Evolutionary Microbiology,* 55, 1129-1131.

GHOSH, B. C., BOCKELMANN, W. & HELLER, K. J. 2009. Casein degradation by enzymes of micro-organisms isolated from surface-ripened smear cheese. *Milchwissenschaft-Milk Science International*, 64, 55-57.

GOBBETTI, M., SMACCHI, E., SEMERARO, M., FOX, P. F., LANCIOTTI, R. & COGAN, T. 2001. Purification and characterization of an extracellular proline iminopeptidase from *Corynebacterium variabilis* NCDO 2101. *Journal of Applied Microbiology,* 90, 449-456.

GOERGES, S., MOUNIER, J., REA, M. C., GELSOMINO, R., HEISE, V., BEDUHN, R., COGAN, T. M., VANCANNEYT, M. & SCHERER, S. 2008. Commercial ripening starter microorganisms inoculated into cheese milk do not successfully establish themselves in the resident microbial ripening consortia of a South German red smear cheese. *Applied and Environmental Microbiology,* 74, 2210-2217.

GRAPPIN, R. 1986. Milk determination of nitrogen content Kjeldahl method and calculation of crude protein content. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 20A, 1-3.

HICKEY, D. K., KILCAWLEY, K. N., BERESFORD, T. P. & WILKINSON, M. G. 2006. Starter bacteria are the prime agents of lipolysis in cheddar cheese. *Journal of Agricultural and Food Chemistry,* 54, 8229-8235.

HYNES, E., OGIER, J. C., SON, O. & DELACROIX-BUCHET, A. 2003. Influence of starter and adjunct lactobacilli culture on ripening of miniature washed-curd cheeses. *Lait,* 83, 17-29.

IDF 1983. Milk, cream and evaporated milk - determination of total solids content (reference method)-provisional international IDF standard 21A-1982. *Milchwissenschaft-Milk Science International,* 38, 21-22.

IDF 1986. Cheese and processed cheese products determination of fat content gravimetric method reference method. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 1-5.

KENNY, O., FITZGERALD, R. J., O'CUINN, G., BERESFORD, T. & JORDAN, K. 2006. Autolysis of selected *Lactobacillus helveticus* adjunct strains during Cheddar cheese ripening. *International Dairy Journal,* 16, 797-804.

KUCHROO, C. N. & FOX, P. F. 1982. Fractionation of the water-soluble nitrogen from Cheddar cheese: chemical methods. *Milchwissenschaft-Milk Science International,* 37, 651-653.

LANE, C. N., FOX, P. F., WALSH, E. M., FOLKERTSMA, B. & McSWEENEY, P. L. H. 1997. Effect of compositional and environmental factors on the growth of indigenous non-starter lactic acid bacteria in Cheddar cheese. *Lait,* 77, 561-573.

LECLERCQ-PERLAT, M. N., OUMER, A., BUONO, F., BERGERE, J. L., SPINNLER, H. E. & CORRIEU, G. 2000. Behavior of *Brevibacterium linens* and *Debaryomyces hansenii* as ripening flora in controlled production of soft smear cheese from reconstituted milk: protein degradation. *Journal of Dairy Science,* 83, 1674-1683.

LEUSCHNER, R. G., HEIDEL, M. & HAMMES, W. P. 1998. Histamine and tyramine degradation by food fermenting microorganisms. *International Journal of Food Microbiology,* 39, 1-10.

LYNCH, C. M., McSWEENEY, P. L. H., FOX, P. F., COGAN, T. M. & DRINAN, F. D. 1996. Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. *International Dairy Journal,* 6, 851-867.

LYNCH, C. M., MUIR, D. D., BANKS, J. M., McSWEENEY, P. L. H. & FOX, P. F. 1999. Influence of Adjunct Cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *Journal of Dairy Science,* 82, 1618-1628.

MADKOR, S. A., TONG, P. S. & EL SODA, M. 2000. Ripening of Cheddar cheese with added attenuated adjunct cultures of lactobacilli. *Journal of Dairy Science,* 83, 1684-1691.

McSWEENEY, P. L. H., FOX, P. F., LUCEY, J. A., JORDAN, K. N. & COGAN, T. M. 1993a. Contribution of the indigenous microflora to the maturation of Cheddar cheese. *International Dairy Journal,* 3, 613-634.

McSWEENEY, P. L. H., OLSON, N. F., FOX, P. F., HEALY, A. & HOJRUP, P. 1993b. Proteolytic specificity of chymosin on bovine alpha-s_1-casein. *Journal of Dairy Research,* 60, 401-412.

MILESI, M. M., McSWEENEY, P. L. H. & HYNES, E. R. 2008. Viability and contribution to proteolysis of an adjunct culture of *Lactobacillus plantarum*

in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology,* 105, 884-892.

ONG, L., HENRIKSSON, A. & SHAH, N. P. 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus, Lb. casei*, *Lb. paracasei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal,* 16, 446-456.

PIRAINO, P., PARENTE, E. & McSWEENEY, P. L. H. 2004. Processing of chromatographic data for chemometric analysis of peptide profiles from cheese extracts: A novel approach. *Journal of Agricultural and Food Chemistry*, 52, 6904-6911.

POLYCHRONIADOU, A. 1988. A simple procedure using trinitrobenzenesulfonic acid for monitoring proteolysis in cheese. *Journal of Dairy Research,* 55, 585-596.

SHAKEEL-UR-REHMAN, FOX, P. F. & McSWEENEY, P. L. H. 2000. Methods used to study non-starter microorganisms in cheese: a review. *International Journal of Dairy Technology,* 53, 113-119.

SHAKEEL-UR-REHMAN, McSWEENEY, P. L. H., BANKS, J. M., BRECHANY, E. Y., MUIR, D. D. & FOX, P. F. 2000. Ripening of Cheddar cheese made from blends of raw and pasteurised milk. *International Dairy Journal,* 10, 33-44.

SHALABI, S. I. & FOX, P. F. 1987. Electrophoretic analysis of cheese - comparison of methods. *Irish Journal of Food Science and Technology,* 11, 135-151.

SOUSA, M. J., ARDÖ, Y. & McSWEENEY, P. L. H. 2001. Advances in the study of proteolysis during cheese ripening. *International Dairy Journal,* 11, 327-345.

STRAUB, B. W., KICHERER, M., SCHILCHER, S. M. & HAMMES, W. P. 1995. The formation of biogenic-amines by fermentation organisms. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung,* 201, 79-82.

SWEARINGEN, P. A., O'SULLIVAN, D. J. & WARTHESEN, J. J. 2001. Isolation, characterization, and influence of native, nonstarter lactic acid bacteria on Cheddar cheese quality. *Journal of Dairy Science,* 84, 50-59.

UPADHYAY, V. K., SOUSA, M. J., RAVN, P., ISRAELSEN, H., KELLY, A. L. & McSWEENEY, P. L. H. 2004. Use of exogenous streptokinase to accelerate proteolysis in Cheddar cheese during ripening. *Lait,* 84, 527-538.

VALDESSTAUBER, N., SCHERER, S. & SEILER, H. 1997. Identification of yeasts and coryneform bacteria from the surface microflora of brick cheeses. *International Journal of Food Microbiology,* 34, 115-129.

WILHELM, B. 2002. Development of defined surface starter cultures for the ripening of smear cheeses. *International Dairy Journal,* 12, 123-131.

YVON, M. & RIJNEN, L. 2001. Cheese flavour formation by amino acid catabolism. *International Dairy Journal,* 11, 185-201.

4. Acceleration of Cheddar cheese ripening using microfluidized yeasts

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4.1. Abstract

In this study, microfluidized strains of *Yarrowia lipolytica* DPC 6266, *Yarrowia lipolytica* DPC 6268 and *Candida intermedia* DPC 6271 were used to accelerate the ripening of Cheddar cheese. The strains were grown in YG broth and treated at 0.17 MPa in a microfluidizer® for four times. The extract was collected after the fourth pass, freeze-dried, stored in an aluminum foil bag and then added to the curd during salting at level of 2% w/w. Control cheese (C1), was made with 2% (w/w) of freeze-dried YG broth, cheese 2 (C2) contained 2% freeze-dried extract of *Yarrowia lipolytica* DPC 6266,, cheese 3 (C3) contained 2% freeze-dried extract of *Yarrowia lipolytica* DPC 6268, and cheese 4 (C4) contained 2% freez-dried extract of *Candida intermedia* DPC 6271. The yeasts positively affected proteolysis and lipolysis of cheeses and had aminopeptidase, dipeptidase, esterase and 5' phosphodiestere activities that contributed to accelerate the ripening of cheese. *Y. lipolytica* and *C. intermedia* also produced biogenic amines such as phenylethylamine, putrescine, cadaverine, tyramine spermidine and spermine.

4.2. Introduction

Yeasts occur naturally in many varieties of cheeses; in some, they are deliberately added as secondary cultures and contribute to flavor, texture development and ripening by the production of proteolytic and lipolytic enzymes (Beresford and Williams, 2004). However, in other cheeses they are undesirable since they contribute to the fruity, bitter, rancid and yeasty off-flavours and gassy, open texture (Fleet et al., 1990). The yeast species mostly found in cheese are *Debaryomyces hansenii*, *Geotrichum candidum*, *Trichosporon beigelii*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Zygosaccaromyces rouxii* (Wouters et al., 2002; Brennan et al., 2004). Other species found less commonly include *Candida catenulata*, *C. intermedia*, *C. rugosa*, *C. sake*, *C. vini* and *C. zeylanoides* (Chamba and Irlinger, 2004).

Yeasts are mainly found in smear cheeses such as Tilsit or Limburger, French cheeses such as Beaufort and Münster, Brie, Camembert, and Italian varieties such as Taleggio and in blue veined cheeses (Chamba and Irlinger, 2004). The low pH, low moisture content, low temperature and high salt concentration of these cheeses enhance the growth of yeasts during the early stage of ripening. They metabolize lactic acid produced by the starter bacteria to CO_2 and H_2O and produce NH_3 by deamination of amino acids, leading to a de-acidification of the cheese surface. The increase in pH permits the growth of a secondary microbiota manly composed of staphylococci, micrococci and corynefoms, enhances the activity of enzymes involved in ripening and affects the texture, making the cheese softer (Brennan et al., 2004). Moreover, yeasts produce stimulatory compounds that promote the growth of smear bacteria such as *Brevibacterium linens*, *Micrococcus caseolyticus, Mc. freudenreichii* and *Mc. caseolyticus* (Brennan et al., 2004), and the mould *Penicillium roqueforti* (Wouters et al., 2002).

Yeasts contribute to cheese ripening by producing proteolytic and lipolytic enzymes. They have caseinolytic, aminopeptidase, carboxypeptidase and lipase activity,

depending on the species and strain. (Chamba and Irlinger, 2004). *Yarrowia lipolytica*, is a yeast found in several foods such as cheese, yogurt, kefir, shoyu, meat and poultry products (Bankar et al., 2009) and known for its proteolytic and strong lipolytic activities (Van den Tempel and Jakobsen, 2000; Guerzoni et al., 2001; Suzzi et al., 2001). *Y. lipolytica* has been isolated from many cheeses such as Picante da Beira Baixa (Freitas et al., 1996), Brick (Valdes-Stauber et al., 1997), Gouda (Welthagen and Viljoen, 1998), Cheddar (Welthagen and Viljoen, 1999), Camembert and Brie (Viljoen et al., 2003), Caprino Lombardo (Foschino et al., 2006), Pecorino Crotonese (Gardini et al., 2006), Turkish white cheese (Yalcin and Ucar, 2009) and Livarot (Mounier et al., 2009).

Y. lipolytica secretes RNase, phosphatase, lipase, esterase and protease enzymes under certain growth conditions (Hernández-Montañez et al., 2007). Due to these properties, *Y. lipolytica* has been used in the chemical and food industries for the production of single-cell protein from *n*-alkanes, production of organic acid such as citric, isocitric and 2-ketoglutaric acids and the conversion of fatty acids into aroma compounds such as γ-decalactone, characterized by an oily-fruity aroma (Fickers et al., 2005). Some lipases from *Y. lipolytica* have been also used for the degradation of waste water from olive oil processing (De Felice et al., 1997; Scioli et al., 1997, Lanciotti et al., 2005). *Y. lipolytica* also produces many volatile sulphur flavor compounds such as *S*-methyl thioacetate, dimethyl disulphide, ethyl-3-methylthio-1-propanoate and methional (Liu and Crow, 2010) which are important for the flavor development in cheese. In spite of its positive effects, in some cases *Y. lipolytica* might be responsible of defects in cheeses such as Gorgonzola-type and some Portuguese raw ewes' milk cheeses due the production of a pyomelanin pigment from tyrosine which result in a brown discoloration of the cheese rind (Williams and Withers, 2007). High numbers of *Y. lipolytica* might also affect negatively the texture of cheese due to a strong degradation of fat (Yalcin and Ucar, 2009). *Y. lipolytica* has been used as adjunct culture in the manufacture of dry-salted, washed-curd cheese and it was found to increased the concentration of free fatty acids, free conjugated linoleic acid isomers (Das et al., 2005). Kesenkas and Akbulut (2008) reported that *Y. lipolytica* had no effect on the concentration of free fatty acids in white brined cheese, but it increased the ripening index. In a study by Bintsis and Robinson (2004), the adjunct of *Y. lipolytica* in the brine used for the manufacture of Feta-type cheese resulted in the production of undesirable aroma compounds, whereas Ferreira and Viljoen (2003) reported that a fruity aroma developed after 2 months of ripening in Cheddar cheese made with *Y. lipolytica*, but off-flavours were detected after 9 months. Wyder et al. (1999) showed that *Y. lipolytica* was capable of improving the overall sensory characteristics of foil-wrapped Raclette cheese and increased the level of free fatty acids when inoculated in combination with *Pichia jadinii*.

Candida intermedia has been isolated from Camembert and blue-veined cheese (Roostita and Fleet, 1996), Rokpol cheese (Wojtatowicz et al., 2001), Manteca cheese (Suzzi et al., 2003), Camembert and Brie (Viljoen et al., 2003) and Livarot cheese (Mounier et al., 2009). This yeast was also found in Spanish fermented sausages (Encinas et al., 2000). Owing to their ability to impact on cheese ripening, *Y. lipolytica* and *C. intermedia* can be used as adjunct cultures for cheesemaking.

Microbial cells are sources of valuable enzymes, proteins and other bioproducts that can be excreted into a growth medium (extracellular) or retained inside the cell's cytoplasm (intracellular). For these reasons microorganisms such as *Aspergillus niger* and *Kluyveromyces fragilis*, *Saccharomyces lactis*, *Kluyveromyces lactis*, *Streptomyces* sp. and *Penicilium notatum* have been used for the production of several microbial enzymes. When the enzymes are retained inside the cell, the isolation of intracellular material requires a mechanical or a non-mechanical disruption. The first method subjects the cells to high stresses produced by high pressure (Hughes press, French press, microfluidizer) and abrasion due to agitation with glass beads or ultrasound. Non-mechanical cell disruption includes physical (thermolysis, decompression, osmotic shock), chemical (antibiotics, chelating agents, solvents, detergents) or enzymatic (lytic enzymes, autolysis) methods (Geciova et al., 2002). In the past years, attenuated starter cultures have been used in cheese in order to accelerate ripening and to enhance the production of flavour compounds without producing lactic acid. Attenuated starter cultures are produced by heat shocking, freeze/thawing, lysozyme treatment, and spray or freeze drying (Klein and Lortal, 1999). In a study by Thompson et al. (1979), a heat-treated culture of *Lc. lactis* subsp. *cremoris* was used to enhance the flavor of low-fat Cheddar cheese; the authors reported no effect on proteolysis but increased levels of volatile fatty acids and of mature flavor. Farkye et al. (1995) used heat-treated mesophilic and thermophilic starters in a Cheddar cheese slurry system and reported an increase in the level of water-soluble nitrogen (N-soluble), trichloroacetic acid-soluble nitrogen (N-TCA), phosphotungstic acid-soluble nitrogen (N-PTA) and free amino acids. Also freeze-shocked starters have been used in Cheddar cheese: El-Soda et al. (1991) and Johnson et al. (1995) used *L. casei*, *Pediococcus* sp. and *Lb. helveticus* in low-fat Cheddar cheese and showed that the attenuated cultures increased the level of proteolysis and the flavour intensity.

Microfluidization is a type of high pressure homogenization used to reduce fat globule size and produce fine emulsions or fat substitutes. It has been applied in many products such as milk used for Cheddar manufacture in order to whiten the cheese (Lemay et al., 1994), in cream liqueurs (Heffernan et al., 2011) and it is often used to create emulsions before encapsulation of microparticles (Takahashi et al., 2009; Wang et al., 2011). This technique reduces particle size by pressing the milk through micro channels into an interaction chamber of fixed geometry to produce two fine jets that are directed against each other under pressure up to 1400 bar. An increase in the numbers of passes through the microfuidizer reduces the mean particle size. The particle size is reduced by crushing forces of the particles hitting one another, shear forces within the interaction chamber and cavitational forces due to extreme velocity changes in the material stream (Sunil and Ashwanl, 2008). Microfluidization has been also applied for the large-scale disintegration of microorganisms. One of the most important factors to take into account is choosing optimal equipment and conditions for cell distruption which are specific for each microorganism (White and Marcus, 1988).

In this work, *Yarrowia lipolytica* DPC 6266, *Yarrowia lipolytica* DPC 6268 and *Candida intermedia* DPC 6271 were grown in broth and cells were disrupted by microfluidization and then freeze-dried. The three yeast preparations were used separately to produce Cheddar cheeses in order to accelerate the ripening process.

4.3. Materials and methods

4.3.1. Strains

The strains used for cheesemaking were *Yarrowia lipolytica* DPC 6266, *Yarrowia lipolytica* DPC 6268 and *Candida intermedia* DPC 6271 and were obtained from the collection of the Teagasc Food Research Centre, Moorepark, Fermoy, Ireland. The strains were grown for 4 d at 30°C under agitation (120 rpm) in 5 L flask containing 2 L of YG broth (yeast extract 10g/l, glucose 20 g/l, Oxoid, South County Business Park, Leopardstown, Dublin 18).

4.3.2. Microfluidization

The yeasts grown cultures were individually treated at 0.17 MPa in a microfluidizer® (Microfluidics M110-EH-30, Newton, MA, USA). The machine was equipped with a Z chamber and each sample was passed through the system four times. During the treatment, the chamber and the pipes system were covered with ice to keep the temperature low (≤ 10°C). After each pass samples were taken and lysis rate was monitored by measuring viable yeast count (YGC agar, Merck, Darmstadt, Germany, incubated at 30° C for 3 days) and alkaline phosphatase activity. The extract was collected after the fourth pass, freeze-dried and stored in an aluminum foil bag until cheesemaking.

4.3.3. Cheesemaking

Cheesemaking was carried out in triplicate on different days. Whole milk was standardized to casein: fat ratio of 0.7:1.0, pasteurised at 72ºC for 15 s and cooled to 30ºC. DVS starter culture (R604 Chr-Hansen, Hørsholm, Denmark) was added at a rate of level 0.03% w/v to cheesemilk. Chymax-180 (Chr. Hansen, Hørshom, Denmark) was used as coagulant. Whey was drained at pH 6.2 and the curd cheddared, milled at pH 5.2. The freeze-dried extract (2% w/w) was mixed with salt (2.5 % w/w) in a sterile bag, added to the milled curd and mixed manually for both control and experimental cheeses. After pressing the curd at 150 kPa for 18 h, the cheese were vacuum packed in a vacuum bag and ripened for 180 days at 8°C. Control cheese (C1), was made with 2% (w/w) of freeze-dried sterile YG broth, cheese 2 (C2) contained 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract, cheese 3 (C3) contained 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract and cheese 4 (C4) contained 2% freeze-dried *Candida intermedia* DPC 6271 extract.

4.3.4. Microbiological analysis

Samples were taken under aseptic conditions using a cheese trier at day 1, 14 and every 30 days for 6 months. Ten g of cheese were homogenized in 90 ml of sterile trisodium citrate (2% w/v) in a stomacher bag (Stomacher 400, Seward Limited, Worthing, West Sussex, UK) for 5 min. Starter cells were enumerated on LM17 agar after incubation at 30° C for 3 days, non-starter lactic acid bacteria (NSLAB) were enumerated on Rogosa agar after 5 days at 30°C, yeast were enumerated on YGC agar (Merck, Darmstadt, Germany) incubated at 30° C for 3 days.

4.3.5. Compositional analysis

Protein (Grappin, 1986), fat (Gerber method; IDF 1986), moisture (oven drying at 102°C; IDF 1983), salt (Fox, 1963) and pH were determined in triplicate after 14 days of ripening. The pH was measured in cheese slurry (1:1 cheese:water) using a combined glass electrode (PHC3001-8, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standard pH Meter, Radiometer, Copenhagen, Denmark).

4.3.6. Biogenic amines

Lyophilized 4.6-soluble fractions samples (obtained from 1 ml of extract) were diluted using perchloric acid (0.6 $mol.l^{-1}$) and 0.5 mL of obtained mixtures (vigorously vortexted) were subjected to derivatisation with dansylchloride according to the method of Dadáková et al. (2009); 1,7-heptanediamine was used as an internal standard. The derivatised samples were filtered (0.22 μm) and applied on a column (Zorbax Eclipse XDB-C18, 150 x 4.6 mm, 3.5μm, Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA, USA). The concentration of eight biogenic amines (histamine, tyramine, phenylethylamine, tryptamine, putrescine, cadaverine, spermidine, spermine) was monitored by an high performance liquid chromatography system equipped with a binary pump; an autosampler (LabAlliance, 349 Science Park Rd, State College, Pennsylvania, PA, USA); a column thermostat; a UV/VIS diode array detector (λ = 254 nm); and a degasser (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA). The conditions for separation of the monitored BA are described by Smělá et al. (2004). Each of the lyophilized samples was derivatised three times. Standards, reagents and eluents were obtained from Sigma Aldrich (St. Louis, MO, USA).

4.3.7. Analysis of cheese

The pH 4.6-soluble and –insoluble fractions of the cheeses were prepared according to the method of Kuchroo and Fox (1982). The extraction was carried out in triplicate for each cheese. The N content of the pH 4.6-soluble fraction of the cheeses was measured by the macro-Kjeldahl method (Grappin, 1986) and expressed as a % of the total N content of the cheeses. Urea-polyacrylamide gel electrophoresis (urea-PAGE) was carried out on the samples after 60 d and 180 d of ripening, using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Andrews (1983) with modifications. Gels were stained directly by the method of Blakesley and Boezi (1977).

Peptide profiles of the pH 4.6-soluble fractions were determined by UPLC Waters Acquity UPLC H-Class Core System with Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A (Waters Corporation, Milford, MA, USA). The core system included an Acquity UPLC H-Class quaternary solvent manager, a H-Class Sample Manager-FTN and a CH-A column heater. The column used was an Acquity UPLC BEH C18 1.7 μm, 2.1 x 50 mm. Samples were made up at 10 mg/ml in buffer A (0.1% TFA in Milli-Q water) and centrifuged at 18,000 g for 10 min. The supernatant was filtered through a 0.22 μm filter (Pall Filters, Dublin, Ireland); Buffer B was made up with 0.1% TFA in acetonitrile. Elution was monitored at 214 nm.

Total free amino acids (FAA) were determined for the 60 d and 180 d old cheeses by the trinitrobenzenesulphonic (TNBS) acid method (Polychroniadou, 1988). Individual free amino acids were quantified at 180 d using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Welwyn Garden City, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column.

Extraction of free individual fatty acids (FFA) for GC analysis was carried out by using a solid-phase extraction technique according to the method described by De Jong and Badings (1990); 500 mg Varian Bond Elut-NH_2 cartriges were used. FFAs were quantified using a gas chromatograph consisting of a Varian Star 3400 CX instrument with Varian 8200 CX autosampler and flame ionization detector (300ºC) interfaced with Star Chromatography Workstation 5.0 software for data acquisition (Varian Analytical Instruments, Harbor City, CA, USA). A wall-coated open tubular fused silica capillary column (25 m length x 0.32 mm internal diameter) coated with FFAP-CB was used. FFAs were separated and identified by reference to known standards and quantified by peak area. A standard calibration mix of 17 fatty acids (including internal standards, valeric acid C5:0; pelargonic acid C9:0; margaric acid C17:0) was prepared at concentrations 1000, 500, 300, 200, 100 and 50 ppm.

One-way analysis of variance of data from of the composition of the cheeses was performed using SPSS Version 18.0 for Windows XP (SPSS Inc., Chicago, IL, USA). Significance was declared at $P \leq 0.05$. The data for individual amino acids, from RP-HPLC peptide profile and individual free fatty acid and biogenic amines concentrations for the cheeses were analysed using multivariate statistical techniques to evaluate the effect of microfluidized yeasts on the parameters of ripening. The HPLC peak height data were pre-processed according to the method of Piraino et al. (2004). Principal component analysis was performed on this pre-processed data using SPSS Version 18.0 for Windows XP.

4.3.8. Enzyme assays

Enzyme assays were performed on freeze-dried yeast extracts and on the pH 4.6-soluble fractions extracted after 60 and 180 d of ripening. pH 4.6-soluble fractions were used directly for enzyme assays whereas freeze-dried yeast extracts were previously dissolved in distilled water to a final concentration of 100 mg/ml and the protein content was measured according to the method of Lowry et al. (1951). The pH 4.6-soluble fractions and the diluted enzyme extracts were tested for the following enzyme activities:

4.3.8.1. Determination of aminopeptidase and dipeptidyl aminopeptidase activity

The aminopeptidase activity was measured using L-glutamic acid-*p*-nitroanilide (Glu-*p*NA) and L-lysine-*p*-nitroanilide (Lys-*p*NA) (Sigma, Dublin, Ireland) according the method described by El-Soda et al. (1978). The incubation mixture contained 800 µl of 0.1 M K-phosphate buffer, pH 7.0, 100 µl of sample and 100 µl ml of substrate (10 mM). The mixture was incubated at 30°C for 30 min and the absorbance was read at 410 nm using a double beam spectrophotometer (Cary, Bio 300, Varian Australia Ltd, Mulgrave, Victoria, Australia). Dipeptidyl aminopeptidase activity was determined as for aminopeptidase activity, but Ala-Pro-*p*NA was used as substrate (4.0 mM). One unit of enzyme activity (U) was defined

as the amount of enzyme producing 1 µmol of *p*-nitroaniline min^{-1} under the conditions of the assay. Specific activity was defined as the number of activity units per milligram of protein.

4.3.8.2. Esterase activity

Esterase activity was measured according to the method described by Breinig et al. (2006) with some modifications. The incubation mixture contained 800 µl of buffer (100 mM Tris-HCl buffer, pH 7.0), 100 µl of sample and 100 µl ml of substrate (4.5 mM *p*-nitrophenyl butyrate in acetonitrile, *p*-NPB; Sigma, Dublin, Ireland). The mixture was incubated at 30°C for 15 min and the absorbance was read at 410 nm using a double beam spectrophotometer (Cary, Bio 300, Varian Australia Ltd, Mulgrave, Victoria, Australia). One unit of enzyme activity (U) was defined as the amount of enzyme producing 1 µmol of *p*-nitrophenol min^{-1} under the conditions of the assay. Specific activity was defined as the number of activity units per milligram of protein.

4.3.8.3. 5' Phosphodiesterase activity

5' Phosphodiesterase activity (5'PDE) was measured according to the method described by Razzell (1963). The incubation mixture contained 800 µl of Tris buffer, 100mM, pH 8.9, 100 µl of sample and 100 µl ml of substrate (10 mM thymidine-5'-monophosphate *p*-nitrophenyl ester, 5'TMP-*p*NP, Sigma, Dublin, Ireland). The mixture was incubated at 37°C for 10 min and the absorbance was read at 400 nm using a double beam spectrophotometer (Cary, Bio 300, Varian Australia Ltd, Mulgrave, Victoria, Australia). One unit of enzyme activity (U) was defined as the amount of enzyme producing 1 µmol of *p*-nitrophenol min^{-1} under the conditions of the assay. Specific activity was defined as the number of activity units per milligram of protein.

4.3.8.4. Alkaline phosphatase

Alkaline phospatase activity (ALP) was measured on the yeast extract according to the method described by Galabova et al. (1996). The incubation mixture contained 800 µl of buffer (0.1M Tris-HCl, pH 8.5), 100 µl of diluted yeast extract and 100 µl ml of substrate (3.8mM *p*-nitrophenyl phosphate, *p*NPP; Sigma, Dublin). The mixture was incubated at 37°C for 15 min and the absorbance was read at 410 nm using a double beam spectrophotometer (Cary, Bio 300, Varian Australia Ltd, Mulgrave, Victoria, Australia). One unit of enzyme activity (U) was defined as the amount of enzyme producing 1 µmol of *p*-nitrophenol min^{-1} under the conditions of the assay. Specific activity was defined as the number of activity units per milligram of protein.

4.4. Results and discussion

Yeasts occur naturally in many cheeses varieties and in some they are used as adjunct cultures. Their proteolytic and lipolytic activities, ability to produce sulphur compounds, ethanol, acetaldehyde and CO_2 play an important role in aroma and flavor formation (Chamba and Irlinger, 2004). In this work, the effect of microfluidized strains of *Yarrowia lipolytica* DPC 6266, *Yarrowia lipolytica* DPC 6268 and *Candida intermedia* DPC 6271 on Cheddar cheese ripening was evaluated. High pressure (0.17 MPa) and shearing forces were imposed by microfluidization in order to release the intracellular enzymes from the yeast cells and the extracts obtained were then freeze-dried and used as ingredient for cheesemaking.

4.4.1. Cell lysis during microfluidization

The number of viable yeasts cells in each batch after growth (0 passes) was ~10^7 cfu/ml. After each pass through the microfluidizer, the number of the viable yeast cells decreased by 1 log cycle and after 4 passes the yeast count in each preparation was ~10^3 log cfu/ml (**Table 4.1**). The cell lysis ratio was also monitored after each pass by measuring the release of alkaline phosphatase (**Table 4.2**) that increased by increasing in number of passes through the microfluidizer. After the fourth pass the extract was collected, freeze-dried and stored in aluminum foil bags. The powder obtained was then added to the cheese with the salt (2% w/w).

Alkaline phosphatase activity (ALP) was measured on the yeast broth before and after each pass through the microfluidizer in order to evaluate the extent of yeast cell lysis. As shown in **Tables 4.1** and **4.2**, as the number of passes increased, the number of viable cells decreased and the activity of free ALP increased. It is known that a part of alkaline phosphatase activity is internally located (Galabova et al., 1996). Its increasing during microfluidization indicates that the treatment was efficient enough to damage the yeasts cells.

Table 4.1. Numbers of yeasts (cfu/ml) on YGC agar incubated at 30° C for 3 days after each pass through the microfluidizer at 0.17 MPa.

	Microbiological counts (CFU/ml)				
	n° of passes (0.17 MPa)				
Strains	0	1	2	3	4
Y. lipolytica DPC 6266	$5.10x10^7$	$2.30x10^6$	$5.23x10^5$	$7.71x10^4$	$3.43x10^3$
Y. lipolytica DPC 6268	$4.60x10^7$	$3.70x10^6$	$4.71x10^5$	$6.63x10^4$	$4.38x10^3$
C. intermedia DPC 6271	$1.20x10^7$	$2.20x10^6$	$2.44x10^5$	$4.68x10^4$	$4.27x10^3$

Table 4.2. **Alkaline phosphatase (ALP) activity after each pass through the microfluidizer at 0.17 MPa.**
Results are expressed as activity units per milligram of protein (U/mg protein).

	Number of passes (0.17 MPa)				
Strains	0	1	2	3	4
Y. lipolytica DPC 6266	161.70±52.34	422.75±13.77	440.28±44.08	467.56±33.06	490.94±5.51
Y. lipolytica DPC 6268	291.55±5.81	519.46±8.71	537.93±34.84	603.64±23.22	624.17±5.81
C. intermedia DPC 6271	277.77±7.22	369.79±12.04	390.24±2.4	426.02±19.27	429.43±4.82

The results are shown as an average of three replicates for each strain ± standard deviation.

4.4.2. Compositional analysis

The composition of each cheese at 14 days was typical for Cheddar cheese in all trials indicating that the use of yeast extract did not have a significant effect on composition (**Table 4.3**). Similar results were obtained by Shakeel-Ur-Rehman et al. (2003) who studied the effect of a food grade dry yeast on reduced-fat Cheddar cheese, Kesenkas and Akbulut (2008) who used *Y. lipolytica*, *Debaryomyces hansenii* and *Kluyveromyces marxianus* as an adjunct culture in Turkish white brined cheese, and Das et al. (2005) who made a dry-salted, washed-curd cheese using *Y. lipolytica* and *Geotrichum candidum* as adjunct.

Table 4.3. pH and composition of Cheddar cheeses made using microfluidised yeast extracts at 14 days of ripening
C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

	Cheese	*pH*	*% Salt*	*% Fat*	*%Moisture*	*% Protein*
Trial A	C1	5.10±0.01[a]	1.59±0.06[a]	30.00±0.01[a]	39.33±0.70[a]	24.96±0.79[a]
	C2	511±0.22[a]	1.59±0.02[a]	30.66±0.58[a]	39.58±0.06[a]	24.50±0.02[a]
	C3	4.87±0.08[a]	1.58±0.05[a]	30.00±0.02[a]	39.12±0.58[a]	24.57±0.15[a]
	C4	5.12±0.10[a]	1.70±0.03[a]	30.33±0.58[a]	38.57±0.15[a]	24.74±0.03[a]
Trial B	C1	5.13±0.06[a]	1.64±0.20[a]	31.00±0.58[a]	39.58±0.11[a]	25.08±0.73[a]
	C2	5.13±0.18[a]	1.61±0.09[a]	31.33±0.58[a]	40.31±0.61[a]	24.41±0.39[a]
	C3	4.86±0.10[a]	1.62±0.04[a]	31.33±1.00[a]	39.77±0.06[a]	24.38±0.02[a]
	C4	5.06±0.07[a]	1.76±0.10[a]	31.66±0.58[a]	40.72±0.20[a]	24.96±0.34[a]
Trial C	C1	4.94±0.16[a]	1.68±0.27[a]	30.66±1.15[a]	39.17±0.52[a]	24.69±0.04[a]
	C2	4.85±0.12[a]	1.66±0.03[a]	30.33±0.58[a]	40.13±0.05[a]	24.23±0.03[a]
	C3	4.83±0.05[a]	1.44±0.04[a]	31.66±0.58[a]	39.51±0.66[a]	24.22±0.11[a]
	C4	4.91±0.06[a]	1.77±0.32[a]	31.33±0.58[a]	39.57±0.16[a]	24.24±0.87[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

4.4.3. Microbiological analysis

Starter lactic acid bacteria (SLAB) were inoculated at level of ~10^9 cfu/g in all trials and their growth in the cheeses is shown in **Figure 4.1 A, B, C**. In Trial A, SLAB decreased by 1 log cycle after 60 days in C1, C3 and after 90 days in C2 and C4. In Trial B, numbers of SLAB reduced to ~10^8 cfu/g in C1, C2, C3 after 60 days and in C4 after 90 days. In Trial C, they decreased by 1 log cycle after 60 days in C1, after 90 days in C2 and after 120 days in C3 and C4. At the end of the ripening they were present at level of ~10^7 cfu/g in all cheeses in all trials. LAB exhibited a relatively similar pattern in all cheeses, however numbers of LAB in cheese C3 made with 2% *Y. lipolytica* DPC 6268 extract (in Trial C) and cheese C4 made with 2% *C. intermedia* DPC 6271 extract (all trials) were slightly higher than numbers of LAB

the control cheese, suggesting that these strains could have enhanced the growth of the lactic microbiota. The stimulating effect of *Y. lipolytica* on LAB was also reported by Lanciotti et al. (2005).

NSLAB started growing after 30 days in the experimental cheeses in all trials, reaching numbers between 10^4-10^6 cfu/g in Trial A and 10^3-10^4 cfu/g in Trial B and C. NSLAB in control cheeses reached levels of 10^4 cfu/g after 60 days in Trials A and B and after 90 days in Trial C (**Figure 4.2 A, B, C**). These results suggest that the use of yeast extract enhanced the growth of the secondary microbiota in cheese and are in agreement with those of Shakeel-Ur-Rehman et al. (2003) in a study conducted on reduced-fat Cheddar cheese made with a food grade active dry yeast. Since yeast cells are a good source of proteins, lipids, RNA, vitamins and minerals, the extract obtained by the disruption of these cells are rich in amino acids, peptides, nucleotides and other soluble cell components (Chae et al., 2001) that could enhance the growth of NSLAB.

No viable yeast cells were present at day 1 in the cheeses in all trials, but after 14 d of ripening a yeast population began to grow at very low levels (10^2-10^4 cfu/g) in all cheeses in Trial A (**Figure 4.3 A, B, C**). In Trial B and C, only cheese C2 contained a yeast population at levels of 10^5 cfu/g and 10^3 cfu/g, respectively, at day 14, but after 30 days of ripening all cheeses contained numbers of viable yeasts between 10^4-10^5 log cfu/g. At the end of ripening the yeast counts were ~10^4 cfu/g in the cheeses in all trials, except for C2 that contained levels of 10^5 cfu/g in Trial A and C.

In a study conducted by Lanciotti et al. (2005), strains of *Y. lipolytica* reached numbers of 7-7.5 log cfu/g in cheeses within two days of ripening and remained almost stable at the same level over 30 days. After 60 days, numbers of *Y. lipolytica* decreased by 1-1.5 log cfu/g depening on the strain. It is known that yeasts occur both in raw and pasteurized milk at very low numbers and their growth in cheese is enhanced by its low pH, low moisture, elevated salt content and low temperature (Fleet, 1990). Some studies on the microbiota of Cheddar cheese did not report the occurrence of yeast (Law and Sharpe, 1977; Cromie et al., 1987). On the other hand, Fleet and Mian (1987) reported a yeast population of 10^4-10^6 cfu/g in 48% of 23 samples of Australian Cheddar cheese, whereas Welthagen and Viljoen (1999), who examined 42 South African Cheddar cheeses, reported that all of them contained yeasts at levels of 10^2-10^7 cfu/g. Also, Lanciotti et al. (2005) reported the presence of a yeasts population up to 10^5 cfu/g in cheese made with pasteurized milk. Hence, it could be possible that some yeasts cells survived the pasteurization process of milk and grew in both control and experimental cheeses during ripening without being affected by the ripening conditions. In case of experimental cheeses is also likely that low numbers of viable cells survived microfluidization and grew in cheese during ripening.

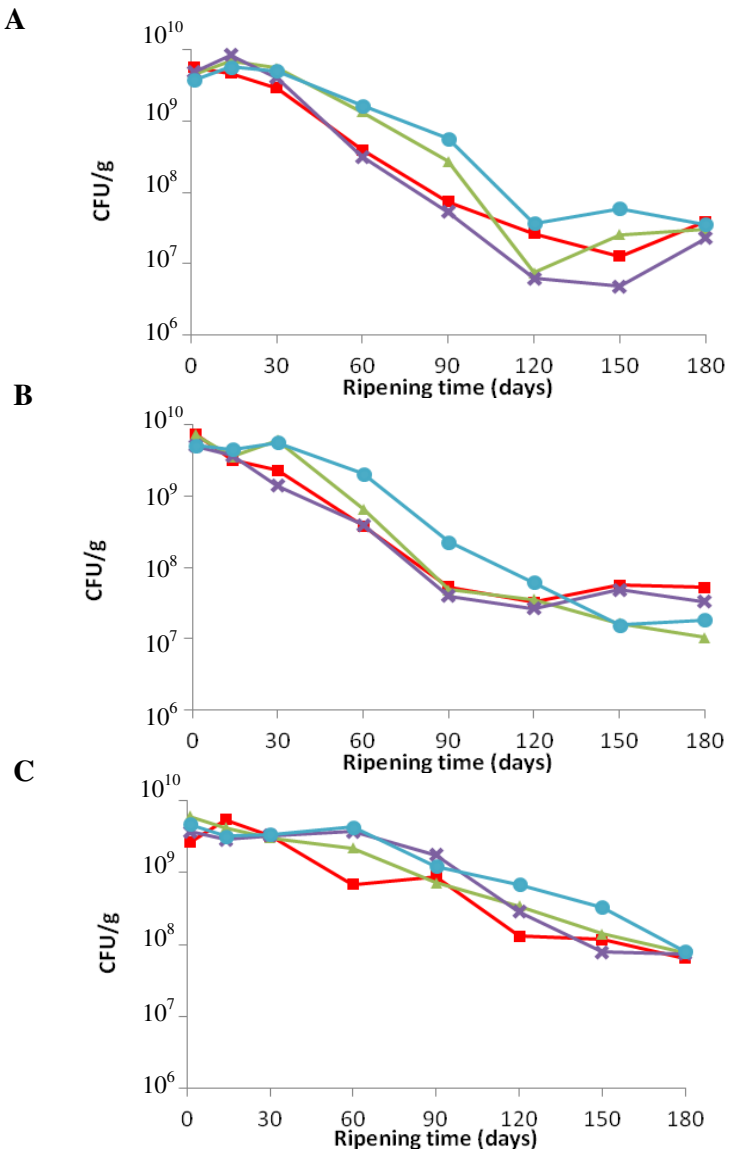

Figure 4.1. Growth of starter lactic acid bacteria (SLAB) on LM17 agar incubated 30°C for 3 days during ripening of Cheddar cheeses made with microfluidised yeast extracts in Trials A, B, C. C1 (■), Control cheese; C2 (▲) made with 2% freze-dried *Yarrowia lipolytica* DPC 6266 extract; C3 (×), cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, (●) cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

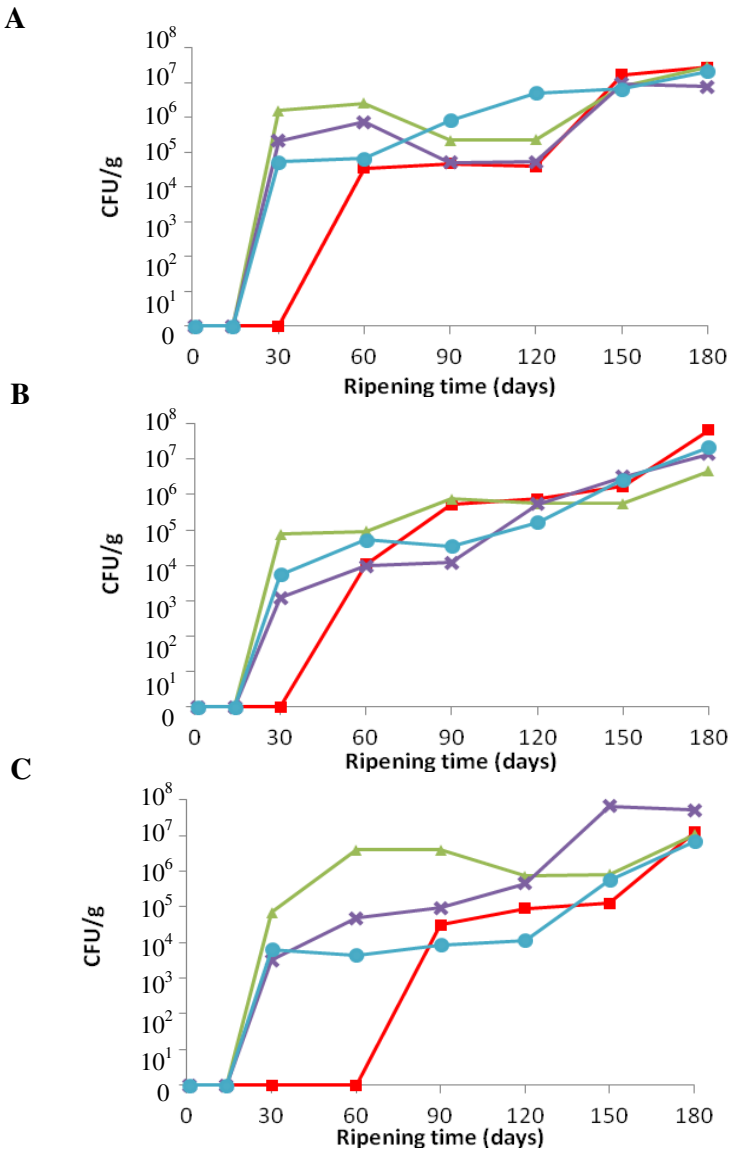

Figure 4.2. Growth of non-starter lactic acid bacteria (NSLAB) enumerated on Rogosa agar incubated at 30°C for 5 days during ripening of Cheddar cheeses made with microfluidised yeast extracts in Trials A, B, C. C1 (■), Control cheese; C2 (▲) made with 2% freze-dried *Yarrowia lipolytica* DPC 6266 extract; C3 (×), cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, (●) cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

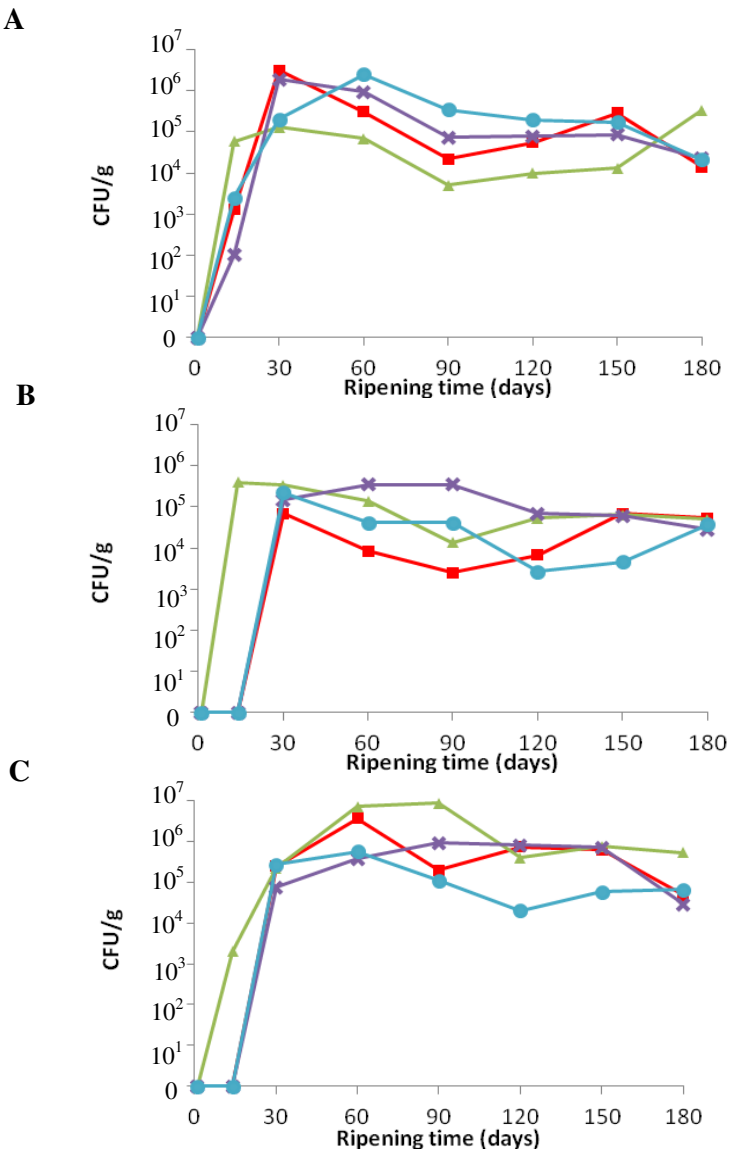

Figure 4.3. Growth of yeasts enumerated on YGC agar incubated at 30° C for 3 days during ripening of Cheddar cheeses made with microfluidised yeast extracts in Trial A, B, C. C1 (■), Control cheese; C2 (▲) made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3 (×), cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, (●) cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

4.4.4. Assesment of proteolysis

4.4.4.1. Urea-PAGE

Electrophoretograms of the cheeses after 60 and 180 days of ripening are shown in **Figures 4.4** and **4.5**. No differences were observed in the hydrolysis of α_{S1}- and β-caseins between control and experimental cheeses suggesting that yeasts extract did not influence proteolysis and confirming that rennet is responsible for the degradation of casein fractions during ripening. Similar results were obtained by Shakeel-Ur-Rehman (2003) who used food grade dry yeast to accelerate Cheddar cheese ripening and Lanciotti et al. (2005) who showed a higher hydrolysis of α_{S1}- and β-caseins in the electrophoretograms of cheeses obtained using *Y. lipolytica* as adjunct culture.

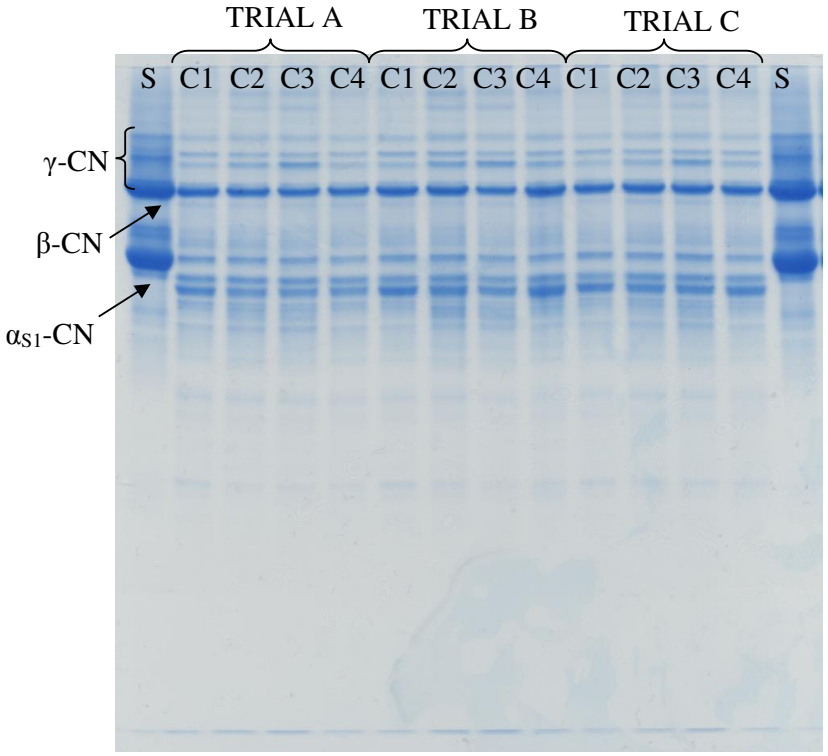

Figure 4.4. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses made with microfluidised yeast extracts at 60 d of ripening in Trial A, B, C. C1, Control cheese; C2, cheese made with 2% *Yarrowia lipolytica* DPC 6266 freeze-dried extract; C3, cheese made with 2% *Yarrowia lipolytica* DPC 6268 freeze-dried extract; C4, cheese made with 2% *Candida intermedia* DPC 6271 freeze-dried extract.

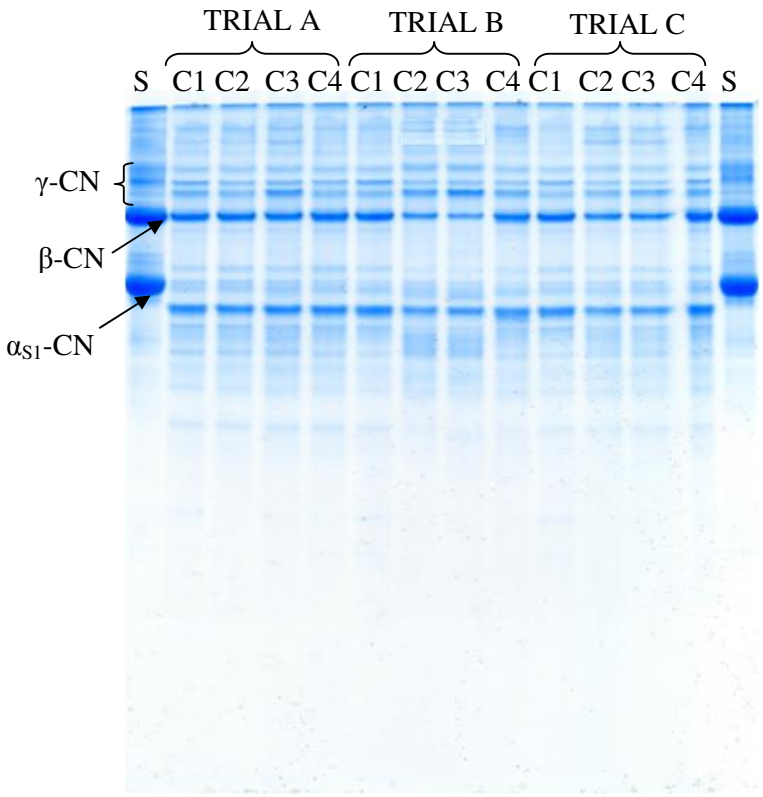

Figure 4.5. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and cheddar Cheeses made with microfluidised yeast extracts at 180 d of ripening in Trial A, B, C. C1, Control cheese; C2, cheese made with 2% *Yarrowia lipolytica* DPC 6266 freeze-dried extract; C3, cheese made with 2% *Yarrowia lipolytica* DPC 6268 freeze-dried extract; C4, cheese made with 2% *Candida intermedia* DPC 6271 freeze-dried extract.

4.4.4.2. Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed on the pH 4.6-soluble fraction of the 60 and 180 days old cheeses. Only results from Trial A are shown in **Figure 4.6** and **4.7**.
At 60 days of cheese ripening chromatograms were similar for all the cheeses; however, minor variations in certain peak height and shape were seen after ~5 min in cheese C3 made with 2% *Y. lipolytica* DPC 6268 freeze-dried extract that showed a higher peak and after 5.5 min when cheeses C2 and C4 made with 2% *Yarrowia lipolytica* DPC 6266 and 2% *C. intermedia* DPC 6271 freeze-dried extract respectively, showed a lower peak than the other cheeses.

No major differences were seen in chromatograms after 180 days of ripening between control and experimental cheeses. As also showed by chromatograms after 60 days of ripening, cheese C3 made with 2% *Y. lipolytica* DPC 6268 freeze-dried extract showed a higher peak after ~5 min.

Figure 4.8 A, B and **4.9 A, B** shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for of the processed peak height data from RP-HPLC of the pH 4.6 soluble extract at 60 and 180 days of ripening in Trial A, B and C. At 60 days, cheeses C3 from all trials are well grouped on the first principal component. After 180 days of ripening only control cheeses C1 from Trial B and C are grouping well, whereas there is a big trial effect between the other samples.

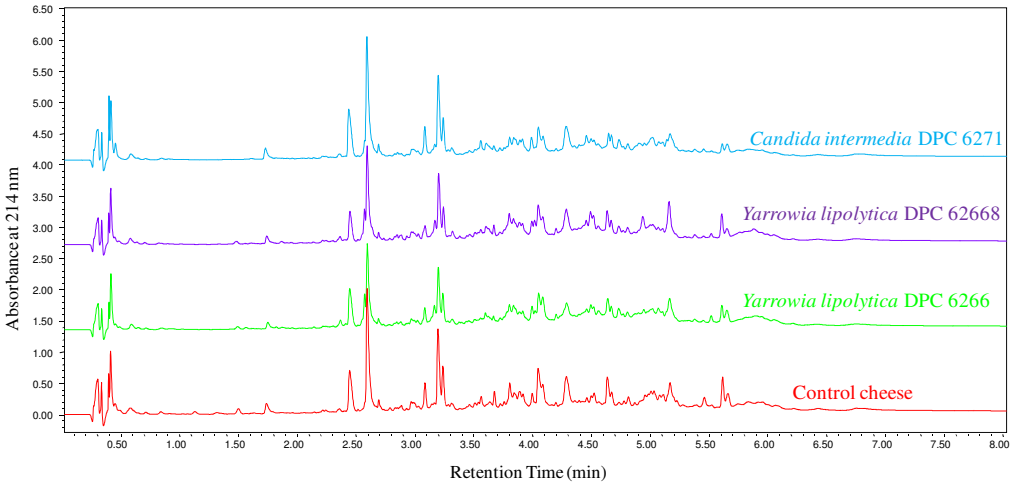

Figure 4.6. Reversed-phase HPLC chromatograms of Control and experimental Cheddar cheeses made with microfluidized yeasts extract at 60 days of ripening in Trial A.

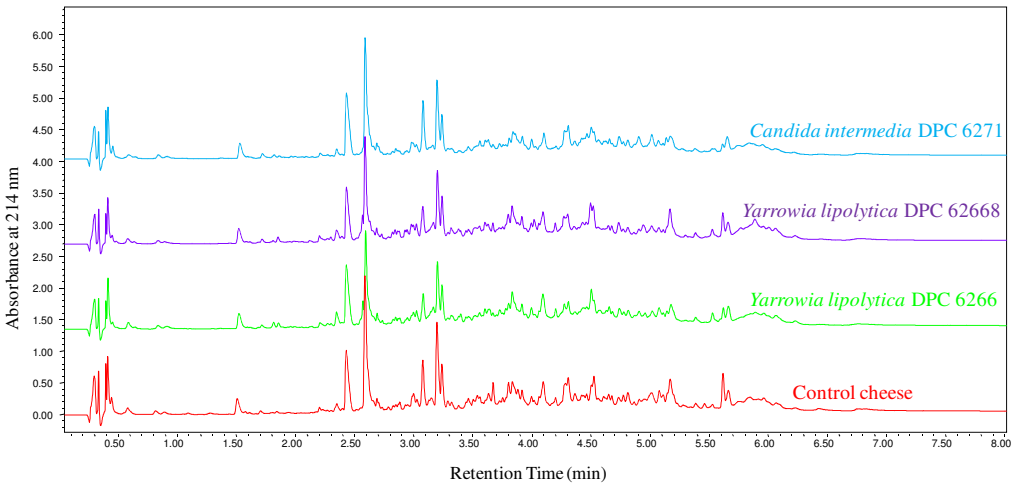

Figure 4.7. Reversed-phase HPLC chromatograms of Control and experimental Cheddar cheeses made with microfluidized yeasts extract at 180 days of ripening in Trial A.

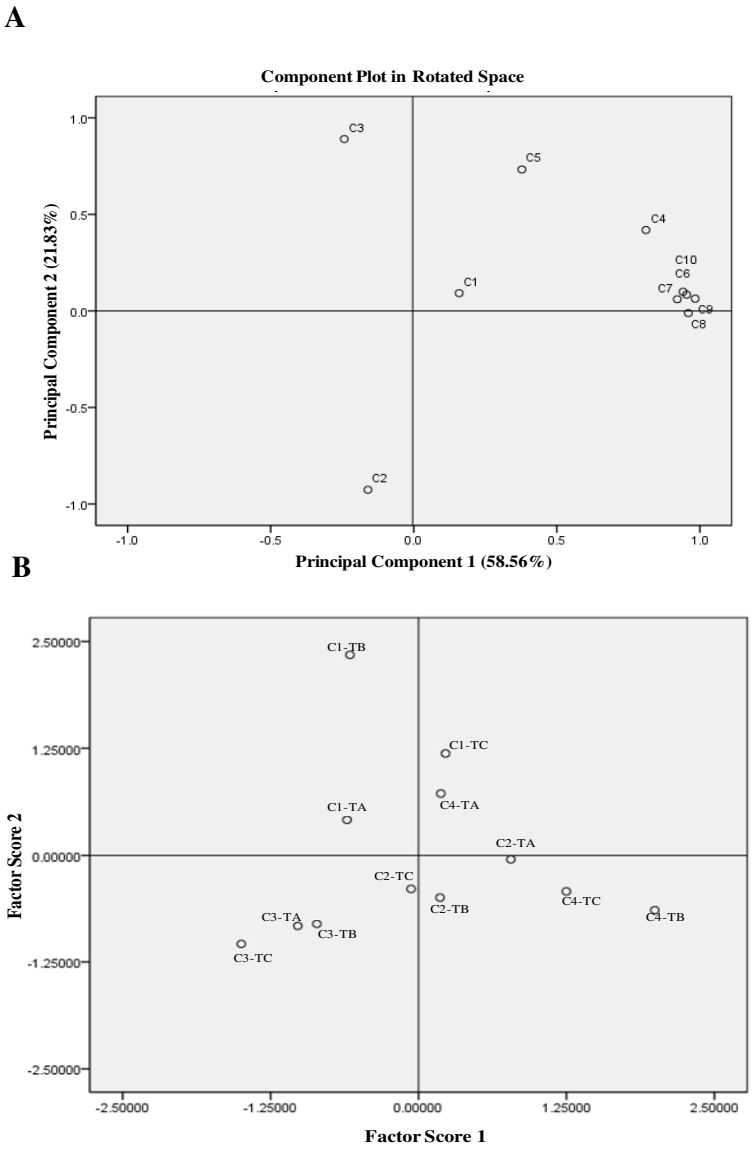

Figure 4.8. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheese at 60 d of ripening in Trials A, B and C. C1, Control cheese; C2, cheese made with 2% *Yarrowia lipolytica* DPC 6266 freeze-dried extract; C3, cheese made with 2% *Yarrowia lipolytica* DPC 6268 freeze-dried extract; C4, cheese made with 2% *Candida intermedia* DPC 6271 freeze-dried extract.

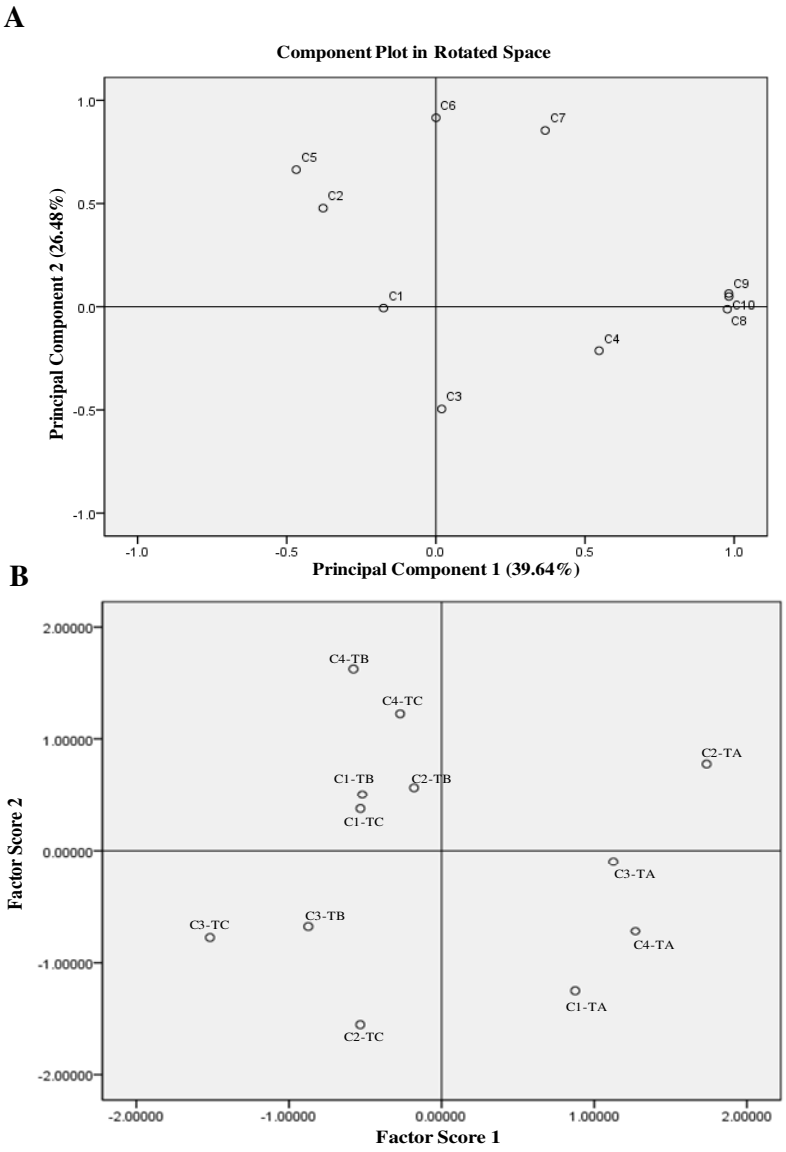

Figure 4.9. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheese at 180 d of ripening in Trials A, B and C. C1, Control cheese; C2, cheese made with 2% *Yarrowia lipolytica* DPC 6266 freeze-dried extract; C3, cheese made with 2% *Yarrowia lipolytica* DPC 6268 freeze-dried extract; C4, cheese made with 2% *Candida intermedia* DPC 6271 freeze-dried extract.

4.4.4.3. pH 4.6-soluble nitrogen

Mean values of pH 4.6-soluble nitrogen expressed as % of total nitrogen at days 60 and 180 are indicated in **Table 4.4**. After 60 and 180 days of ripening, the experimental cheeses had a significantly higher level ($P \leq 0.05$) of pH 4.6-soluble nitrogen than the control cheese, in all trials. In particular, cheese C3, made with 2% extract of *Y. lipolytica* DPC 6268 had the highest level of pH 4.6-soluble nitrogen after 180 days, in all trials. Similar results were reported by Wyder and Puhan (1999) who showed that *Y. lipolytica* was the species with the strongest proteolytic activity in cheese increasing the water-soluble fraction by up to more than 100%. These results are also in agreement with those reported by De Wit et al. (2005) in a study conducted on Cheddar cheese made with *D. hansenii* and *Y. lipolytica* and by Kesenkas and Akbulut (2008) in a study on Turkish white brined cheese made with *Y. lipolytica*, *D. hansenii*, and *K. marxianus*. However, no differences in the level of pH 4.6-soluble nitrogen were observed by Shakeel-Ur-Rehman et al. (2003) who used dry yeasts. Some authors have reported that *Y. lipolytica* was able to hydrolyze all components of casein under the environmental conditions of cheese ripening, suggesting its possible contribution to cheese proteolysis (Van den Tempel and Jakobsen, 2000; Vannini et al., 2001). In this study, it was shown that also adjunct cultures such as yeasts can contribute to the degradation of casein. Generally, in many cheese varieties, proteolysis is mainly caused by the action of coagulant and plasmin (McSweeney et al., 1993; Sousa et al., 2001) and it has previously been shown not to be influenced by addition of many adjunct cultures (McSweeney et al., 1994; Fernandez-Espla and Fox, 1998; Gardiner et al. 1998; Kenny et al., 2006).

Table 4.4. Mean levels of pH 4.6-soluble nitrogen as percentage of total nitrogen in Cheddar cheeses made with microfluidised yeast extracts at 60 and 180 days of ripening.

C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

%WSN	Cheese	Ripening time (days)	
		60d	180d
Trial A	C1	14.02±0.21[a]	22.25±0.21[a]
	C2	16.64±0.85[b]	24.07±0.42[b]
	C3	17.23±0.24[b]	25.14±0.19[c]
	C4	16.22±0.16[b]	23.39±0.51[b]
Trial B	C1	14.41±0.15[a]	20.99±0.93[a]
	C2	16.59±0.32[b]	24.55±0.10[b]
	C3	17.16±0.27[b]	26.47±0.67[c]
	C4	16.57±0.24[b]	23.05±0.79[b]
Trial C	C1	14.34±0.45[a]	21.10±0.26[a]
	C2	16.86±0.60[b]	24.13±0.13[b]
	C3	17.40±0.44[b]	25.34±0.26[c]
	C4	16.91±0.85[b]	23.77±0.07[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

4.4.4.4. Total free amino acids

The levels of total free amino acids expressed as mg leucine/g cheese at days 60 and 180 are shown in **Table 4.5**. After 60 days of ripening, only cheese C4 contained a significantly higher level ($P \leq 0.05$) of total free amino acids than the control cheese in Trials A and B, whereas in Trial C all the experimental cheeses contained significantly higher level ($P \leq 0.05$) of total free amino acids than the control cheese. After 180 days of ripening, it was found that the level of total free amino acids in the experimental cheeses, in all trials, was significantly higher ($P \leq 0.05$) than in the control cheese. In particular, cheese C2, containing 2% extract of *Yarrowia lipolytica* DPC 6266, contained the highest level. In a study conducted by De Wit et al. (2005), Cheddar cheese made with a culture mix of *Y. lipolytica* and *D. hansenii* had a significantly higher level of FAA than the control cheese. Similary, Shakeel-Ur-Rehman et al. (2003) showed that Cheddar cheese made with a food-grade dry yeast had higher levels of FAA than the control cheese throughout ripening. Others authors have shown that bacterial adjuncts such as *L. plantarum* and *L. paracasei* increased the level of free individual amino acids in cheese (Milesi et al. 2008; Burns et al. 2012).

Table 4.5. Level of total free amino acids in Cheddar cheeses made with microfluidised yeast extracts at 60 and 180 of ripening.
C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

mg Leu/g cheese	Cheese	Ripening time	
		60d	180d
Trial A	C1	9.15±0.95[a]	22.88±1.07[a]
	C2	12.13±1.94[ab]	27.92±1.13[b]
	C3	11.36±1.35[ab]	26.98±2.10[b]
	C4	14.78±1.02[b]	26.97±0.65[b]
Trial B	C1	13.53±0.47[a]	23.93±1.46[a]
	C2	16.52±1.57[ab]	29.76±0.66[b]
	C3	15.82±2.28[a]	28.22±0.58[b]
	C4	20.79±1.78[b]	29.60±0.14[b]
Trial C	C1	13.69±0.55[a]	25.86±0.73[a]
	C2	18.82±1.75[b]	32.31±0.68[c]
	C3	20.18±1.99[b]	28.98±0.27[b]
	C4	17.76±0.98[b]	31.50±1.71[bc]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

4.4.4.5. Individual Free amino acids

Levels of individual free amino acids at day 180 are shown as an average of results from Trial A, B and C in **Table 4.6**. Generally, Leu, Phe and Lys were the most abundant amino acids in all cheeses. Control cheese (C1) contained the highest amount of Thr, Ser, Glu, Gly, Ala, Val, Met and Ile; Cheese C2 had significantly higher level ($P \leq 0.05$) of Gly, Phe, GABA, Lys, Arg and Pro than the control cheese; C3 had significantly higher amount ($P \leq 0.05$) of Tyr and His than the control cheese; cheese C4 exhibited significantly higher concentration ($P \leq 0.05$) of Glu, Val, Leu, Phe, Lys, Arg and Pro than the control cheese.

De Wit et al. (2005) showed that Leu, Phe, Glu and Asn were the most abundant amino acids in Cheddar cheese made with yeasts. In particular, Cheddar cheese made with *Y. lipolytica* contained higher levels of Ala, Pro and Tyr than control cheese, while Cheddar cheese made a combination of *Y. lipolytica* and *D. hansenii* had higher levels of all amino acids than control cheese, except for His and Trp.

Figure 4.10 A, B shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for individual free amino acids at 6 months of ripening in Trial A, B and C.

Results suggest that there is no trial effect between all cheeses in all trials. Only cheese C4 from Trial B does not group well with the other cheeses from Trial A and C.

Table 4.6. Levels of individual free amino acids in Cheddar cheeses made with microfluidised yeast extracts at 180 days of ripening.
C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

IFA	**Cheese**			
mg/100gCheese	C1	C2	C3	C4
Asp	28.63±1.72^{bc}	22.95±1.11^{b}	14.60±0.98^{a}	33.85±5.83^{c}
Thr	27.25±0.74^{c}	22.50±1.30^{bc}	15.97±2.08^{a}	21.19±3.85^{ab}
Ser	25.40±0.28^{bc}	22.29±0.88^{ab}	17.88±1.71^{a}	27.68±3.60^{c}
Glu	70.41±4.35^{a}	81.61±4.46^{ab}	47.63±5.07^{a}	159.01±63.46^{b}
Gly	20.65±1.12^{b}	23.68±1.04^{c}	11.84±0.80^{a}	14.14±1.42^{a}
Ala	62.71±3.42^{b}	27.90±0.54^{a}	24.19±1.58^{a}	27.27±3.16^{a}
Val	65.56±6.36^{b}	74.27±2.93^{bc}	49.25±6.63^{a}	83.38±1.01^{c}
Met	26.10±2.99^{ab}	29.27±2.85^{ab}	24.27±3.56^{a}	33.36±2.72^{b}
Ile	31.43±1.90^{b}	21.13±0.80^{a}	17.06±2.53^{a}	23.08±5.17^{a}
Leu	146.19±20.86^{ab}	195.99±0.75^{bc}	139.03±23.77^{a}	216.53±23.87^{c}
Tyr	43.40±7.60^{a}	51.56±6.78^{ab}	65.71±5.16^{b}	39.41±5.52^{a}
Phe	122.37±23.16^{a}	161.73±7.81^{b}	129.08±3.68^{ab}	157.71±6.26^{b}
GABA	47.24±3.23^{a}	64.94±2.31^{b}	49.64±5.31^{a}	53.82±1.82^{a}
His	59.88±6.46^{ab}	62.94±2.07^{b}	68.60±3.45^{b}	50.89±2.66^{a}
Lys	55.07±6.79^{a}	112.59±7.73^{b}	75.69±1.78^{a}	135.14±21.15^{b}
Arg	n.d.	4.36±0.12^{b}	4.80±0.65^{b}	7.63±0.94^{c}
Pro	11.62±1.82^{a}	38.01±5.08^{b}	13.18±5.85^{a}	15.62±0.68^{a}

The results are shown as an average between trials (A, B, C) ± standard deviation.
[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).
n.d.: not detected

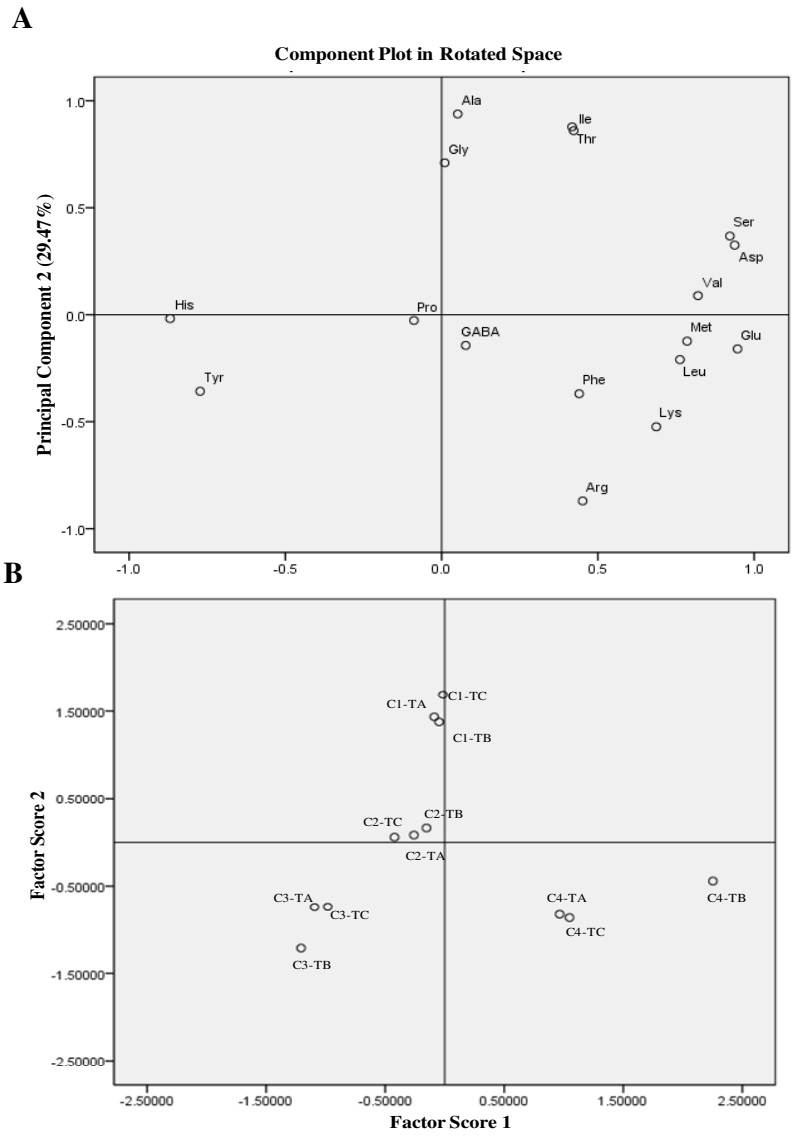

Figure 4.10. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for individual free amino acids at 6 months of ripening in Trials A, B and C. C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract

4.4.5. Assessment of lipolysis

4.4.5.1. Free fatty acid profile

Levels of total and each free fatty acids (from butyric, C4:0 to arachidic, C20:0) of cheeses at 180 days are shown in **Table 4.7** and are expressed as mg/kg of cheese. In Trial A, total lipolysis was significantly higher ($P \leq 0.05$) in all experimental cheeses than the control cheese. In particular, cheese C3 and C4, made with *Y. lipolytica* DPC 6268 and *C. intermedia* DPC 6271, respectively, had the highest values. All experimental cheeses had significantly higher ($P \leq 0.05$) levels of caproic acid (C6:0), capric acid (C10:0), myristic acid (C14:0) and oleic acid (C18:1) than the control cheese, whereas only cheeses C3 and C4 had significantly higher ($P \leq 0.05$) levels of butyric acid (C4:0), lauric acid (C12:0) and stearic acid (C18:0) than the control cheese. In Trial B, total lipolysis was significantly higher ($P \leq 0.05$) in all experimental cheeses than the control cheese and cheese C3 made with *Y. lipolytica* DPC 6268 had the highest value. All experimental cheeses had significantly higher ($P \leq 0.05$) levels of capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0) and oleic acid (C18:1) than the control cheese. Levels of butyric acid (C4:0) were significantly higher ($P \leq 0.05$) in cheeses C3 and C4, made with with *Y. lipolytica* DPC 6268 and *C. intermedia* DPC 6271 extracts, respectively, than the control cheese, whereas levels of stearic acid (C18:0) were significantly higher ($P \leq 0.05$) in cheeses C2 and C4 made with *Y. lipolytica* DPC 6266 and *C. intermedia* DPC 6271 extracts, respectively, than the control cheese. Also in Trial C, total lipolysis was significantly higher ($P \leq 0.05$) in all experimental cheeses than the control cheese; cheese C4 made with *C. intermedia* DPC 6271 extract had the highest value. All the experimental cheeses had significantly higher ($P \leq 0.05$) levels of caproic acid (C6:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0) and stearic acid (C18:0). Levels of butyric acid (C4:0) were significantly higher ($P \leq 0.05$) only in cheese C4 made with *C. intermedia* DPC 6271, whereas levels of oleic acid (C18:1) were significantly higher ($P \leq 0.05$) in cheeses C2 and C3 made with *Y. lipolytica* DPC 6266 and *Y. lipolytica* DPC 6268, respectively, than the control cheese. Caprylic acid (C8:0) was not detected in any cheeses, probably because it was present at a concentration too low to be detected.

These results confirm the ability of yeasts such as *Y. lipolytica* and *C. intermedia* to produce lipase and therefore contribute to lipolyisis in cheese. Similar results were also reported by Das et al. (2005) who showed that the concentration of free fatty acids was higher in dry-salted, washed-curd cheese made with *G. candidum* and *Y. lipolytica* than the control cheese and Lanciotti et al. (2005) in a study conducted on cheese made with *Y. lipolytica* as an adjunct culture. On the other hand, Kesenkas and Akbulut (2008), showed that *Y. lipolytica* had no effect on the free fatty acids composition of Turkish white brined cheese. In cheese made from pasteurised milk, lipolysis is the result of the action of microbial lipases (Wyder et al., 1999). It is known that *C. intermedia* and *Y. lipolytica* have a strong lipolytic activity (Welthagen and Viljoen, 1999; Van den Tempel and Jakobsen, 2000; Suzzi et al., 2001) and for this reason can positively affect cheese ripening.

Figure 4.11 A, B shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for free fatty acids at 6 months of ripening in Trial A, B and C. Only control cheese C1 are clustered together in the lower left quadrant of the

score plot, whereas there is a big trial effect in the experimental cheeses from all trials. Only C2 from Trial A and B grouped together in the upper left quadrant of the score plot.

Table 4.7. Levels of free fatty acids in Cheddar cheeses made with microfluidised yeast extracts at 180 days of ripening.
C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

FFA	***Trial A***			
mg/kg cheese	C1	C2	C3	C4
C4:0	0.50±0.01^{a}	0.52±0.01^{a}	0.73±0.05^{b}	0.92±0.02^{c}
C6:0	0.1±0.01^{a}	0.31±0.03^{c}	0.45±0.02^{d}	0.17±0.01^{b}
C8:0	n.d.	n.d.	n.d.	n.d.
C10:0	0.18±0.03^{a}	0.61±0.09^{b}	1.15±0.10^{d}	0.83±0.02^{c}
C12:0	0.45±0.04^{a}	0.54±0.02^{a}	1.26±0.08^{c}	1.09±0.01^{b}
C14:0	0.67±0.05^{a}	1.37±0.27^{b}	1.61±0.05^{b}	2.18±0.10^{c}
C16:0	0.11±0.01^{a}	0.12±0.01^{a}	0.16±0.01^{b}	0.10±0.01^{a}
C18:0	0.27±0.02^{a}	0.32±0.01^{ab}	0.34±0.02^{b}	0.45±0.02^{c}
C18:1	0.7±0.04^{a}	1.47±0.15^{bc}	1.30±0.16^{b}	1.72±0.11^{c}
C18:2	n.d.	0.25±0.02	0.24±0.04	0.18±0.01
C18:3	n.d.	n.d.	0.13	n.d.
C20:0	n.d.	0.11	n.d	n.d.
Total	3.75±0.13^{a}	6.14±0.57^{b}	7.93±0.32^{c}	8.32±0.17^{c}

FFA	***Trial B***			
mg/kg cheese	C1	C2	C3	C4
C4:0	0.44±0.07^{a}	0.51±0.01^{a}	0.81±0.01^{b}	0.87±0.03^{b}
C6:0	n.d.	0.33±0.03	0.43±0.03	0.17±0.03
C8:0	n.d.	n.d.	n.d.	n.d.
C10:0	0.20±0.04^{a}	0.62±0.08^{b}	1.2±0.13^{c}	0.74±0.02^{b}
C12:0	0.28±0.02^{a}	0.68±0.09^{b}	1.29±0.18^{c}	1.12±0.03^{c}
C14:0	0.63±0.02^{a}	1.4±0.14^{b}	2.4±0.11^{c}	2.28±0.03^{c}
C16:0	0.11±0.01^{b}	0.13±0.01^{b}	0.11±0.01^{b}	0.07±0.01^{a}
C18:0	0.27±0.05^{a}	0.37±0.02^{bc}	0.32±0.01^{ab}	0.44±0.01^{c}
C18:1	0.63±0.14^{a}	1.47±0.19^{b}	1.58±0.48^{b}	1.54±0.14^{b}
C18:2	n.d.	0.25	0.23	0.14
C18:3	n.d.	n.d.	0.13	n.d.
C20:0	n.d.	0.10	n.d.	n.d.
Total	3.28±0.30^{a}	6.39±0.52^{b}	9.14±0.30^{d}	7.93±0.22^{c}

FFA	*Trial C*			
mg/kg cheese	C1	C2	C3	C4
C4:0	0.56±0.04[b]	0.43±0.05[a]	0.59±0.06[b]	0.90±0.01[c]
C6:0	0.11±0.01[a]	0.38±0.06[b]	0.29±0.09[b]	0.33±0.02[b]
C8:0	n.d.	n.d.	n.d.	n.d.
C10:0	0.30±0.02[a]	0.72±0.01[b]	0.88±0.02[c]	0.98±0.01[c]
C12:0	0.38±0.02[a]	0.77±0.02[b]	0.97±0.01[c]	1.40±0.14[d]
C14:0	0.93±0.02[a]	1.65±0.11[b]	1.56±0.06[b]	2.24±0.17[c]
C16:0	n.d.	n.d.	0.13±0.01	0.15±0.01
C18:0	0.25±0.01[a]	0.40±0.01[b]	0.44±0.01[b]	0.51±0.03[c]
C18:1	0.71±0.09[a]	1.68±1.16[b]	1.52±0.34[b]	1.44±0.48[ab]
C18:2	n.d.	0.12±0.02	0.16±0.01	0.31±0.01
C18:3	n.d.	n.d.	n.d.	0.14±0.03
C20:0	n.d.	0.12	0.10	n.d.
Total	3.99±0.22[a]	6.83±0.28[b]	7.22±0.52[b]	9.02±0.50[c]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

n.d.: not detected

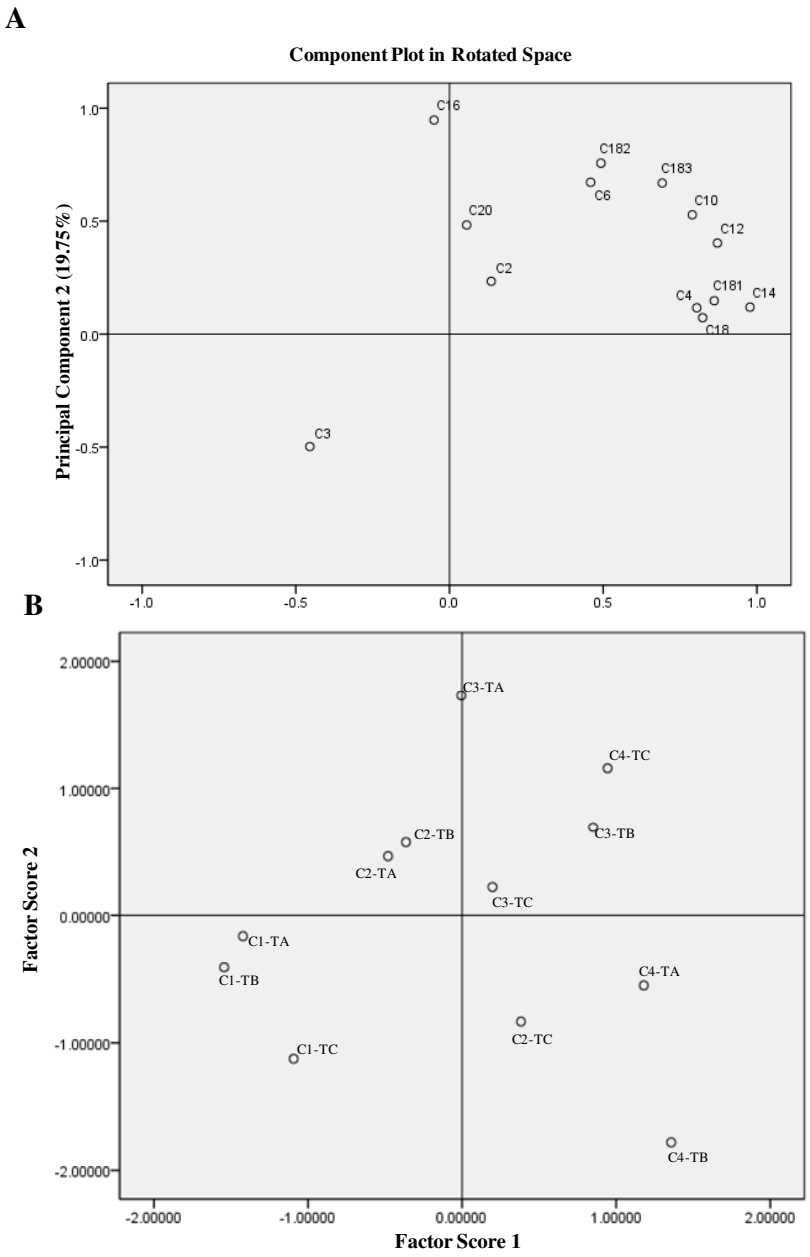

Figure 4.11. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for free fatty acids at 6 months of ripening in Trials A, B and C. C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

4.4.6. Enzymatic assays in yeast extracts

The assays carried out on the freeze-dried yeast extracts showed that all the strains had aminopeptidase activity against Glu-*p*NA and Lys-*p*NA, dipeptidyl aminopeptidase activity against Ala-Pro-*p*NA, 5'phosphodiesterase activity (5'PDE) and esterase activity against *p*-nitrophenyl butyrate (**Table 4.8**). *Y. lipolytica* DPC 6266 had the highest activity on Lys-*p*NA, whereas *C. intermedia* DPC 6271 had the highest activity on Glu-*p*NA and Ala-Pro-*p*NA. *Y. lipolytica* DPC 6268 and *C. intermedia* DPC 6271 exhibited significantly higher ($P \leq 0.05$) esterase activity than *Y. lipolytica* DPC 6266, whereas *Y. lipolytica* DPC 6266 and *C. intermedia* DPC 6271 had significantly higher ($P \leq 0.05$) 5'PDE activity than *Y. lipolytica* DPC 6268. These results indicated that enzyme activity in the freeze-dried yeast extract was not affected by the microfluidization and freeze-drying treatments and could be used for cheesemaking.

Table 4.8. Specific activity of freeze-dried yeasts extract powder after production and before addiction to the cheeses.

Strain	SPECIFIC ACTIVITY (U/mg protein)				
	Glu-pNA	Lys-pNA	Ala-Pro-pNA	Esterase (p-NPB)	5'PDE (T5'MP-pNP)
Y. lipolytica DPC 6266	68.34±3.60[b]	142.09±1.08[c]	35.61±0.36[b]	1616.97±19.48[a]	727.31±13.68[b]
Y. lipolytica DPC 6268	56.86±3.79[a]	56.87±3.03[a]	17.82±1.14[a]	3223.50±349.04[b]	175.20±54.53[a]
C. intermedia DPC 6271	78.66±3.15[c]	92.87±1.17[b]	72.37±3.15[c]	2811.76±119.29[b]	831.60±68.93[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation and expressed as unit per milligram of protein (U/mg protein).
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

4.4.7. Enzymatic assays in cheeses

Aminopeptidase, dipeptidase, esterase and 5'phosphodiestere activity were measured on the pH 4.6-soluble extract from the cheeses after 60 and 180 days of ripening.
Assays on pH 4.6-soluble extract for enzyme activity after 60 days, showed that all experimental cheeses had significantly higher ($P \leq 0.05$) esterase activity than the control cheese, in all trials (**Table 4.9**). 5'PDE activity was detected in all experimental cheeses whereas no activity was found in control cheeses. No significant differences were found in Glu-*p*NA, Lys-*p*NA and Ala-Pro-*p*NA activities between the control and the experimental cheeses in Trials A and B, except for C2 that had Lys-pNA activity higher than the control. In Trial C, significantly higher levels ($P \leq 0.05$) of Glu-*p*NA and Ala-Pro-*p*NA activity were found in C2, C3 and C4 than the control cheese C1, whereas levels of Lys-*p*NA in the experimental cheeses were similar to the control cheese C1. After 180 d of ripening, all the experimental cheeses had significantly higher levels ($P \leq 0.05$) of aminopeptidase,

dipeptidase and esterase activity than the control cheese C1. 5'PDE activity was detected only in the experimental cheeses (**Table 4.10**).

Similar results were also reported by Hernández-Montañez et al. (2007) who showed that *Y. lipolytica* had aminopeptidase, dipeptidyl aminopeptidase and carboxypeptidase activity. Some strains of *Candida* isolated from Manteca cheese have aminopeptidase and X-prolyl-dipeptidyl aminopeptidase activity against Arg-βNA, Lys-βNA and Leu-βNA, Phe-Pro-βNA and Pro-βNA (Suzzi et al., 2003). In yeast, the nucleic acid fraction consists mainly of RNA and DNA (Belem et al., 1997). A high 5'phosphodiestere activity against RNA is desirable for the production of 5'ribonucleotides such as guanosine-5'-monophosphate (GMP) and inosine 5'-monophosphate (IMP) that can strongly enhance the umami taste. Umami, the savory taste of glutamate, is one of the 5 basic tastes, in addition to sweet, bitter, salty and sour, that humans can detect; it was first discovered in 1908 by K. Ikeda, but only recently accepted as a basic taste quality (Zhang et al., 2008). The results of this study confirmed that *C. intermedia* and *Y. lipolytica* have aminopeptidase, dipeptidase, esterase and 5'phosphodiestere activity that can contribute to accelerate the ripening and improve the flavor of Cheddar cheese.

Table 4.9. Enzymatic activity of pH 4.6-soluble extracts from Cheddar cheeses made with microfluidised yeast extracts at 60 d.
C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

	Cheese	SPECIFIC ACTIVITY (U/mg protein)				
		Glu-pNA	Lys-pNA	Ala-Pro-pNA	Esterase (p-NPB)	5'PDE (T5'MP-pNP)
Trial A	C1	0.42±0.03^{a}	0.89±0.07^{a}	0.58±0.05^{a}	7.71±0.19^{a}	n.d.
	C2	0.44±0.02^{a}	1.12±0.07^{a}	0.69±0.02^{a}	17.32±0.18^{b}	4.95±0.05^{b}
	C3	0.45±0.03^{a}	0.95±0.05^{a}	0.70±0.03^{a}	21.11±0.04^{c}	5.37±0.12^{c}
	C4	0.53±0.05^{a}	0.79±0.03^{a}	0.69±0.04^{a}	39.38±0.36^{d}	2.64±0.04^{a}
Trial B	C1	0.57±0.03^{a}	0.51±0.01^{a}	0.52±0.01^{a}	3.81±0.13^{a}	n.d.
	C2	0.61±0.01^{a}	0.94±0.06^{b}	0.56±0.02^{a}	25.69±6.52^{c}	4.60±0.05^{b}
	C3	0.63±0.02^{a}	0.56±0.02^{a}	0.54±0.09^{a}	16.64±0.45^{b}	3.83±0.12^{a}
	C4	0.56±0.07^{a}	0.55±0.02^{a}	0.45±0.04^{a}	18.82±0.17^{bc}	4.58±0.09^{b}
Trial C	C1	0.01±0.03^{a}	0.53±0.04^{a}	0.10±0.01^{a}	8.52±0.88^{a}	n.d.
	C2	0.23±0.01^{b}	0.84±0.01^{a}	0.18±0.02^{b}	14.34±1.98^{b}	2.77±0.24^{a}
	C3	0.22±0.01^{b}	0.67±0.01^{a}	0.17±0.02^{b}	21.01±1.53^{c}	2.14±0.07^{a}
	C4	0.22±0.06^{b}	0.56±0.04^{a}	0.26±0.01^{c}	22.73±1.03^{c}	2.39±0.13^{a}

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation and expressed as unit per milligram of protein (U/mg protein).
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).
n.d.: not detected

Table 4.10. Enzymatic activity of pH 4.6-soluble extracts from Cheddar cheeses made with microfluidised yeast exttacts at 180 d

C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

		SPECIFIC ACTIVITY (U/mg protein)				
	Cheese	**Glu-pNA**	**Lys-pNA**	**Ala-Pro-pNA**	**Esterase (p-NPB)**	**5'PDE (T5'MP-pNP)**
Trial A	C1	0.45±0.03^{a}	0.77±0.01^{a}	0.65±0.04^{a}	4.89±0.12^{a}	n.d.
	C2	0.61±0.09^{b}	0.85±0.03^{b}	0.81±0.01^{b}	13.54±0.12^{bc}	6.47±0.76^{ab}
	C3	0.62±0.03^{b}	0.81±0.00^{ab}	0.80±0.01^{b}	13.44±0.02^{b}	5.76±0.18^{a}
	C4	0.53±0.06^{ab}	0.82±0.02^{ab}	0.89±0.01^{c}	13.91±0.25^{c}	7.10±0.05^{b}
Trial B	C1	0.65±0.03^{a}	0.73±0.02^{a}	0.63±0.04^{a}	2.56±0.11^{a}	n.d.
	C2	0.82±0.01^{c}	0.80±0.02^{b}	0.82±0.01^{b}	14.46±0.61^{c}	6.67±0.31^{b}
	C3	0.76±0.01^{b}	0.74±0.03^{ab}	0.76±0.02^{b}	11.28±0.20^{b}	4.79±0.08^{a}
	C4	0.79±0.03^{bc}	0.86±0.01^{c}	0.84±0.04^{b}	13.52±0.79^{c}	8.58±0.07^{c}
Trial C	C1	0.61±0.05^{a}	0.51±0.02^{a}	0.46±0.05^{a}	5.79±0.60^{a}	n.d.
	C2	0.80±0.01^{b}	0.74±0.02^{b}	0.84±0.02^{b}	12.12±0.58^{b}	8.25±0.45^{ab}
	C3	0.80±0.02^{b}	0.70±0.01^{b}	0.81±0.02^{b}	13.39±0.28^{bc}	7.63±0.12^{a}
	C4	0.84±0.02^{b}	0.74±0.01^{b}	0.84±0.03^{b}	13.56±0.64^{c}	8.96±0.82^{b}

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation and expressed as unit per milligram of protein (U/mg protein).

[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

n.d.: not detected

4.4.8. Biogenic amines

Levels of biogenic amines (BA) are shown in **Table 4.11** and expressed as µg of BA per g of cheese. BA are the result of decarboxylation of amino acids, a process in which decarboxylases remove the carboxyl group of the amino acid and liberate the corresponding amine. BA play an important role as flavour components and also as food poisoning agents causing the release of adrenaline and noradrenaline, causing migraine, tachycardia, gastric acid secretion, increase of cardiac output, blood sugar levels, and blood pressure (Wyder et al., 1999; Linares et al., 2011). In Trial A, only cheese C4 made with *C. intermedia* DPC 6271 extract had significantly higher ($P \leq 0.05$) levels of spermidine and spermine than the control cheese, while only C3 made with *Y. lipolytica* DPC 6268 extract had significantly higher ($P \leq 0.05$) levels of spermine than the control cheese. Tryptamine and histamine were not detected in any of the cheeses, while cadaverine was found only in control cheese C1 and cheese C3 made with *Y. lipolytica* DPC 6268 extract. In Trial B, cheese C2 had significant higher ($P \leq 0.05$) level of phenylethylamine and tyramine than the control cheese, cheese C3 had only significant higher ($P \leq 0.05$) levels of spermine, while cheese C4 made with *C. intermedia* DPC 6271 extract had significantly higher ($P \leq 0.05$) levels of phenylethylamine, putrescine, tyramine and spermidine than the control cheese C1.

Histamine is the most toxic amine detected in foods (Santos, 1996). Histamine and tryptamine were not detected in any of the cheeses, while cadaverine was found only in cheese C4 at a very low level. In Trial C, significantly higher ($P \leq 0.05$) levels of phenylethylamine and tyramine were found in all experimental cheeses, whereas only cheese C4 had a significant higher ($P \leq 0.05$) level of spermidine than the control cheese. Tryptamine, histamine and cadaverine were not detected in any of the cheeses. In a study conducted by Gardini et al. (2006), nine strains of *Candida intermedia*, isolated from Pecorino Crotonese cheese, were able to produce putrescine *in vitro* and one of the strains also produced phenylethylamine, while three strains of *Y. lipolytica* produced putrescine, phenylethylamine, tyramine and cadaverine *in vitro*. Wyder et al. (1999) showed that the interaction between *Pichia jadinii* and *Y. lipolytica* in foil wrapped Raclette cheese led to high levels of total BA (118.63 mg/kg), histamine (19.23 mg/kg) and putrescine (9.58 mg/kg). Similarly, the interaction between *D. hansenii* B and *Y. lipolytica* resulted in elevated levels of histamine (22.63 mg/kg) and tryptamine (15.28 mg/kg), whereas *Galactomyces geotrichum* inhibited the production of BA by *P. jadinii*, *Y. lipolytica* and *D. hansenii*. *C. rugosa* isolated from Manteca cheese produced phenylethylamine, putrescine, cadaverine, spermine and spermidine *in vitro* after 7 days of incubation in milk with added precursor amino acids (Suzzi et al., 2003).

Biogenic amines levels in cheese vary as a function of ripening period and microbiota; usually highest levels are found in cheeses contaminated with spoilage microorganisms (Fox et. al, 2000). After fish, cheese is the second most common food associated with histamine poisoning. The toxicological level of amines is very difficult to establish because it depends on individual characteristics and the presence of other amines, however, values of 100-800 mg/kg of tyramine and 30 mg/kg of phenylethylamine have been reported as toxic doses in food (Santos, 1996). There is little specific legislation concerning BA content in foods. Upper limits for BA have only been recommended or suggested (e.g., 100 mg of histamine

per kg of food, or 2 mg of histamine per liter of alcoholic beverage). In the case of tyramine, a limit of between 100 and 800 mg kg^{-1} has been recommended, and a limit of 30 mg kg^{-1} of β-phenylethylamine has been proposed (Linares et al., 2011). In a study by Min et al. (2004), tyramine was the major BA (44.46±0.83 μg/g), followed by histamine (29.37 μg/g) detected in Cheddar cheese. Higher level of phenylethylamine, putrescine, tyramine and spermidine were detected in Cheddar cheeses in this study than those obtained by Min et al. (2004). In a study by Rea et al. (2004), levels of tyramine in Cheddar cheeses made with some strains of enterococci ranged from 72.00 to 197.00 mg/kg after 36 weeks of ripening, whereas the concentration of tyramine in the experimental cheeses of this study ranged from 10.37 to 86.03 mg/kg. As reported by EFSA's scientific opinion on risk-based control of biogenic amine formation in fermented foods (EFSA, 2011), high occurrence values of biogenic amines (mg/kg) in cheese are as follows: histamine (130), tyramine (440), putrescine (143), cadaverine (470), phenylethylamine (18.8), tryptamine (‹50). According to those data, levels of tyramine in all cheeses, in all trials of this study can be considered low, whereas level of putrescine in cheeses C1 (173.85 mg/kg) and C3 (182.16) (Trial A) and in cheese C4 (Trial A and B) can be considered high.

Phenylethylamine was detected at levels close to those reported by the EFSA's scientific opinion (EFSA, 2011) in all cheeses in Trial A, whereas in Trial B and C, only cheese C2 had high levels of phenylethylamine (22.21 and 23.25, respectively). Tryptamine and histamine were not detected in any of the cheeses, while cadaverine was present only in few cheeses at concentration much lower than those indicated by the EFSA's scientific opinion (EFSA, 2011).

Table 4.11. Levels of biogenic amines in Cheddar cheeses made using microfluidised yeast extracts at 180 days of ripening.
C1, Control cheese; C2, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6266 extract; C3, cheese made with 2% freeze-dried *Yarrowia lipolytica* DPC 6268 extract; C4, cheese made with 2% freeze-dried *Candida intermedia* DPC 6271 extract.

BA	***Trial A***			
(µg/1g of cheese)	C1	C2	C3	C4
tryptamine	n.d.	n.d.	n.d.	n.d.
phenylethylamine	34.83±7.88[a]	36.93±4.37[a]	38.04±4.42[a]	34.55±2.67[a]
putrescine	347.70±93.86[b]	101.96±9.23[a]	364.31±58.18[b]	255.78±33.38[b]
cadaverine	2.93±0.25	n.d.	4.94±0.92	n.d.
histamine	n.d.	n.d.	n.d.	n.d.
tyramine	133.87±3.84[a]	144.64±14.18[a]	128.31±12.15[a]	149.72±11.51[a]
spermidine	118.77±7.08[a]	136.58±7.17[a]	119.73±9.03[a]	191.83±7.20[b]
spermine	9.60±0.33[a]	11.72±1.00[a]	15.36±0.33[b]	20.45±2.53[c]

BA	***Trial B***			
(µg/1gr of cheese)	C1	C2	C3	C4
tryptamine	n.d.	n.d.	n.d.	n.d.
phenylethylamine	15.85±1.33[a]	44.42±0.14[c]	12.55±1.97[a]	30.19±2.64[b]
putrescine	22.91±4.27[a]	24.92±6.95[a]	23.02±3.23[a]	497.84±10.82[b]
cadaverine	n.d.	n.d.	n.d.	1.97±0.14
histamine	n.d.	n.d.	n.d.	n.d.
tyramine	88.16±6.91[a]	130.47±1.51[b]	88.17±7.39[a]	172.06±6.45[c]
spermidine	116.40±7.30[a]	121.80±1.56[a]	131.84±16.69[a]	202.67±15.94[b]
spermine	13.78±3.73[ab]	12.05±0.73[a]	21.26±3.76[c]	20.40±1.53[bc]

BA	***Trial C***			
(µg/1gr of cheese)	C1	C2	C3	C4
tryptamine	n.d.	n.d.	n.d.	n.d.
phenylethylamine	3.34±0.83[a]	46.49±0.95[c]	19.38±1.95[b]	20.10±0.42[b]
putrescine	139.03±6.11[b]	22.73±1.62[a]	18.28±1.00[a]	21.12±7.65[a]
cadaverine	n.d.	n.d.	n.d.	n.d.
histamine	n.d.	n.d.	n.d.	n.d.
tyramine	20.74±6.19[a]	150.60±3.66[d]	104.08±7.43[b]	124.57±7.93[c]
spermidine	151.43±11.32[ab]	173.09±8.65[b]	145.85±4.69[a]	234.39±7.15[c]
spermine	47.06±1.94[c]	15.22±1.43[a]	20.49±3.32[a]	39.86±1.64[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).
n.d.: not detected

4.5. Conclusions

Y. lipolytica and *C. intermedia* play an important role during cheese ripening thanks to their high proteolytic and lipolytic activities that lead to the formation of aroma precursors such as amino acids, fatty acids and esters. In this study two strains of *Y. lipolytica* and one strain of *C. intermedia* were grown in broth, microfluidized at high pressure, freeze-dried and then added during Cheddar cheese manufacture. The yeasts positively affected the proteolysis and lipolysis of cheeses and had aminopeptidase, dipeptidase, esterase and 5'phosphodiestere activities that can contribute to accelerate the ripening and improve the flavor of cheese. *Y. lipolytica* and *C. intermedia* also produced high levels of biogenic amines such as phenylethylamine and putrescine. Histamine, which is one of the most toxic biogenic amines, together with tyramine, was not detected in any of the cheese, whereas tyramine was present at low levels. Therefore, it can be concluded that the use of microfluidized strains of *Y. lipolytica* and *C. intermedia* increased only the production of few biogenic amines and that the most toxic ones (histamine and tyramine) were not produced or produced at very low level.

4.6. References

ANDREWS, A. T. 1983. Proteinases in normal bovine-milk and their action on caseins. *Journal of Dairy Research,* 50, 45-55.

BANKAR, A. V., KUMAR, A. R. & ZINJARDE, S. S. 2009. Environmental and industrial applications of *Yarrowia lipolytica*. *Applied Microbiology and Biotechnology,* 84, 847-865.

BELEM, M. A. F., GIBBS, B. F. & LEE, B. H. 1997. Enzymatic production of ribonucleotides from autolysates of *Kluyveromyces marxianus* grown on whey. *Journal of Food Science,* 62, 851-&.

BERESFORD, T. & WILLIAMS, A. 2004. The Microbiology of Cheese Ripening. *In:* PATRICK F. FOX, P. L. H. M. T. M. C. & TIMOTHY, P. G. (eds.) *Cheese: Chemistry, Physics and Microbiology.* Academic Press.

BERESFORD, T. & WILLIAMS, A. 2004. The microbiology of cheese ripening. *Cheese: chemistry, physics and microbiology*, Vol. 1. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 287-317.

BINTSIS, T. & ROBINSON, R. K. 2004. A study of the effects of adjunct cultures on the aroma compounds of Feta-type cheese. *Food Chemistry,* 88, 435-441.

BLAKESLEY, R. W. & BOEZI, J. A. 1977. New staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry,* 82, 580-582.

BREINIG, F., DIEHL, B., RAU, S., ZIMMER, C., SCHWAB, H. & SCHMITT, M. J. 2006. Cell surface expression of bacterial esterase A by *Saccharomyces cerevisiae* and its enhancement by constitutive activation of the cellular unfolded protein response. *Applied and Environmental Microbiology*, 72, 7140-7147.

BRENNAN, N. M., COGAN, T. M., LOESSNER, M. & SCHERER, S. 2004. Bacterial surface-ripened cheeses. *In: Cheese: Chemistry, Physics and Microbiology.* Academic Press.

BRENNAN, N. M., COGAN, T. M., LOESSNER, M. & SCHERER, S. 2004. Bacterial surface-ripened cheeses. C*heese: chemistry, physics and microbiology*, Vol. 2. Edited by P. F. Fox, P. L. H. McSweeney, T. M. Cogan, T. P. Guinee, Academic Press, London, 199-225.

BURNS, P., CUFFIA, F., MILESI, M., VINDEROLA, G., MEINARDI, C., SABBAG, N. & HYNES, E. 2012. Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheeses. *Food Microbiology*, 30, 45-50.

CHAE, H. J., JOO, H. & IN, M. J. 2001. Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1. effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology,* 76, 253-258.

CHAMBA, J. F. & IRLINGER, F. 2004. Secondary and adjunct cultures. C*heese: chemistry, physics and microbiology*, . Vol. 1. Edited by Patrick P. F. Fox, Paul P. L. H. McSweeney, Timothy T. M. Cogan, Timothy,. P. Guinee, Academic Press, LondonVol. 1, 191-206.

CROMIE, S. J., GILES, J. E. & DULLEY, J. R. 1987. Effect of elevated ripening temperatures on the microflora of Cheddar cheese. *Journal of Dairy Research,* 54, 69-76.

DADAKOVA, E., KRIZEK, M. & PELIKANOVA, T. 2009. Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC). *Food Chemistry,* 116, 365-370.

DAS, S., HOLLAND, R., CROW, V. L., BENNETT, R. J. & MANDERSON, G. J. 2005. Effect of yeast and bacterial adjuncts on the CLA content and flavour of a washed-curd, dry-salted cheese. *International Dairy Journal,* 15, 807-815.

DE FELICE, B., PONTECORVO, G. & CARFAGNA, M. 1997. Degradation of waste waters from olive oil mills by *Yarrowia lipolytica* ATCC 20255 and *Pseudomonas putida*. *Acta Biotechnologica*, 17, 231-239.

DE FELICE, B., PONTECORVO, G. & CARFAGNA, M. 1997. Degradation of waste waters from olive oil mills by *Yarrowia lipolytica* ATCC 20255 and *Pseudomonas putida*. *Acta Biotechnologica*, 17, 231-239.

DE FELICE, B., PONTECORVO, G. & CARFAGNA, M. 1997. Degradation of waste waters from olive oil mills by Yarrowia lipolytica ATCC 20255 and Pseudomonas putida. *Acta Biotechnologica*, 17, 231-239.

DE JONG, C. & BADINGS, H. T. 1990. Determination of free fatty acids in milk and cheese procedures for extraction clean up and capillary gas chromatographic analysis. *HRC Journal of High Resolution Chromatography*, 13, 94-98.

DE JONG, C. & BADINGS, H. T. 1990. Determination of free fatty acids in milk and cheese procedures for extraction clean up and capillary gas chromatographic analysis. *HRC Journal of High Resolution Chromatography*, 13, 94-98.

DE WIT, M., OSTHOFF, G., VILJOEN, B. C. & HUGO, A. 2005. A comparative study of lipolysis and proteolysis in Cheddar cheese and yeast-inoculated Cheddar cheeses during ripening. *Enzyme and Microbial Technology,* 37, 606-616.

DE WIT, M., OSTHOFF, G., VILJOEN, B. C. & HUGO, A. 2005. A comparative study of lipolysis and proteolysis in Cheddar cheese and yeast-inoculated Cheddar cheeses during ripening. *Enzyme and Microbial Technology,* 37, 606-616.

DEFELICE, B., PONTECORVO, G. & CARFAGNA, M. 1997. Degradation of waste waters from olive oil mills by Yarrowia lipolytica ATCC 20255 and Pseudomonas putida. *Acta Biotechnologica*, 17, 231-239.

EFSA 2011. Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal*, 9.

EFSA 2011. Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal*, 9, 2393.

EL-SODA, M., BERGERE, J. L. & DESMAZEAUD, M. J. 1978. Detection and localization of peptide-hydrolases in *Lactobacillus casei. Journal of Dairy Research,* 45, 519-524.

ENCINAS, J.-P., LÓPEZ-DíAZ, T.-M. A., GARCíA-LÓPEZ, M. A.-L., OTERO, A. & MORENO, B. 2000. Yeast populations on Spanish fermented sausages. *Meat Science*, 54, 203-208.

FARKYE, N. Y., MADKOR, S. A. & ATKINS, H. G. 1995. Proteolytic abilities of some lactic-acid bacteria in a model cheese system. *International Dairy Journal,* 5, 715-725.

FERREIRA, A. D. & VILJOEN, B. C. 2003. Yeasts as adjunct starters in matured Cheddar cheese. *International Journal of Food Microbiology,* 86, 131-140.

FICKERS, P., BENETTI, P. H., WACHÉ, Y., MARTY, A., MAUERSBERGER, S., SMIT, M. & NICAUD, J. M. 2005. Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. *FEMS Yeast Research,* 5, 527-543.

FLEET, G. H. & MIAN, M. A. 1987. The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology*, 4, 145-155.

FLEET, G. H. 1990. Yeasts in dairy-products. *Journal of Applied Bacteriology,* 68, 199-211.

FOSCHINO, R., PICOZZI, C., BORGHI, M., CERLIANI, M. C. & CRESCI, E. 2006. Investigation on the microflora of Caprino Lombardo cheese from raw goat milk. *Italian Journal of Food Science,* 18, 33-49.

FOX, P. F. 1963. Potentiometric determination of salt in cheese. *Journal of Dairy Science,* 46, 744-745.

FOX, P. F., GUINEE, T. P., COGAN, T. M. & McCSWEENEY, P. L. H. 2000. *Fundamentals of Cheese Science*: Aspen Publishers, Gaithersburg, MD.

FREITAS, A. C., PAIS, C., MALCATA, F. X. & HOGG, T. A. 1996. Microbiological characterization of Picante da Beira Baixa cheese. *Journal of Food Protection,* 59, 155-160.

GALABOVA, D., TULEVA, B. & SPASOVA, D. 1996. Permeabilization of *Yarrowia lipolytica* cells by Triton X-100. *Enzyme and Microbial Technology,* 18, 18-22.

GARDINER, G., ROSS, R. P., COLLINS, J. K., FITZGERALD, G. & STANTON, C. 1998. Development of a probiotic cheddar Cheddar cheese containing human-derived *Lactobacillus paracasei* strains. *Applied and Environmental Microbiology*, 64, 2192-2199.

GARDINI, F., TOFALO, R., BELLETTI, N., IUCCI, L., SUZZI, G., TORRIANI, S., GUERZONI, M. E. & LANCIOTTI, R. 2006. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiology,* 23, 641-648.

GECIOVA, J., BURY, D. & JELEN, P. 2002. Methods for disruption of microbial cells for potential use in the dairy industry—a review. *International Dairy Journal,* 12, 541-553.

GRAPPIN, R. 1986. Milk determination of nitrogen content kjeldahl Kjeldahl method and calculation of crude protein content. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 20A, 1-3.

GUERZONI, M. E., LANCIOTTI, R., VANNINI, L., GALGANO, F., FAVATI, F., GARDINI, F. & SUZZI, G. 2001. Variability of the lipolytic activity in *Yarrowia lipolytica* and its dependence on environmental conditions. *International Journal of Food Microbiology,* 69, 79-89.

HEFFERNAN, S. P., KELLY, A. L., MULVIHILL, D. M., LAMBRICH, U. & SCHUCHMANN, H. P. 2011. Efficiency of a range of homogenisation technologies in the emulsification and stabilization of cream liqueurs. *Innovative Food Science & Emerging Technologies,* 12, 628-634.

HERNÁNDEZ-MONTAÑEZ, Z., ARAUJO-OSORIO, J., NORIEGA-REYES, Y., CHÁVEZ-CAMARILLO, G. & VILLA-TANACA, L. 2007. The intracellular proteolytic system of *Yarrowia lipolytica* and characterization of an aminopeptidase. *FEMS Microbiology Letters,* 268, 178-186.

IDF 1983. Milk, cream and evaporated milk - determination of total solids content (reference method)-provisional international IDF standard 21A-1982. *Milchwissenschaft-Milk Science International,* 38, 21-22.

IDF 1986. Cheese and processed cheese products determination of fat content gravimetric method reference method. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 1-5.

JOHNSON, J. A. C., ETZEL, M. R., CHEN, C. M. & JOHNSON, M. E. 1995. Accelerated ripening of reduced-fat Cheddar cheese using 4 attenuated *Lactobacillus helveticus* CNRZ 32 adjuncts. *Journal of Dairy Science,* 78, 769-776.

KENNY, O., FITZGERALD, R. J., O'CUINN, G., BERESFORD, T. & JORDAN, K. 2006. Autolysis of selected *Lactobacillus helveticus* adjunct strains during Cheddar cheese ripening. *International Dairy Journal*, 16, 797-804.

KESENKAS, H. & AKBULUT, N. 2008. Yeasts as ripening adjunct cultures in Turkish white brined cheese production. *Turkish Journal of Veterinary & Animal Sciences,* 32, 327-333.

KLEIN, N. & LORTAL, S. 1999. Attenuated starters: an efficient means to influence cheese ripening: a review. *International Dairy Journal,* 9, 751-762.

KUCHROO, C. N. & FOX, P. F. 1982. Fractionation of the water-soluble nitrogen from cheddar cheese: - chemical methods. *Milchwissenschaft-Milk Science International,* 37, 651-653.

LANCIOTTI, R., GIANOTTI, A., BALDI, D., ANGRISANI, R., SUZZI, G., MASTROCOLA, D. & GUERZONI, M. E. 2005. Use of *Yarrowia lipolytica* strains for the treatment of olive mill wastewater. *Bioresource Technology,* 96, 317-322.

LAW, B. A. & SHARPE, M. E. 1977. Influence of microflora of Cheddar cheese on flavor development. *Dairy Industries International,* 42, 10-&.

LEMAY, A., PAQUIN, P. & LACROIX, C. 1994. Influence of microfluidization of milk on Cheddar cheese composition, color, texture, and yield. *Journal of Dairy Science,* 77, 2870-2879.

LINARES, D. M., MARTIN, M. C., LADERO, V., ALVAREZ, M. A. & FERNANDEZ, M. 2011. Biogenic aAmines in dDairy pProducts. *Critical Reviews in Food Science and Nutrition*, 51, 691-703.

LIU, S.-Q. & CROW, V. L. 2010. Production of dairy-based, natural sulfur flavor concentrate by yeast fermentation. *Food Biotechnology,* 24, 62 - 77.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry,* 193, 265-275.

McSWEENEY, P. L. H., OLSON, N. F., FOX, P. F., HEALY, A. & HOJRUP, P. 1993. Proteolytic specificity of chymosin on bovine alpha-s_1-casein. *Journal of Dairy Research*, 60, 401-412.

McSWEENEY, P. L. H., WALSH, E. M., FOX, P. F., COGAN, T. M., DRINAN, F. D. & CASTELO-GONZALEZ, M. 1994. A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. *Irish Journal of Agricultural and Food Research*, 33, 183-192.

MILESI, M. M., McSWEENEY, P. L. H. & HYNES, E. R. 2008. Viability and contribution to proteolysis of an adjunct culture of *Lactobacillus plantarum* in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology*, 105, 884-892.

MIN, J. S., LEE, S. O., JANG, A., LEE, M. & KIM, Y. 2004. Quantitative analysis of biogenic amines in raw and processed foods of animal origin on Korean domestic market. *Asian-Australasian Journal of Animal Sciences*, 17, 1764-1768.

MOUNIER, J., MONNET, C., JACQUES, N., ANTOINETTE, A. & IRLINGER, F. 2009. Assessment of the microbial diversity at the surface of Livarot cheese using culture-dependent and independent approaches. *International Journal of Food Microbiology,* 133, 31-37.

PIRAINO, P., PARENTE, E. & McCSWEENEY, P. L. H. 2004. Processing of chromatographic data for chemometric analysis of peptide profiles from cheese extracts: A novel approach. *Journal of Agricultural and Food Chemistry*, 52, 6904-6911.

POLYCHRONIADOU, A. 1988. A simple procedure using trinitrobenzenesulfonic acid for monitoring proteolysis in cheese. *Journal of Dairy Research*, 55, 585-596.

RAZZELL, W. E. 1963. Phosphodiesterases. *Methods in Enzymology,* 6, 236-258.

REA, M. C., FRANZ, C., HOLZAPFEL, W. H. & COGAN, T. M. 2004. Development of enterococci and production of tyramine during the manufacture and ripening of Cheddar cheese. *Irish Journal of Agricultural and Food Research*, 43, 247-258.

ROOSTITA, R. & FLEET, G. H. 1996. The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. *International Journal of Food Microbiology,* 28, 393-404.

SANTOS, M. H. S. 1996. Biogenic amines: their importance in foods. *International Journal of Food Microbiology*, 29, 213-231.

SCIOLI, C. & VOLLARO, L. 1997. The use of *Yarrowia lipolytica* to reduce pollution in olive mill wastewaters. *Water Research*, 31, 2520-2524.

SHAKEEL- UR-REHMAN, R., , FARKYE, N. Y., VEDAMUTHU, E. R. & DRAKE, M. A. 2003. A preliminary study on the effect of adding yeast extract to cheese curd on proteolysis and flavour development of reduced-fat Cheddar. *Journal of Dairy Research,* 70, 99-103.

SOUSA, M. J., ARDÖ, Y. & McSWEENEY, P. L. H. 2001. Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, 11, 327-345.

SUNIL, P. & ASHWANL, R. 2008. Microfluidization: A new concept in dairy and food industry. *Indian Dairyman,* 60, 37-42.

SUZZI, G., LANORTE, M. T., GALGANO, F., ANDRIGHETTO, C., LOMBARDI, A., LANCIOTTI, R. & GUERZONI, M. E. 2001. Proteolytic, lipolytic and molecular characterisation of *Yarrowia lipolytica* isolated from cheese. *International Journal of Food Microbiology,* 69, 69-77.

SUZZI, G., SCHIRONE, M., MARTUSCELLI, M., GATTI, M., FORNASARI, M. E. & NEVIANI, E. 2003. Yeasts associated with Manteca. *FEMS Yeast Research,* 3, 159-166.

TAKAHASHI, M., KITAMOTO, D., ASIKIN, Y., TAKARA, K. & WADA, K. 2009. Liposomes encapsulating *Aloe vera* leaf gel extract significantly enhanced proliferation and collagen synthesis in human skin cell lines. *Journal of Oleo Science,* 58, 643-650.

THOMPSON, M. P., SOMKUTI, G. A., FLANAGAN, J. F., BENCIVENGO, M., BROWER, D. P. & STEINBERG, D. H. 1979. Thermally shocked bacteria in the preparation of low-fat cheese. *Journal of Dairy Science,* 62, 68-69.

VALDES-STAUBER, N., SCHERER, S. & SEILER, H. 1997. Identification of yeasts and coryneform bacteria from the surface microflora of brick cheeses. *International Journal of Food Microbiology,* 34, 115-129.

VAN DEN TEMPEL, T. & JAKOBSEN, M. 2000. The technological characteristics of *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starter cultures for production of Danablu. *International Dairy Journal,* 10, 263-270.

VANNINI, L., BALDI, D. & LANCIOTTI, R. 2001. Use of Fourier transform infrared spectroscopy to evaluate the proteolytic activity of *Yarrowia lipolytica* and its contribution to cheese ripening. *International Journal of Food Microbiology,* 69, 113-123.

VILJOEN, B. C., KHOURY, A. R. & HATTINGH, A. 2003. Seasonal diversity of yeasts associated with white-surface mould-ripened cheeses. *Food Research International,* 36, 275-283.

WANG, R. X., TIAN, Z. G. & CHEN, L. Y. 2011. Nano-encapsulations liberated from barley protein microparticles for oral delivery of bioactive compounds. *International Journal of Pharmaceutics,* 406, 153-162.

WELTHAGEN, J. J. & VILJOEN, B. C. 1998. Yeast profile in Gouda cheese during processing and ripening. *International Journal of Food Microbiology,* 41, 185-194.

WELTHAGEN, J. J. & VILJOEN, B. C. 1999. The isolation and identification of yeasts obtained during the manufacture and ripening of Cheddar cheese. *Food Microbiology,* 16, 63-73.

WHITE, M. D. & MARCUS, D. 1988. Disintegration of microorganisms. *Advances in biotechnological Biotechnological processesProcesses,* 8, 51-96.

WILLIAMS, A. G. & WITHERS, S. E. 2007. Tyrosine metabolism in pigment-forming *Yarrowia lipolytica* strains isolated from English and European speciality mould-ripened cheese exhibiting a brown discolouration defect. *International Journal of Dairy Technology,* 60, 165-174.

WOJTATOWICZ, M., CHRZANOWSKA, J., JUSZCZYK, P., SKIBA, A. & GDULA, A. 2001. Identification and biochemical characteristics of yeast microflora of Rokpol cheese. *International Journal of Food Microbiology,* 69, 135-140.

WOUTERS, J. T. M., AYAD, E. H. E., HUGENHOLTZ, J. & SMIT, G. 2002. Microbes from raw milk for fermented dairy products. *International Dairy Journal,* 12, 91-109.

WYDER, M. T. & PUHAN, Z. 1999. Role of selected yeasts in cheese ripening: an evaluation in aseptic cheese curd slurries. *International Dairy Journal,* 9, 117-124.

WYDER, M. T., BACHMANN, H. P. & PUHAN, Z. 1999. Role of selected yeasts in cheese ripening: An evaluation in foil wrapped Raclette cheese. *Lebensmittel-Wissenschaft & Technologie,* 32, 333-343.

YALCIN, H. T. & UCAR, F. B. 2009. Isolation and characterization of cheese spoiler yeast isolated from Turkish white cheeses. *Annals of Microbiology,* 59, 477-483.

ZHANG, F., KLEBANSKY, B., FINE, R. M., XU, H., PRONIN, A., LIU, H. T., TACHDJIAN, C. & LI, X. D. 2008. Molecular mechanism for the umami taste synergism. *Proceedings of the National Academy of Sciences of the United States of America,* 105, 20930-20934.

5. Effect of *Hafnia alvei* on Cheddar cheese ripening

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5.1. Abstract

In this study, *Hafia alvei* was used as an adjunct in the manufacture of Cheddar cheese. Control cheese C1 was made 1% of sterile broth (121C° x 15 min), cheese C2 was made with *Hafia alvei* at levels of 10^7 cfu/ml and cheese C3 was made with *Hafia alvei* at levels of 10^8 cfu/ml. Numbers of *H. alvei* in Cheddar cheese dramatically reduced after the first days of ripening and then remained constant at level of ~10^3 cfu/g until the end of ripening (180 d). The strain significantly increased the level of pH 4.6-soluble nitrogen, total free amino-acids, and some individual free amino-acids of Cheddar cheese, whereas no differences in the urea-polacrylamide gel electrophoresis of the cheeses were detected. *H. alvei* was able to contribute to lipolysis by increasing the level of free fatty acids (only in Trial A). Levels of some biogenic amines were significantly higher in cheese made with *H. alvei* than the control cheese, apart from in Trial C, where only spermine was present at significantly higher level in the experimental cheeses than the control cheese. The results of this study suggest that *H. alvei* can be used as an adjunct in order to accelerate the ripening of Cheddar cheese, although this strains showed an effect on lipolysis only in Trial A.

5.2. Introduction

Hafnia alvei is a facultatively anaerobic, Gram-negative, oxidase negative and catalase positive bacterium belonging to the family of the Enterobacteriaceae. It occurs in the faeces of humans and in a wide range of animals including birds, in sewage, soil, water, and dairy products (Brenner et al., 2005). *H. alvei* has been found to be an opportunistic pathogen that produces infections in patients with some underlying illness or predisposing factors such as diabetes, chronic renal failure, chronic obstructive pulmonary disease, malignancy, and HIV infection. It is also associated with cases of diarrhea in humans, but its enteropathogenesis is still under discussion (Brenner et al., 2005; Donato et al., 2008).

Hafnia alvei has been isolated from several foods such as Turkish meat products (Noveir et al., 2000), Spanish fermented meat products (Hortensia Silla Santos, 1998), surface of Livarot cheese (Mounier et al., 2009), artisan kid rennet (Florez et al., 2006), Spanish farmhouse cheese (Abriouel et al., 2008), Iberian dry-cured ham (Martin et al., 2008), cold-smoked salmon (Hansen and Huss, 1998), tuna sandwiches (Kung et al., 2009), Tunisian meat products (Najjari et al., 2008), raw cows' milk (Ercolini et al., 2009), goats' milk cheeses from Sierra de Aracena (Martin-Platero et al., 2009) and San Simon cheese (Tornadijo et al., 2001).

Gram-negative bacteria are very common in cheese, but their impact on cheese ripening and flavor is not well understood since they are often regarded as indicators of poor hygiene (Irlinger et al., 2012; Delbes-Paus et al., 2012). Gram-negative bacteria such as *Pseudomonas* spp. are considered as cheese contaminants because of their production of volatile compounds which may negatively contribute to the sensory quality of cheese; *Enterobacter aerogenes* and *Escherichia coli* causes defects in cheese texture and flavor including early blowing; *Serratia* spp. and *Kluyvera* spp. may negatively affect the cheese sensory quality because of their lipolytic and proteolytic activities (Coton et al., 2012). Delbes-Paus et al. (2012)

showed that some strains of Enterobacteriaceae had no significant effect on the colour, odour and volatile compounds composition of an uncooked pressed type model cheese, but slightly affected its texture.

In some cases, Gram-negative bacteria can accelerate cheese ripening because of their proteolytic enzymes which degrade casein and peptides and contribute to the liberation of free amino acids (Morales et al., 2003; Chaves-Lopez et al., 2006). Distribution of enterobacteriaceae in the cheese core and on the surface is likely influenced by the physico-chemical conditions of cheese, including pH increase due to yeast growth, oxygen availability, water activity, proteolysis and salt content (Coton et al., 2012). They usually start growing in cheese during the first week of ripening and then decrease at a variable rate depending on the species, cheese microbiota and physical/chemical characteristics of cheeses (Deetae et al., 2009a; Irlinger et al., 2012). However, some authors have reported the presence of *H. alvei* at the end of ripening of Livarot cheese (Mounier et al., 2009) and in a Spanish farmhouse cheese up to levels of 10^5 cfu/g (Abriouel et al., 2008).

Deetae et al. (2009a) showed that *Proteus vulgaris* was able to grow up to 10^9 cfu/g in a cheese model after two weeks and remained stable until the end of ripening (21 days); *Proteus vulgaris* was also found to impair the growth of *H. alvei*, *Arthrobacter arilaitensis* and *Brevibacterium aurantiacum* and had a strong impact on cheese flavor by producing branched-chain aldehydes and their corresponding alcohols and esters. One of the risks associated with enterobacteria is the production of biogenic amines (BA), low molecular weight basic nitrogenous compounds that can be produced by bacteria and accumulate in foods containing free amino acids (Coton et al., 2012). BA are generally associated with the decarboxylase activity of lactobacilli, enterococci, micrococci, some strains of Enterobacteriaceae and their presence is sometimes regarded as a sign of spoilage or a cause of poisoning (Pattono et al., 2008). These compounds can cause the release of adrenaline and noradrenaline, provoke gastric acid secretion, increase cardiac output, migraine, tachycardia, blood sugar levels, and blood pressure (Linares et al., 2011).

Many studies have reported the production of BA by several species of enterobacteria *in vitro* (Martuscelli et al., 2005; Curiel et al., 2011). Cheese is the dairy product that accumulates the highest BA concentrations (Linares et al., 2011). Pattono et al. (2008) showed that some Enterobacteriaceae in dairy products produced large amounts of putrescine and cadaverine. Similar results were obtained by Delbes-Paus et al. (2012) who showed that putrescine and cadaverine were the only BA produced in significant amounts in model cheese made with added Gram-negative bacteria such as *Citrobacter freundii*, *Hafnia alvei*, *Proteus vulgaris*, *Halomonas venusta*, *Morganella morganii* and *Klebsiella Oxytoca*, Marino et al. (2000) observed a correlation between high counts of Enterobacteriaceae and high concentrations of cadaverine and putrescine in blue-veined cheese; most of the organisms isolated were *Enterobacter* spp. followed by *Serratia liquefaciens* and *Escherichia coli. H. alvei* has been found to produce significant amounts of some BA *in vitro* (Pattono et al., 2008; Coton et al., 2012) and *in situ* when used as adjunct culture in model cheese (Delbes-Paus et al., 2012).

Adjunct cultures can be defined as selected strains of cheese-related microorganisms that are added to the milk cheese to improve development of cheese sensory quality

and to accelerate its ripening (El Soda et al., 2000). Adjunct cultures used in cheese are usually lactic acid bacteria such as *Lactobacillus casei*, *Lactobacillus paracasei, Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus curvatus, Lactobacillus rhamnosus* or *Bifidobacterium* sp. (Puchades et al., 1989; Milesi et al., 2008). Other secondary cultures traditionally involved in the ripening of many cheese varieties include *Propionibacterium* in Swiss-type cheese, smear bacteria and yeasts such as *Brevibacterium linens*, *Geotrichum candidum* and *Debaryomyces hansenii* in smear cheeses and moulds such *Penicillium roqueforti* and *Penicillium camemberti* in mould ripened cheeses (Beresford et al., 2001). In recent years, the impact of bacteria belonging to the family of Enterobacteriaceae such as *Hafnia alvei*, *Psychrobacter celer* and *Proteus vulgaris* (Morales et al., 2003; Deetae et al., 2009a, b, Delbes-Paus et al., 2012; Irlinger et al., 2012) has been studied in cheese.

In this study, *H. alvei* was used as an adjunct in the manufacture of Cheddar cheese and its effect during ripening was evaluated.

5.3. Materials and methods

5.3.1. Strain

A concentrated (5 x 10^9 cfu/ml) culture of a commercially available food-grade strain (AF036) of *Hafnia alvei* was obtained as a gift from Bioprox Company (Proxis-Developpement Group, Levallois France).

5.3.2. Cheesemaking

Cheesemaking was carried out in triplicate on different days. Whole milk was standardized to casein: fat ratio of 0.7:1.0, pasteurised at 72ºC for 15 s and cooled to 30ºC. DVS starter culture (R604 Chr-Hansen, Hørsholm, Denmark) was added at a rate of level 0.03% w/v to cheesemilk. *Hafnia alvei* culture was added directly to cheesemilk to give a final concentration of ~5 x 10^7 cfu/ml in cheese batch C2 and ~5 x 10^8 cfu/ml in cheese batch C3. Control cheese C1 was made with 1% of sterile broth (121°C x 15min), cheese 2 (C2) contained 0.1% of *Hafnia alvei* culture and 0.9 % of sterile broth and cheese 3 (C3) contained 1% of *Hafnia alvei* culture. Chymax-180 (Chr. Hansen, Hørshom, Denmark) was used as coagulant. Whey was drained at pH 6.2 and the curd cheddared, milled at pH 5.2 and salted to a level of 2.5 % (w/w). After pressing the curd at 150 kPa for 18 h, the cheese were vacuum packed in a vacuum bag and ripened for 180 days at 8°C.

5.3.3. Microbiological analysis

Samples were taken under aseptic conditions using a cheese trier. Ten g of cheese were homogenized in 90 ml of sterile trisodium citrate (2% w/v) in a stomacher bag (Stomacher 400, Seward Limited, Worthing, West Sussex, UK) for 5 min. Starter cells were enumerated on LM17 agar after incubation at 30° for 3 days, NSLAB were enumerated on Rogosa medium after 5 days at 30°C, *Hafnia alvei* was enumerated on VRBGA after 3 d at 37°C under anaerobic conditions. Large,

translucent, circular, and colorless colonies were considered as *H. alvei*. All media were obtained from Oxoid (South County Business Park, Leopardstown, Dublin 18).

5.3.4. Compositional analysis

Protein (Grappin, 1986), fat (Gerber method; IDF 1986), moisture (oven drying at 102°C; IDF 1983), salt (Fox, 1963) and pH were determined in triplicate after 14 days of ripening. The pH was measured in cheese slurry (1:1 cheese:water) using a combined glass electrode (PHC3001-8, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Copenhagen, Denmark).

5.3.5. Analysis of cheese

The pH 4.6-soluble and insoluble fractions of the cheeses were prepared according to the method of Kuchroo and Fox (1982). The extraction was carried out in triplicate for each cheese. The N content of the pH 4.6-soluble fraction of the cheeses was measured by the macro-Kjeldahl method (Grappin, 1986) and expressed as a % of the total N content of the cheeses.

Urea-polacrylamide gel electrophoresis (PAGE) was carried out on the samples after 120 d and 180 d of ripening, using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Andrews (1983) with modifications. Gels were stained directly by the method of Blaksely and Boezi (1977).

Peptide profiles of the pH 4.6-soluble fractions were determined by UPLC Waters Acquity UPLC H-Class Core System with Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A (Waters Corporation, Milford, MA, USA). The core system included an Acquity UPLC H-Class quaternary solvent manager, a H-Class Sample Manager-FTN and a CH-A column heater. The column used was an Acquity UPLC BEH C18 1.7 µm, 2.1 x 50 mm. Samples were made up at a concentration of 10 mg/ml in buffer A (0.1% TFA in Milli-Q water) and centrifuged at 18,000 g for 10 min. The supernatant was filtered through a 0.22 µm filter (Pall Filters, Dublin, Ireland); Buffer B was made up with 0.1% TFA in acetonitrile. Elution was monitored at 214 nm.

Total free amino acids (FAA) were determined for 120 d and 180 d old cheeses by the trinitrobenzenesulphonic (TNBS) acid method (Polychroniadou, 1988). Individual free amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Welwyn Garden City, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column.
Free fatty acids were quantified by gas chromatograpy (GC) at 180 d as described by De Jong and Badings (1990). Extraction of free individual fatty acids (FFA) for GC analysis was carried out by using a solid-phase extraction technique; 500 mg Varian Bond Elut-NH_2 cartridges were used. FFAs were quantified using a gas chromatograph (Varian Star 3400 CX with Varian 8200 CX autosampler and flame ionization detector (300ºC) interfaced with Star Chromatography Workstation 5.0 software for data acquisition; Varian Analytical Instruments, Harbor City, CA, USA). A wall-coated open tubular fused silica capillary column (25 m length x 0.32

mm internal diameter) coated with FFAP-CB was used. FFAs were separated and identified by reference to known standards and quantified by peak area. A standard calibration mix of 17 fatty acid (including internal standards, valeric acid C5:0; pelargonic acid C9:0; margaric acid C17:0) was prepared at concentrations 1000, 500, 300, 200, 100 and 50 ppm. One-way analysis of variance of data from of the composition of the cheeses was performed using SPSS Version 18.0 for Windows XP (SPSS Inc., Chicago, IL, USA). Significance was declared at $P \leq 0.05$.

The data for individual amino acids, from RP-HPLC peptide profile and individual free fatty acid and biogenic amines concentrations for the cheeses were analysed using multivariate statistical techniques to evaluate the effect of *H. alvei* on the parameters of ripening. The HPLC peak height data were pre-processed according to the method of Piraino et al. (2004). The output of this pre-processing consisted of classes of retention time within which peak heights were accumulated using the distance from the centre of the class as a weight. Principal component analysis was performed on this pre-processed data using SPSS Version 18.0 for Windows XP.

5.3.6. Biogenic amines

Lyophilized 4.6-soluble fractions samples (obtained from 1 ml of extract) were diluted using perchloric acid (c = 0.6 $mol.l^{-1}$) and 0.5 mL of obtained mixtures (vigorously vortexted) were subjected to derivatisation with dansylchloride according to Dadáková et al. (2009); 1,7-heptanediamine was used as an internal standard. The derivatised samples were filtered (porosity 0.22 μm) and applied on a column (ZORBAX Eclipse XDB-C18, 150 x 4.6 mm, 3.5μm, Agilent Technologies, Santa Clara, CA, USA). The concentration of eight biogenic amines (histamine, tyramine, phenylethylamine, tryptamine, putrescine, cadaverine, spermidine, spermine) was monitored by an high performance liquid chromatography system equipped with a binary pump; an autosampler (LabAlliance, State College, USA); a column thermostat; a UV/VIS DAD detector (λ = 254 nm); and a degasser (1260 Infinity, Agilent Technologies, Santa Clara, USA). The conditions for separation of the monitored BA were described by Smělá et al. (2004). Each of the lyophilized samples was derivatised three times. Standards, reagents and eluents were obtained from Sigma Aldrich (St. Louis, MO, USA).

5.4. Results and discussion

5.4.1. Compositional analysis

Compositions of control Cheddar cheeses and cheeses made using adjunct *H. alvei* are shown in **Table 5.1**. The addition of *H. alvei* to the cheese did not affected the gross compositions. However, moisture content was higher and pH much lower than the values expected for Cheddar cheese (Fox et al., 2000), although no significant differences ($P \leq 0.05$) were observed between the control and experimental cheeses. Other bacteria such as *Enterococcus faecium Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus plantarum* have been used in Cheddar cheese and generally adjuncts had no effect on composition (Gardiner et al., 1999; Lynch et al., 1999; Milesi et al., 2008; Burns et al., 2012). Burns et al. (2012) reported no differences in gross composition between control and experimental cheeses made with *L. plantarum* I91 and *L. paracasei* I90 except for pH values that were significantly lower in the experimental cheeses than the control.

Table 5.1. pH and composition of Cheddar cheeses at 14 days of ripening.

C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

	Cheese	*pH*	*% Salt*	*% Fat*	*% Moisture*	*% MNFS*	*% Protein*
Trial A	C1	4.70±0.13[a]	1.23±0.13[a]	30.33±0.58[a]	41.59±0.33[a]	59.70±0.35[a]	24.22±1.23[a]
	C2	4.68±0.13[a]	1.21±0.18[a]	30.00±1.00[a]	41.34±0.26[a]	59.06±0.38[a]	23.25±0.53[a]
	C3	4.75±0.09[a]	1.24±0.16[a]	30.67±0.58[a]	42.14±0.27[a]	59.34±0.38[a]	22.76±0.59[a]
Trial B	C1	4.76±0.02[a]	1.14±0.06[a]	30.67±0.58[a]	41.17±1.03[a]	59.38±1.49[a]	22.56±0.44[a]
	C2	4.72±0.26[a]	1.19±0.01[a]	30.33±1.15[a]	41.24±0.09[a]	59.20±0.09[a]	22.21±0.71[a]
	C3	4.75±0.01[a]	1.15±0.04[a]	31.00±1.00[a]	40.87±0.84[a]	59.23±1.21[a]	22.41±1.26[a]
Trial C	C1	4.75±0.08[a]	1.05±0.04[a]	30.33±0.58[a]	40.96±0.78[a]	58.79±1.12[a]	23.03±0.19[a]
	C2	4.74±0.17[a]	1.06±0.05[a]	31.00±1.00[a]	39.76±0.50[a]	56.62±0.72[a]	23.70±0.49[a]
	C3	4.78±0.36[a]	1.08±0.02[a]	30.66±0.57[a]	40.07±0.68[a]	57.79±0.98[a]	23.36±0.31[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

5.4.2. Microbiological analysis

Starter lactic acid bacteria (SLAB) were present at ~10^9 cfu/g at the end of manufacture and their evolution during ripening in Trials A, B and C is shown in **Figure 5.1 A, B, C**. In Trial A, they decreased by ~ 1 log cycle after 60 days and after 90 days in Trials B and C. At the end of ripening they were present at 10^6 cfu/g in Trial A, C and 10^5 cfu/g in Trial B.

In Trial A, numbers of non-starter lactic acid bacteria (NSLAB) started increasing after 60 days of ripening and reached levels of 10^4 cfu/g in C1, 10^2 and 10^5 cfu/g in C2 and C3 respectively (**Figure 5.2 A, B, C**). In Trial B, NSLAB grew up to 10^4 cfu/g in C1 after 60 d and in C2 after 90 d of ripening, whereas in C3 they reached levels of 10^5 cfu/g after 120 d. In Trial C they reached 10^3 cfu/g in C1 after 60 d and 10^3 cfu/g in C2 and C3 after 120 d of ripening (**Figure 5.2 A, B, C**).

Numbers of *Hafnia alvei* in cheeses C2 and C3 at day 1 were ~10^7 and 10^8 cfu/g respectively, in all trials. Generally, numbers decreased by ~2 log cycles during the first week and after 180 d levels of 10^2 cfu/g and 10^3 cfu/g were found in C2 and C3 respectively in Trial A and B, whereas in Trial C, *Hafnia alvei* was present at levels of 10^3 cfu/g in both cheeses C2 and C3 (**Figure 5.3 A, B, C**).

These results showed that numbers of *H. alvei* reduced during the first days of ripening, but it was able to survive in Cheddar cheese throughout ripening even though at low numbers. Enterobacteriaceae numbers in cheese usually reach a peak during the first week of ripening and then start decreasing with time, depending on the species, cheese type and location within the cheese (Delbes-Paus et al., 2012; Irlinger et al., 2012).

Irlinger et al. (2012) inoculated *H. alvei* in the curd of a smear soft cheese at level of 10^2 and 10^6 cfu/g; regardless the inoculation level, the strain was able to grow to 10^8 cfu/g at the end of ripening (35 days), but it became subdominant at the end of ripening because of the growth of other bacterial strains in the community. In the same work, the authors also reported that another Gram-negative bacterium, *Psychrobacter celer*, was able to survive during ripening and reached the highest count when inoculated at the highest level of 10^6 cfu/g. *P. celer* was also shown to be competitive since bacterial populations in cheese decreased when this strain was present at high level.

In a work conducted by Delbes-Paus et al. (2012), *H. alvei* was present at level of ~10^7 cfu/g at the end of the ripening in a model cheese (28 days). In the same work, the strain was then selected for a second experiment and its growth was monitored during ripening. It was inoculated at level of ~10^3 cfu/g and reached a level of ~10^7 cfu/g after 28 days of ripening, the highest among all the tested Gram-negative bacteria in the experiment.

In a study conducted by Deetae et al. (2009a), the growth of *H. alvei* on the surface of a model cheese was influenced by the presence of *Proteus vulgaris*: after 21 days of ripening *H. alvei* was present at level ~10^7 cfu/g in cheese model made with *P. vulgaris* whereas it reached levels of ~10^9 cfu/g in cheese model made this organism, but it dominated during the first 5 days of ripening reaching counts of ~10^8 cfu/g.

The results obtained from these studies suggested that *H. alvei* was able to grow in cheese and survive during ripening. These findings are in contrast to the results observed in this work where numbers of *H. alvei* dramatically reduced after the first days of ripening and then remained constant at level of ~10^3 cfu/g until the end of ripening (180 d).

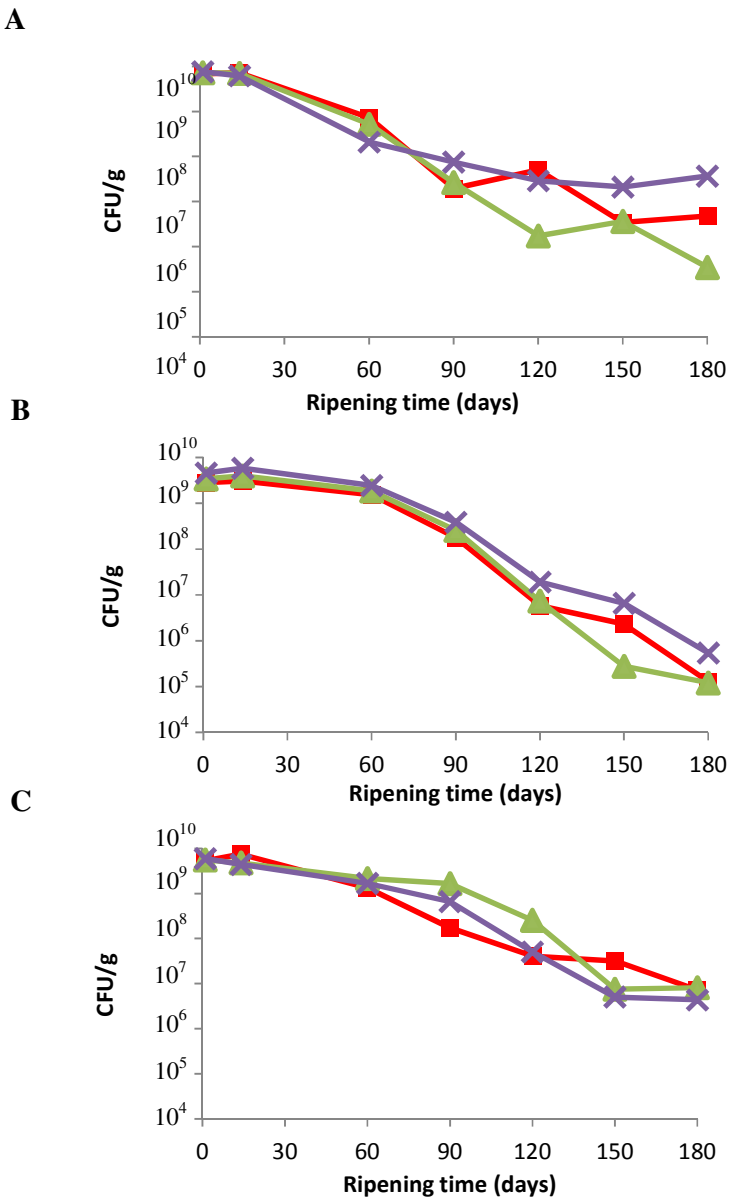


Figure 5.1. Growth of starter lactic acid bacteria (SLAB) on LM17 incubated at 30°C for 3 days during ripening of Cheddar cheeses in Trials A, B, C. C1 (■), Control cheese; C2 (▲), cheese made with 0.1% of *Hafnia alvei*; C3 (×), cheese made with 1% of *Hafnia alvei.*

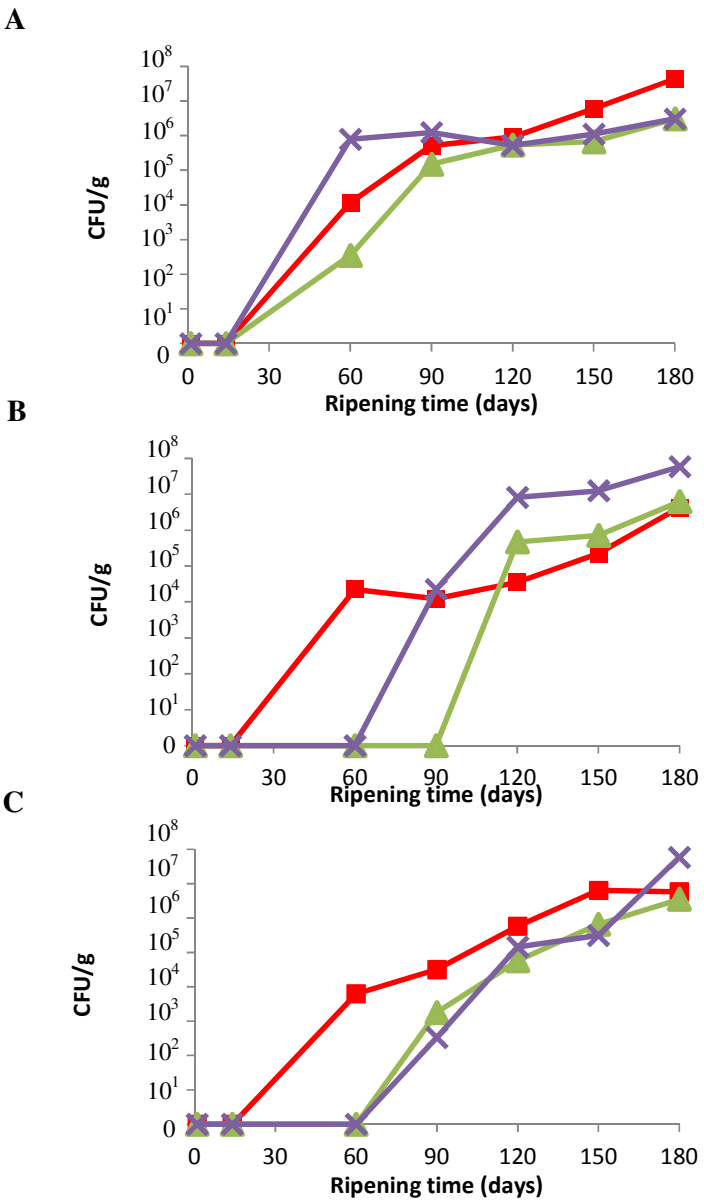


Figure 5.2. Growth of non-starter lactic acid bacteria (NSLAB) on Rogosa agar incubated at 30°C for 5 days during ripening of Cheddar cheeses in Trial A, B, C. C1 (■), Control cheese; C2 (▲), cheese made with 0.1% of *Hafnia alvei*; C3 (×), cheese made with 1% of *Hafnia alvei.*

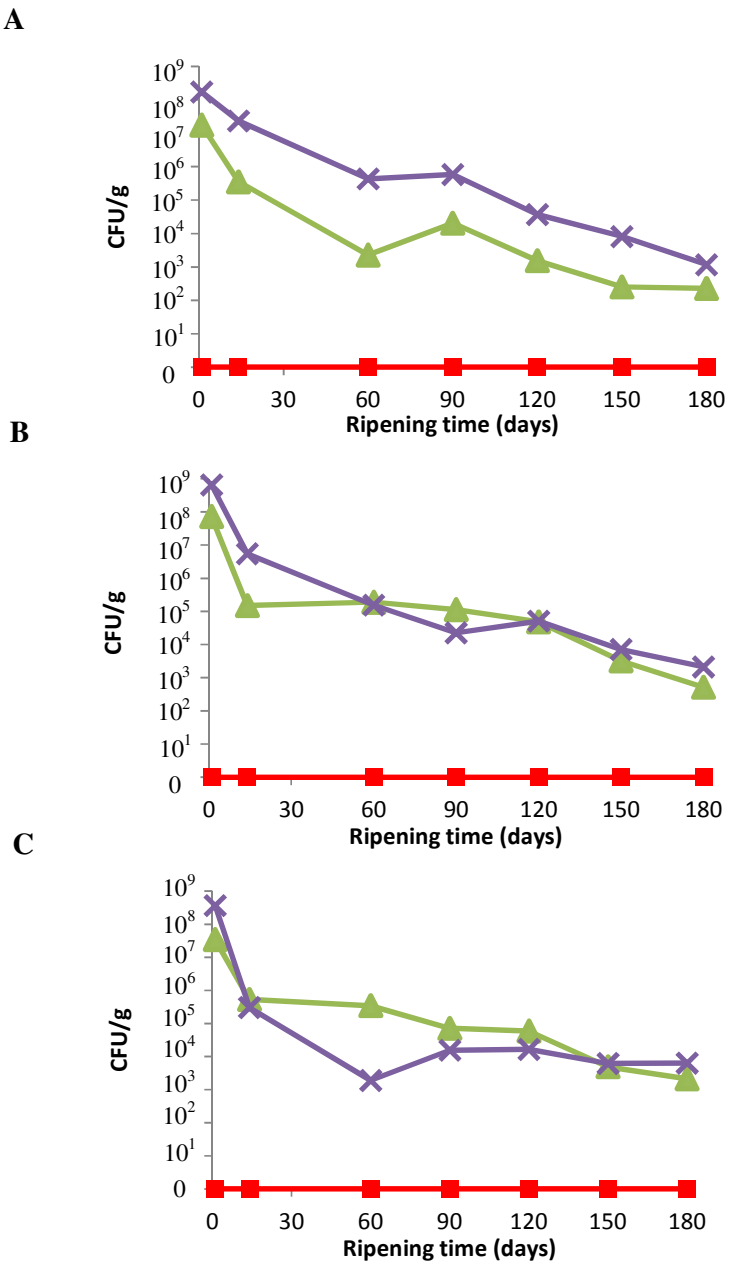


Figure 5.3. Growth of *Hafnia alvei* on VRBGA agar incubated at 37°C during for 3 days ripening of Cheddar cheeses in Trial A, B, C. C1 (■), Control cheese; C2 (▲), cheese made with 0.1% of *Hafnia alvei*; C3 (×), cheese made with 1% of *Hafnia alvei.*

5.4.3. Assessment of proteolysis

5.4.3.1. Urea-PAGE

Urea-PAGE electrophoretograms of the cheeses after 120 and 180 days are shown in **Figures 5.4** and **5.5**.

No differences in proteolysis were observed between the control and the experimental cheeses during ripening. Similar patterns of degradation of α_{s1}-casein and β-casein confirmed that the proteolysis is mainly due to the action of chymosin and plasmin (Lynch et al., 1996).

These results are also in agreement with those reported by other authors (Lynch et al., 1999; Hynes et al., 2003; Poveda et al., 2003; Di Cagno et al., 2006; Milesi et al., 2008) who studied a range of different adjunct cultures. On the other hand, Chaves-Lopez et al. (2006), who studied the proteolytic activity of some strains of Enterobacteriaceae isolated from Pecorino Abruzzese in skim milk after 48 h of fermentation at 30°C, reported that only two of them, *Kluyvera* spp. and *Serratia odorifera*, had casenolytic activity. Morales et al. (2003) showed that strains of Enterobacteriaceae, among which *H. alvei*, had proteolytic activity on caseins under cheese manufacture and ripening conditions.

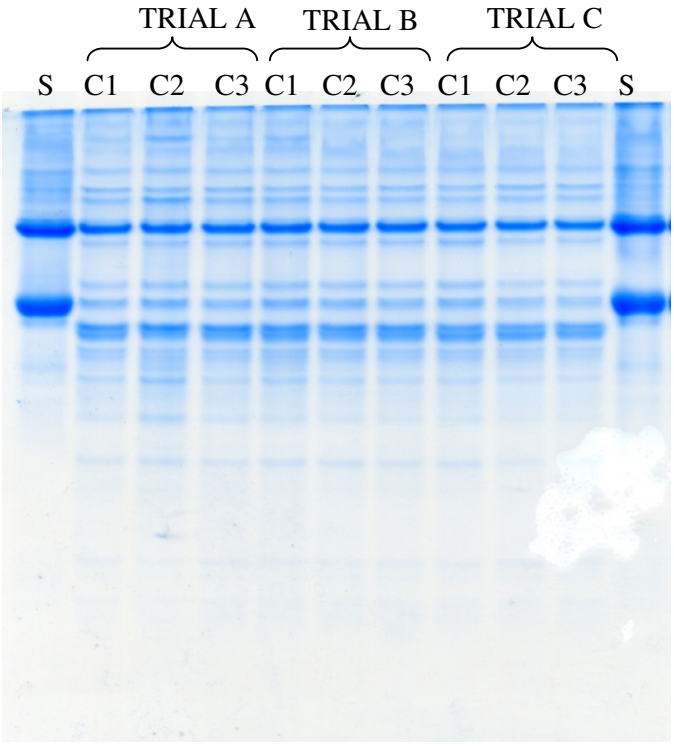


Figure 5.4. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses at 120 d of ripening in Trial A, B and C.
C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

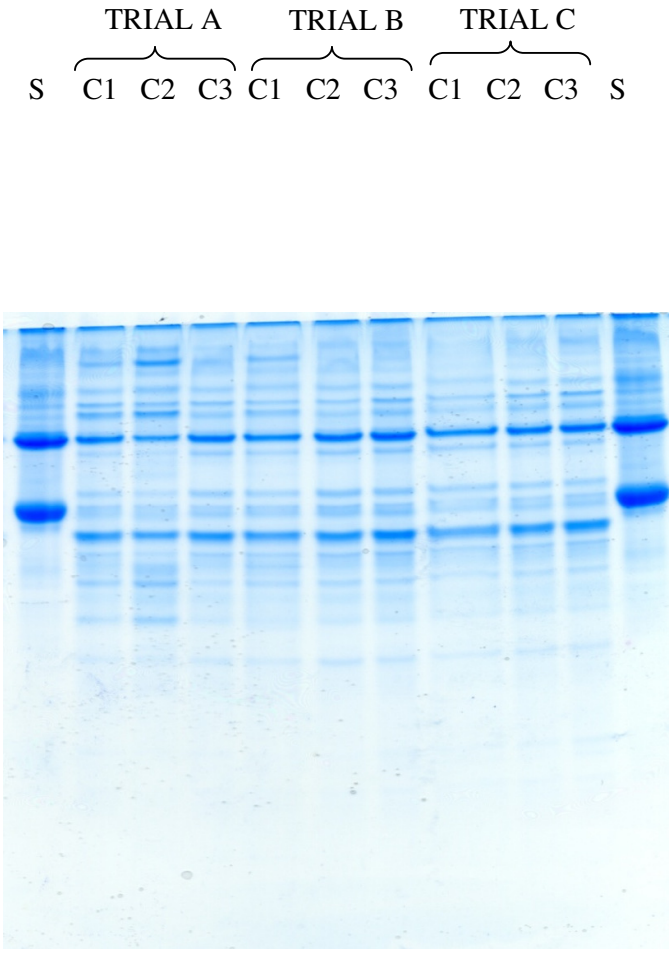


Figure 5.5. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses at 180 d of ripening in Trial A, B and C.
C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

5.4.3.2. Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed on the pH 4.6-soluble fraction of the 120 and 180 days old cheeses. Only results from Trial A are shown in **Figures 5.6** and **5.7**.
No major differences were seen in chromatograms after 120 and 180 days of ripening between control and experimental cheeses.

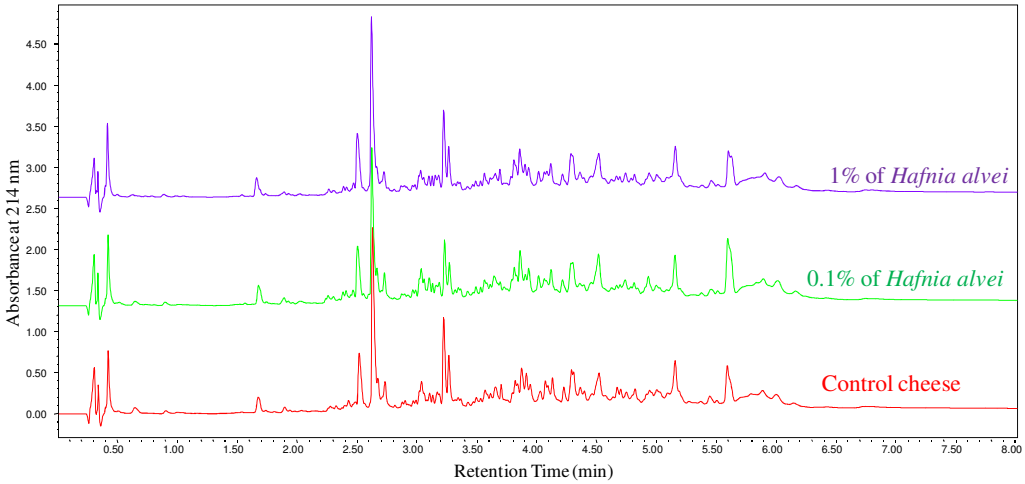


Figure 5.6. Reversed-phase HPLC chromatograms of control and experimental Cheddar cheeses made with 0.1 and 1% of *Hafnia alvei* at 120 days of ripening in Trial A.

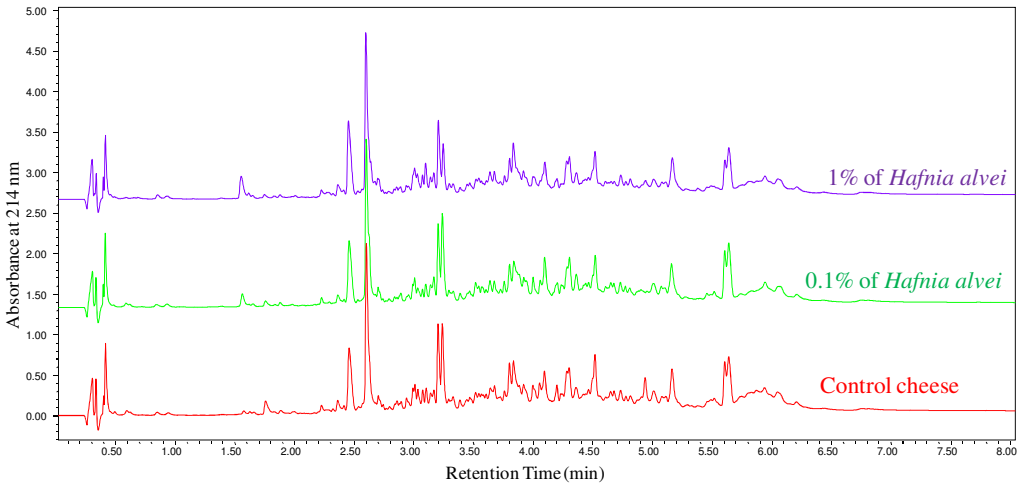
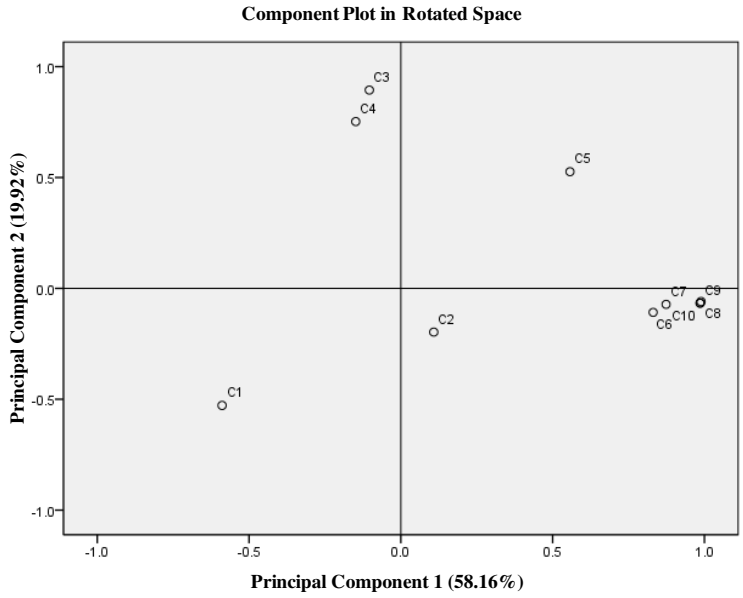


Figure 5.7. Reversed-phase HPLC chromatograms of control and experimental Cheddar cheeses made with 0.1 and 1% of *Hafnia alvei* at 180 days of ripening in Trial A.

Figure 5.8 A, B and **5.9 A, B** shows plot of loadings and scores for the first and second principal component of Principal Component Analysis (PCA) for of the processed peak height data from RP-HPLC of the pH 4.6 soluble extract at 120 and 180 days of ripening in Trial A, B and C. Results show that after 120 days, experimental cheeses C2 and C3 clustered together in the lower and upper left quadrant of the score plot, indicating that there is no trial effect between the cheeses, whereas a trial effect was observed for control cheeses C1.

After 180 days of ripening, a big trial effect was observed in all cheeses. Only cheese C2 from Trial B and C were well grouped together on the lower left quadrant of the score plot.

A



B

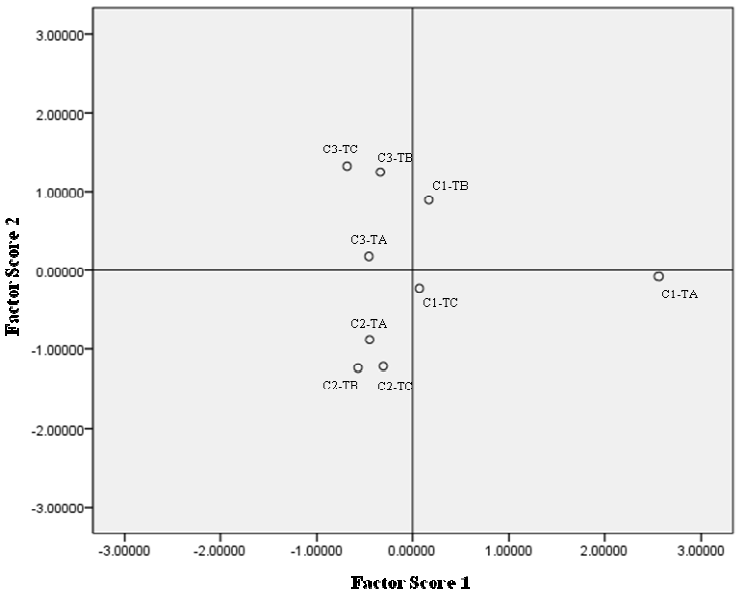
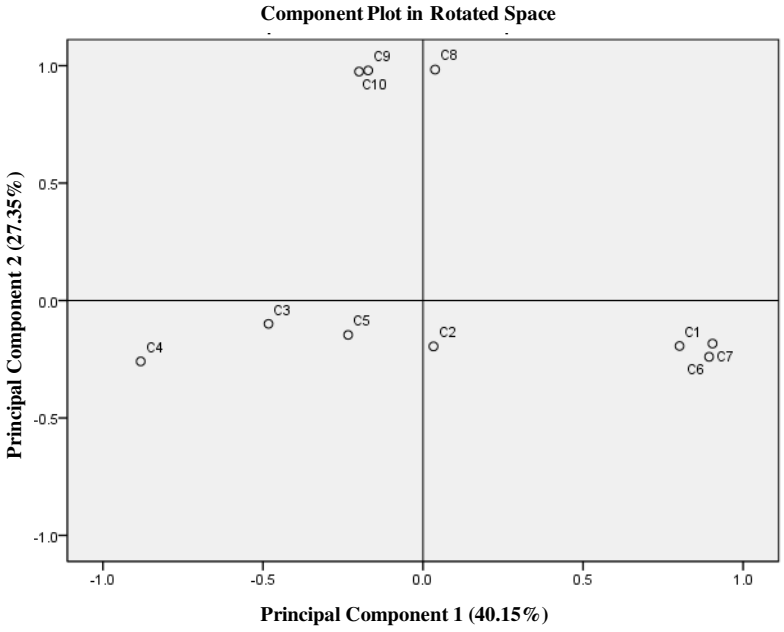


Figure 5.8. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheeses at 120 d of ripening in Trial A, B, C. C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

A



B

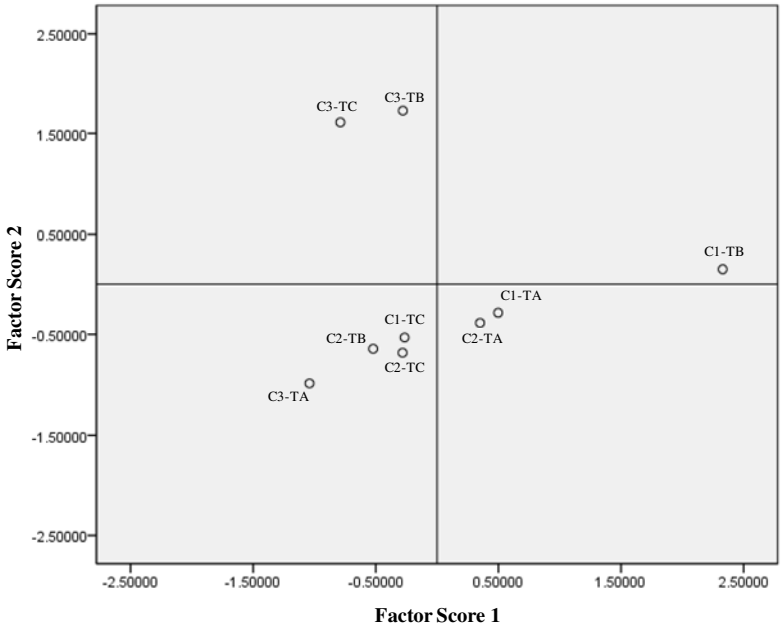


Figure 5.9. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheeses at 180 d of ripening in Trial A, B, C. C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

5.4.3.3. pH 4.6-Soluble nitrogen

Mean levels of pH 4.6-soluble nitrogen at 120 d were statistically significantly higher ($P \leq 0.05$) in cheeses C2 and C3 than the control cheese C1 in Trials A and C, but a statistically significant difference was observed only for cheese C3 in Trial B (**Table 5.2**). After 180 d of ripening, all the experimental cheeses had a significantly higher ($P \leq 0.05$) level of pH 4.6-soluble nitrogen than the control cheese C1.

Liberation of pH 4.6-soluble nitrogen in Cheddar cheese is mainly due to chymosin and plasmin activity. In this work *H. alvei* contributed to proteolysis by increasing the of pH 4.6-soluble nitrogen in the experimental cheeses. No statistically significant differences were observed between the moisture level and or pH of cheese. As the cheeses were made from the same milk, and equal levels of rennet were added, no differences in chymosin or plasmin activity would be expected. Hence, the differences in levels of pH 4.6-soluble N observed must have been due to the action of the adjunct. No data regarding the effect of *H. alvei* on pH 4.6-soluble nitrogen in cheese are available for comparison in the literature. However, previous work has shown that other bacteria such as *E. faecium* increased the proteolytic index in Feta cheese (Sarantinopoulos et al., 2002); Morales et al. (2003) showed that degradation of most of the casein fractions was higher in cheeses made with Enterobacteria than the control cheese. On the other hand, Gardiner et al. (1999) reported that no differences in pH 4.6-soluble nitrogen levels were found between control and experimental cheeses made with *E. faecium*. Similar results were also reported by Lynch et al. (1999), who showed that *Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus plantarum* did not affect the level of pH 4.6-soluble nitrogen in Cheddar cheese and Burns et al. (2012) who reported a similar proteolysis in control and experimental soft cheeses made with *L. plantarum* I91 or *L. paracasei* I90.

Table 5.2. Mean levels of pH 4.6-soluble nitrogen in Cheddar cheese at 120 and 180 days of ripening.
C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

% pH 4.6 SN	Cheese	Ripening time (days)	
		120d	180d
Trial A	C1	18.05±0.48[a]	21.61±0.23[a]
	C2	21.86±0.27[b]	24.29±0.51[b]
	C3	23.35±0.99[b]	24.56±0.52[b]
Trial B	C1	15.91±0.62[a]	18.16±0.77[a]
	C2	18.64±0.87[b]	20.37±0.29[b]
	C3	19.74±0.24[b]	21.74±0.64[b]
Trial C	C1	16.93±0.55[a]	18.91±0.34[a]
	C2	18.52±0.73[b]	21.31±0.17[b]
	C3	18.76±0.62[b]	21.22±0.99[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

5.4.3.4. Total free and individual amino-acids

The level of total free amino acids (FAA) at 120 and 180 days, was determined in cheeses by TNBS assay and was expressed as mg leucine/mg cheese (**Table 5.3**). Statistically significant differences ($P \leq 0.05$) between control and experimental cheeses were found at 120 days in Trial A and B, whereas in Trial C only cheese C2 showed a significantly ($P \leq 0.05$) higher value than the control cheese C1. After 180 d of ripening, all the experimental cheeses in all trials contained a significantly ($P \leq 0.05$) higher level of total free amino acids than the control cheese C1. No significant differences ($P \leq 0.05$) in the levels of total free amino acids were found between the two experimental cheeses made with different concentrations of *Hafnia alvei*. Similar results are reported by Gardiner et al. (1999) in a study conducted on Cheddar cheese and Sarantinopoulos et al. (2002) who showed that *E. faecium* was able to increased significantly the level of total FAA in Feta cheese. Also, Awad et al. (2010) showed that Domiati cheese made with *Lb. delbrueckii* ssp. *lactis*, *Lb. paracasei* ssp. *paracasei*, *Lb. casei*, *Lb. plantarum*, and *E. faecium* had a higher level of FAA than control cheese made without adjunct cultures.

Table 5.3. Level of total free amino acids in Cheddar cheese at 120 and 180 of ripening.
C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

Mg Leu/g cheese	Cheese	Ripening time (days)	
		120d	180d
Trial A	C1	8.29±0.47[a]	11.63±0.23[a]
	C2	12.64±1.20[b]	13.89±0.91[b]
	C3	11.02±0.56[b]	14.88±0.46[b]
Trial B	C1	6.77±0.21[a]	8.98±0.40[a]
	C2	7.94±0.28[b]	10.53±0.33[b]
	C3	7.86±0.35[b]	11.34±0.90[b]
Trial C	C1	5.24±0.67[a]	8.04±0.27[a]
	C2	7.71±1.34[b]	9.87±0.45[b]
	C3	7.19±0.09[ab]	9.46±0.24[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

Concentrations of individual free amino acids (IFA) are shown in **Table 5.4**. In Trial A, both cheese C2 and C3 contained significantly higher levels ($P \leq 0.05$) of Asp, and Gaba than the control cheese C1. Only cheese C2, made with *H. alvei* at levels of 10^7 cfu/ml had significantly higher ($P \leq 0.05$) levels of all other amino acids (apart from Phe) than the control cheese.

No statistically significant differences ($P \leq 0.05$) were observed between cheese C3 and control cheese C1 despite the former cheese containing the highest level of *H. alvei* (10^8 cfu/ml). In Trial B, only cheese C3 showed significantly higher ($P \leq 0.05$) levels of Thr, Ser, Glu, Ala, Val, Gaba, Lys than the control cheese, whereas no differences were observed between cheese C2 and the control cheese.

In Trial C, both experimental cheeses C2 and C3 contained significantly higher levels ($P \leq 0.05$) of Thr, Ser, Glu, Gly and Ala than the control cheese. Only cheese C2 had significantly higher levels ($P \leq 0.05$) of Val, Met, Ile, Leu, Tyr, Phe, Gaba and His than the control cheese.

Lynch et al. (1999) reported that no differences in the level of FAA between control and experimental cheese made with *Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus plantarum* until 3 months of ripening; the level increased thereafter probably due to an increase in the peptidase activity caused by the growth of adjunct lactobacilli. In agreement with the results of this study, Burns et al. (2012) showed that the content of FAA was enhanced in cheeses made with *L. plantarum* I91 and *L. paracasei* I90. These cheeses also contained significantly higher levels of Leu, Phe and Arg than the control cheese. Milesi et al. (2008) reported significantly higher level of FAA in Cheddar-type cheese and a soft cheese made with *L. plantarum* I91 than the control cheeses; at the end of ripening, Cheddar cheeses made with adjuncts had a significantly higher level of many FAA at 60 days of ripening compared to the control. Delbes-Paus et al. (2012) showed that lysine concentration was significantly lower in cheeses inoculated with *H. alvei* than in control cheeses, while non-significant differences were found in the levels of Leu and Phe.

Table 5.4. Levels of individual free amino acids in Cheddar cheeses at 180 days of ripening.

C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

IFA	*Trial A*		
mg/100gCheese	C1	C2	C3
Asp	10.30±0.34^{a}	54.50±1.73^{c}	32.34±1.24^{b}
Thr	9.18±0.23^{b}	13.89±0.36^{c}	7.66±0.57^{a}
Ser	9.78±0.36^{a}	19.24±0.61^{b}	9.48±0.55^{a}
Glu	42.80±3.80^{c}	26.42±1.40^{b}	15.48±0.32^{a}
Gly	6.48±0.15^{b}	10.43±0.27^{c}	5.89±0.26^{a}
Ala	14.19±0.25^{a}	21.70±0.28^{b}	14.65±0.74^{a}
Val	35.63±0.93^{b}	46.66±0.58^{c}	26.21±0.73^{a}
Met	19.36±0.86^{b}	31.34±1.00^{c}	15.69±0.46^{a}
Ile	10.00±0.55^{b}	16.26±0.51^{c}	4.71±0.36^{a}
Leu	111.37±3.33^{b}	159.09±9.65^{c}	92.08±7.42^{a}
Tyr	-	-	-
Phe	83.91±0.67^{a}	127.49±16.78^{a}	103.71±27.61^{a}
GABA	28.33±0.54^{a}	69.45±4.32^{c}	48.77±4.87^{b}
His	54.61±2.69^{b}	87.75±1.87^{c}	43.98±1.69^{a}
Lys	38.17±2.19^{b}	48.52±1.57^{c}	2.44±0.31^{a}
Arg	0.45±0.80	-	-
Pro	8.03±2.46^{a}	20.63±2.82^{b}	12.56±2.08^{a}

IFA	*Trial B*		
mg/100gCheese	C1	C2	C3
Asp	30.02±2.47^{a}	32.38±1.25^{a}	30.63±1.55^{a}
Thr	7.09±0.32^{a}	7.67±0.57^{a}	10.18±1.27^{b}
Ser	7.86±0.73^{a}	9.49±0.56^{ab}	13.58±3.68^{b}
Glu	20.03±1.65^{b}	15.50±0.32^{a}	20.40±1.44^{b}
Gly	4.66±0.40^{a}	5.90±0.26^{a}	7.31±1.91^{a}
Ala	12.60±0.86^{a}	14.67±0.74^{a}	20.19±1.02^{b}
Val	25.66±2.48^{a}	26.24±0.73^{a}	30.55±1.12^{b}
Met	15.23±1.32^{a}	15.71±0.46^{a}	15.21±0.29^{a}
Ile	5.34±0.52^{a}	4.71±0.36^{a}	5.47±0.66^{a}
Leu	85.22±1.46^{a}	92.20±7.43^{a}	98.31±5.17^{a}
Tyr	11.30±1.42	-	-
Phe	116.77±8.66^{a}	103.84±27.64^{a}	133.95±5.16^{a}
GABA	42.82±4.39^{a}	48.84±4.87^{ab}	53.68±0.78^{b}
His	52.99±3.93^{b}	44.04±1.69^{a}	42.33±2.20^{a}
Lys	3.97±0.86^{ab}	2.44±0.31^{a}	4.85±0.20^{b}
Arg	1.08±0.15	-	-
Pro	10.39±1.82^{a}	12.57±2.09^{a}	13.61±2.98^{a}

IFA	*Trial C*		
mg/100gCheese	C1	C2	C3
Asp	26.29±0.90[a]	33.29±1.14[b]	27.12±1.54[a]
Thr	6.56±0.31[a]	9.19±0.73[b]	8.28±0.58[b]
Ser	7.32±0.26[a]	9.67±0.33[b]	10.00±0.49[b]
Glu	21.44±0.70[a]	27.48±1.44[b]	29.08±1.55[b]
Gly	4.09±0.30[a]	5.24±0.16[b]	5.18±0.17[b]
Ala	11.95±0.55[a]	15.33±0.39[b]	16.97±0.77[c]
Val	25.51±0.64[a]	30.33±1.27[b]	27.51±1.62[ab]
Met	12.19±0.27[a]	14.52±0.32[b]	12.07±0.53[a]
Ile	4.26±0.21[a]	5.93±0.31[b]	4.87±0.31[a]
Leu	76.80±2.11[a]	83.57±2.86[b]	79.98±2.28[ab]
Tyr	-	12.11±0.57	7.77±6.75
Phe	118.19±3.27[a]	129.46±5.83[a]	108.10±17.76[a]
GABA	36.76±0.99[a]	44.59±1.13[b]	38.44±2.13[a]
His	40.88±2.50[a]	46.21±0.38[b]	38.15±1.48[a]
Lys	4.36±0.77[a]	3.75±0.10[a]	3.69±0.19[a]
Arg	-	1.24±1.21	2.72±0.04
Pro	11.90±2.34[a]	11.95±4.16[a]	9.07±0.75[a]

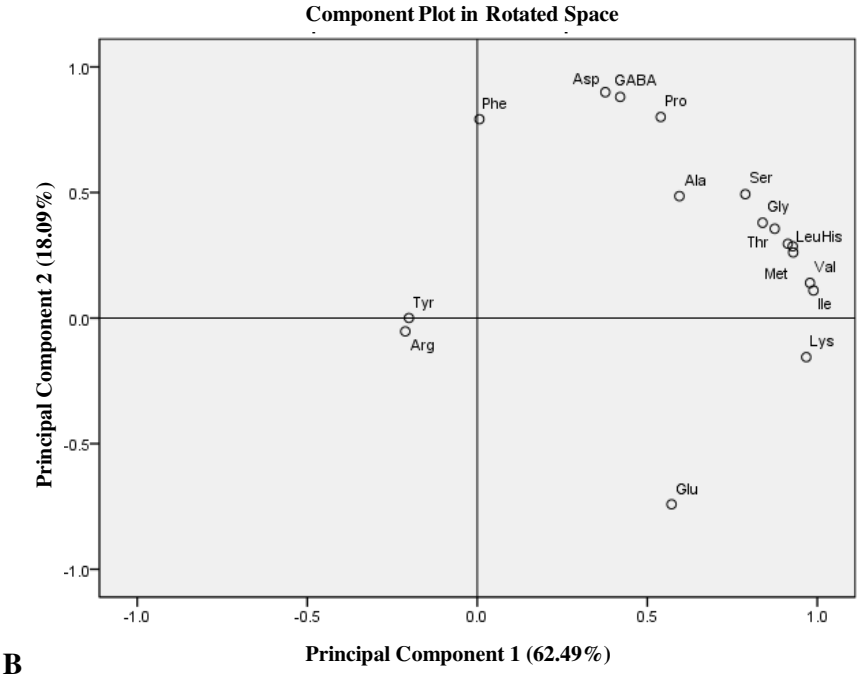
The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

Figure 5.10 A, B shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for individual free amino acids at 6 months of ripening in Trial A, B and C.

Results suggest that there was a small trial effect between cheeses C3 made with 1% *Hafnia alvei* in all trials. Cheese C3 from Trial A and B clustered together in the upper left quadrant of the score plot whereas cheese C3 from Trial C was located in the lower left quadrant of the score plot. All cheeses C3 had a negative scores on PC1. Cheeses C2 and C1 from Trial B and C grouped well together in the left quadrant of the score plot showing had a negative scores on PC1. A big trial effect was found for cheeses C1 and C2 from Trial A. The samples were located in the lower and upper right quadrant of the score plot, respectively. Control cheese C1 from Trial A had higher amount of Glu than control cheeses C1 from Trial B and C.

A



B

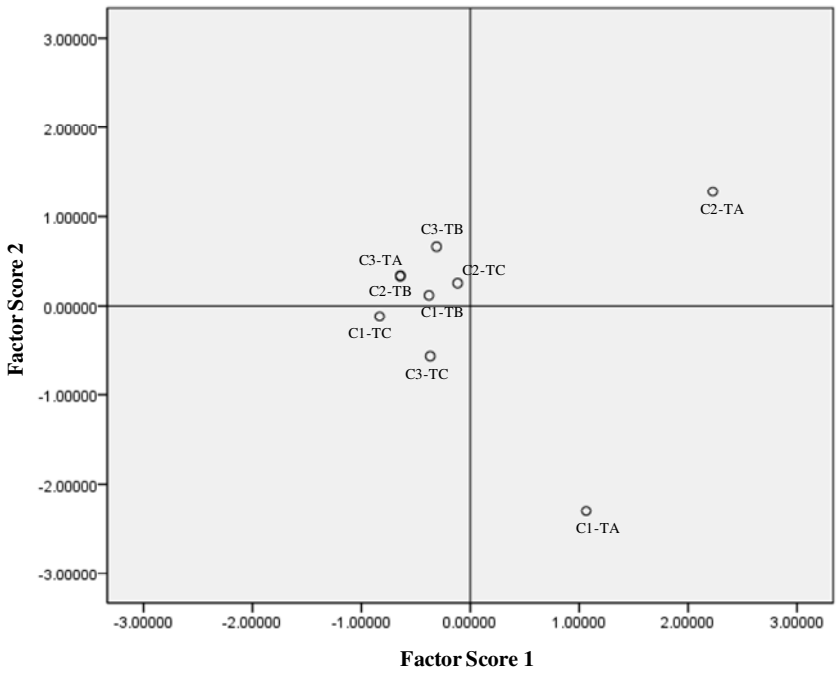


Figure 5.10. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for individual free amino acids at 6 months of ripening in Trial A, B, C.
C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

5.4.4. Assessment of lipolysis

5.4.4.1. Free fatty acid profile

Levels of total and individual free fatty acids (from butyric, C4:0 to arachidic, C20:0) in cheeses at 180 days are shown in **Table 5.5** and are expressed as mg/kg of cheese. In Trial A, both experimental cheeses C2 and C3 showed statistically significantly higher levels ($P \leq 0.05$) of total free fatty acids than the control cheese. In particular, significantly higher levels ($P \leq 0.05$) of butyric acid (C4:0), myristic acid (C14:0), stearic acid (C18:0) and oleic acid (C18:1) were found in cheeses C2 and C3 than in the control cheese. Only cheese C3 had significantly higher levels ($P \leq 0.05$) of capric acid (C10:0) and arachidic acid (C20:0) than the control, whereas cheese C2 did not show any significant difference in the levels of the other fatty acids compared to the control.

In Trial B, no significant differences ($P \leq 0.05$) were found between experimental and control cheeses in the level of total fatty acids. Cheeses C2 and C3 had only significantly ($P \leq 0.05$) higher level of butyric acid (C4:0) than the control cheese respectively.

In Trial C, no significantly higher levels ($P > 0.05$) of total fatty acids were found between cheese C3 and control cheese C1, whereas cheese C2 contained significantly lower levels ($P \leq 0.05$) than the control cheese. Both cheese C2 and C3 contained significantly higher ($P \leq 0.05$) levels of stearic acid (C18:0) than the control cheese. Levels of lauric acid (C12:0) and oleic acid (C18:1) were significantly higher ($P \leq 0.05$) in cheese C3 than in the control cheese.

These results showed that *H. alvei* contributed to lipolysis by increasing the level of free fatty acids only in Trial A. Juven et al. (1981) showed that some strains of enterobacteria increased the level of free fatty acids in milk incubated at 7°C, but they did not show any lipolytic activity on Tween 80 agar. Some enterobacteria isolated from Pecorino Abruzzese cheese reported to have lipase activity in butter-agar and pork fat-agar (Chaves-Lopez et al., 2006). Awad et al. (2010) showed that Domiati cheese made with *L. delbrueckii* ssp. *lactis*, *Lb. paracasei* ssp. *paracasei*, *Lactobacillus casei*, *L. plantarum*, and *E. faecium* had a higher level of FFA than control cheese made without these adjuncts. In a study conducted by Kondyli et al. (2002) the authors showed that low-fat Feta cheese made with a commercial adjunct culture containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* had higher total free fatty acid levels than the control cheese.

Figure 5.11 A, B shows plot of loadings and scores for the first and second Principal Component Analysis (PCA) for free fatty acids at 6 months of ripening in Trial A, B and C. No trial effect was observed in all cheeses that are well grouped together in the left lower quadrant of the score plot, apart from control cheeses C1 and cheese C3 made with 1% of *Hafnia alvei* from Trial C which are located in the right and left upper quadrant of the score plot, respectively. As shown in the plot of loadings, control cheese C1 was richer in all free fatty acids apart from C12:0, C18:0, C16:0 and C18:3 than the other control cheeses from Trials A and B, whereas cheese C3 from Trial C is richer in C12 and C18 than the other cheeses C3 from Trial A and B.

Table 5.5. Levels of free fatty acids in Cheddar cheeses at 180 days of ripening. C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

FFA	***Trial A***		
mg/kg cheese	C1	C2	C3
C4:0	0.37±0.06[a]	0.65±0.07[b]	0.61±0.08[b]
C6:0	0.10±0.01[a]	0.12±0.04[a]	0.13±0.05[a]
C10:0	0.17±0.04[a]	0.30±0.02[ab]	0.38±0.09[b]
C12:0	0.07±0.01[a]	0.11±0.03[a]	0.14±0.05[a]
C14:0	0.77±0.07[a]	1.55±0.39[b]	1.40±0.05[b]
C16:0	0.08±0.05[a]	0.15±0.07[a]	0.18±0.08[a]
C18:0	0.28±0.03[a]	0.44±0.07[b]	0.52±0.05[b]
C18:1	0.32±0.06[a]	0.76±0.04[c]	0.63±0.04[b]
C18:2	0.68±0.06[a]	0.76±0.06[a]	0.72±0.06[a]
C18:3	0.08±0.01[a]	0.08±0.01[a]	0.08±0.01[a]
C20:0	0.02±0.00[a]	0.03±0.01[ab]	0.035±0.01[b]
Total	3.35±0.26[a]	6.1±0.56[b]	5.70±0.21[b]

FFA	***Trial B***		
mg/kg cheese	C1	C2	C3
C4:0	0.57±0.04[a]	0.77±0.05[b]	0.63±0.03[a]
C6:0	0.17±0.01[b]	0.12±0.02[a]	0.10±0.01[a]
C10:0	0.39±0.01[b]	0.40±0.07[b]	0.27±0.03[a]
C12:0	0.08±0.01[a]	0.10±0.02[a]	0.06±0.01[a]
C14:0	1.44±0.04[ab]	1.52±0.24[b]	1.08±0.06[a]
C16:0	0.17±0.01[a]	0.24±0.09[a]	0.15±0.04[a]
C18:0	0.45±0.02[a]	0.51±0.05[a]	0.46±0.06[a]
C18:1	0.65±0.03[b]	0.71±0.06[b]	0.52±0.03[a]
C18:2	1.07±0.02[a]	1.09±0.24[a]	0.87±0.03[a]
C18:3	0.08±0.01[a]	0.07±0.01[a]	0.08±0.01[a]
C20:0	0.05±0.01[a]	0.05±0.00[a]	0.04±0.01[a]
Total	5.52±0.04[a]	6.07±0.78[a]	5.00±0.28[a]

IFA	***Trial C***		
mg/kg cheese	C1	C2	C3
C4:0	0.92±0.06[c]	0.39±0.08[a]	0.58±0.02[b]
C6:0	0.22±0.08[b]	0.10±0.02[ab]	0.10±0.01[a]
C10:0	0.96±0.02[b]	0.29±0.04[a]	0.24±0.03[a]
C12:0	0.16±0.04[a]	0.08±0.02[a]	0.39±0.06[b]
C14:0	2.62±0.40[b]	1.30±0.10[a]	2.25±0.52[ab]
C16:0	0.17±0.05[a]	0.15±0.04[a]	0.10±0.04[a]
C18:0	0.09±0.03[a]	0.55±0.03[b]	0.73±0.03[c]
C18:1	0.72±0.06[a]	0.62±0.02[a]	0.93±0.06[b]
C18:2	1.28±0.44[a]	1.20±0.09[a]	1.24±0.46[a]
C18:3	0.07±0.02[a]	0.08±0.01[a]	0.08±0.02[a]
C20:0	0.04±0.01[a]	0.042±0.00[a]	0.04±0.01[a]
Total	7.71±0.59[a]	5.22±0.43[b]	7.29±0.13[b]

The results are shown as an average of three replicates for each trial (A, B, C ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

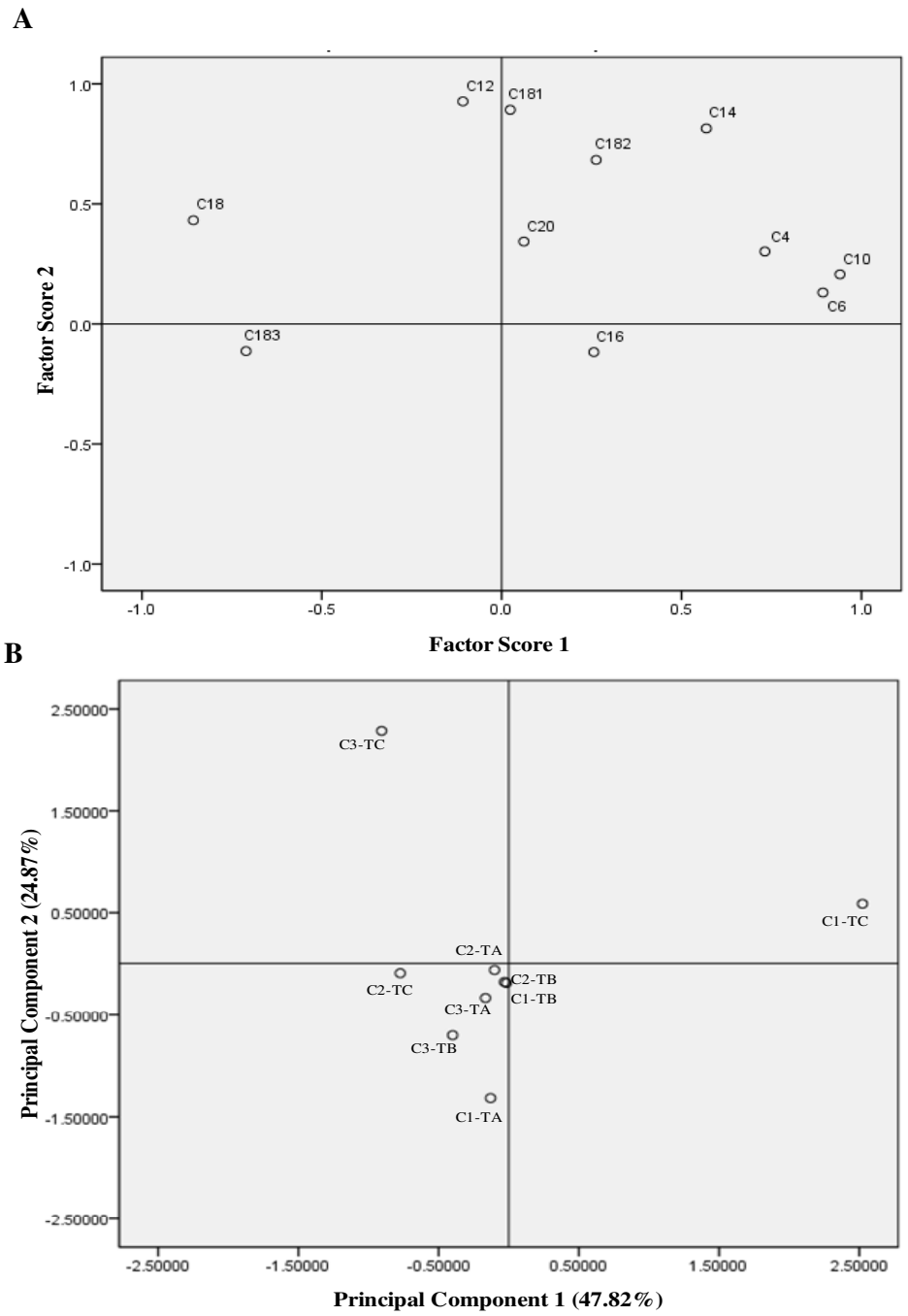


Figure 5.11. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for free fatty acids at 6 months of ripening in Trial A, B, C. C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

5.4.5. Biogenic amines

Levels of biogenic amines (BA) are shown in **Table 5.6** and expressed as µg of BA per g of cheese. In Trial A, tryptamine was not detected in any of the cheeses, whereas phenylethylamine was detected in all cheeses but no significant differences ($P \leq 0.05$) were found between the samples. Putrescine was present at the highest level in cheese C2; cadaverine was detected only in cheeses C2 and C3 and. Histamine was detected only in C3. In cheeses C2 and C3, tyramine, spermidine and spermine was present at significantly higher levels ($P \leq 0.05$) than in the control cheese C1 showing that *H. alvei* contributed to the production of these BA.

In Trial B, tryptamine was not detected in any of the cheeses and histamine was detected only in C3. Phenylethylamine was found only in C2 and C3, the latter cheese contained a higher level. Putrescine was present at a significantly ($P \leq 0.05$) higher level in C3 than control cheese C1 and cheese C2, whereas cadaverine and tyramine were present at a significantly ($P \leq 0.05$) higher level in C2 and C3 than the control cheese C1. No significant differences in the level of spermidine ($P \leq 0.05$) were found in all cheeses whereas a significantly ($P \leq 0.05$) higher level of spermine was detected in control cheese C1 than in C2 and C3.

Also in Trial C, tryptamine was not detected in any of the cheeses, phenylethylamine was detected only in C1 and histamine in C3. Levels of putrescine and tyramime were significantly ($P \leq 0.05$) higher in the control cheese C1 than experimental cheeses C2 and C3. No significant ($P > 0.05$) differences were found in levels of cadaverine and spermidine between all the samples. Cheese C2 and C3 contained significantly ($P \leq 0.05$) higher levels of spermine than control cheese C1.

Results from Trial A and B are in agreement with those reported by Delbes-Paus et al. (2012) who showed that cadaverine and putrescine were present at significantly higher level in cheeses made with *H. alvei* than the control cheese. After 28 days of ripening the authors reported the presence of cadaverine and putrescine at levels of 0.81 and 1.53 mmol kg^{-1} of cheese dry matter respectively. Cadaverine was produced from day 1 and its concentration increased at a fairly constant rate until day 28 whereas putrescine concentration increased slowly until day 7 and then increased at a similar rate as cadaverine.

Generally, the addition of *H. alvei* to the cheese significantly increased the level of most of the BA, apart from in Trial C, where only spermine was present at significantly higher level in the experimental cheeses than the control cheese. In studies conducted *in vitro*, *H. alvei* was found to produce tryptamine, penylethylamine, putrescine, isoamylamine, cadaverine, histamine and tyramine; putrescine, cadaverine, histidine and tyramine (Pattono et al., 2008; Coton et al., 2012).

Similary, Lorenzo et al. (2010) reported that *H. alvei* produced the maximum amount of putrescine and cadaverine during the exponential phase, but the production of putrescine also continued during the stationary phase at lower levels. Chaves-Lopez et al. (2006), showed all the strains of Enterobacteriaceae studied produced histamine, putrescine, spermine and spermidine. Marino et al. (2000) isolated several

Enterobacteria from blue veined cheese, amongst which *H. alvei,* that were able to produce cadaverine, putrescine, tyramine, histamine *in vitro*.

As reported by EFSA's scientific opinion on risk based control of biogenic amine formation in fermented foods (EFSA, 2011), high occurrence values of biogenic amines (mg/kg) in cheese are as follows: histamine (130), tyramine (440), putrescine (143), cadaverine (470), phenylethylamine (18.8), tryptamine (‹50). According to those data, putrescine was produced at high level in all cheeses in Trial A, (particulary in cheese C2 contained 0.1% of *Hafnia alvei*), in cheese 3 (Trial B) and control cheese C1 (Trial C). Levels of tyramine and cadaverine can be considered low, whereas phenylethylamine was present at high level only in cheese C3 in Trial A. Tryptamine was not produced in any of the cheeses and histamine was produced only in cheese C3 in all trials at very low levels (from 4.32 to 11.71).

Table 5.6. Levels of biogenic amines in Cheddar cheeses at 180 days of ripening. C1, Control cheese; C2, cheese made with 0.1% of *Hafnia alvei*; C3, cheese made with 1% of *Hafnia alvei.*

BA	***Trial A***		
(ug/1g of cheese)	C1	C2	C3
tryptamine	n.d.	n.d.	n.d.
phenylethylamine	16.85±0.82[a]	13.86±1.54[a]	20.17±4.60[a]
putrescine	117.00±13.60[a]	532.91±20.59[b]	148.37±9.94[a]
cadaverine	n.d.	114.42±3.06	54.03±14.01
histamine	n.d.	n.d.	4.32±1.70
tyramine	41.59±5.07[a]	171.28±11.34[c]	71.64±6.45[b]
spermidine	80.60±1.93[a]	125.47±14.20[b]	109.45±18.51[ab]
spermine	8.23±0.35[a]	13.37±1.15[b]	11.55±1.07[b]

BA	***Trial B***		
(ug/1g of cheese)	C1	C2	C3
tryptamine	n.d.	n.d.	n.d.
phenylethylamine	n.d.	4.43±0.82	9.65±0.63
putrescine	51.18±7.74[a]	87.39±8.61[a]	226.08±41.13[b]
cadaverine	70.30±6.67[a]	91.79±5.59[b]	91.26±6.77[b]
histamine	n.d.	n.d.	11.72±1.04
tyramine	11.97±0.87[a]	70.59±5.13[b]	73.19±9.79[b]
spermidine	140.90±15.67[a]	151.21±5.93[a]	166.41±16.48[a]
spermine	50.51±6.64[b]	20.86±2.87[a]	21.63±3.52[a]

BA	***Trial C***		
(ug/1g of cheese)	C1	C2	C3
tryptamine	n.d.	n.d.	n.d.
phenylethylamine	4.29±0.30	n.d.	n.d.
putrescine	180.98±2.39[b]	70.84±8.60[a]	74.94±9.13[a]
cadaverine	71.61±2.88[a]	82.51±4.12[a]	90.57±3.96[a]
histamine	n.d.	n.d.	11.47±2.70
tyramine	61.30±0.88[b]	4.42±0.53[a]	3.21±0.25[a]
spermidine	119.02±10.39[a]	131.78±10.49[a]	115.70±10.33[a]
spermine	18.09±1.51[a]	51.90±2.48[b]	47.31±2.34[b]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($P \leq 0.05$).

n.d. not detected

5.5. Conclusions

In this study, *H. alvei* was used as an adjunct in the manufacture of Cheddar cheese and its effect during ripening was evaluated. *H. alvei* numbers in Cheddar cheese dramatically reduced after the first days of ripening and then remained constant at level of ~10^3 cfu/g until the end of ripening. The strain significantly increased the level of pH 4.6-soluble nitrogen, total free amino-acids, and some individual free amino-acids of Cheddar cheese, whereas no differences in the urea-PAGE electrophoretograms of the cheeses were detected. *H. alvei* contributed to lipolysis by increasing the level of free fatty acids only in Trial A, in particular by increasing the level of acetic acid (C2:0), butyric acid (C4:0), myristic acid (C14:0), stearic acid (C18:0) and oleic acid (C18:1). *H. alvei* also significantly increased the level of most of the BA, apart from in Trial C, where only spermine was present at significantly higher level in the experimental cheeses than the control cheese. Anyway, levels of BA were considered low for cheese, except for putrescine and phenylethylamine. Tyramine and histamine, which are considered the most toxic BA, were not produced or present at very low level. This results suggest that *H. alvei* can be used in order to accelerate the ripening of Cheddar cheese, although this strains showed to have an effect on lipolysis only in Trial A.

5.6. References

ABRIOUEL, H., MARTIN-PLATERO, A., MAQUEDA, M., VALDIVIA, E. & MARTINEZ-BUENO, M. 2008. Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology,* 127, 200-208.

ANDREWS, A. T. 1983. Proteinases in normal bovine-milk and their action on caseins. *Journal of Dairy Research,* 50, 45-55.

AWAD, S., AHMED, N. & EL SODA, M. 2010. Influence of microfiltration and adjunct culture on quality of Domiati cheese. *Journal of Dairy Science,* 93, 1807-1814.

BERESFORD, T. P., FITZSIMONS, N. A., BRENNAN, N. L. & COGAN, T. M. 2001. Recent advances in cheese microbiology. *International Dairy Journal,* 11, 259-274.

BLAKESLEY, R. W. & BOEZI, J. A. 1977. New staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry,* 82, 580-582.

BRENNER D. J. & FARMER J.J. III, 2005. Order XIII. "Enterobacteriales", *Bergey's manual of systematic bacteriology*, Vol. 2: the Proteobacteria, part B: the Gammaproteobacteria. Edited by D. J. Brenner, N. R. Krieg, James T. Staley, G. M. Garrity, 587-850.

BURNS, P., CUFFIA, F., MILESI, M., VINDEROLA, G., MEINARDI, C., SABBAG, N. & HYNES, E. 2012. Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheeses. *Food Microbiology,* 30, 45-50.

CHAVES-LOPEZ, C., DE ANGELIS, M., MARTUSCELLI, M., SERIO, A., PAPARELLA, A. & SUZZI, G. 2006. Characterization of the Enterobacteriaceae isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *Journal of Applied Microbiology,* 101, 353-360.

COTON, M., DELBES-PAUS, C., IRLINGER, F., DESMASURES, N., LE FLECHE, A., STAHL, V., MONTEL, M.-C. & COTON, E. 2012. Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiology,* 29, 88-98.

CURIEL, J. A., RUIZ-CAPILLAS, C., DE LAS RIVAS, B., CARRASCOSA, A. V., JIMENEZ-COLMENERO, F. & MUNOZ, R. 2011. Production of biogenic amines by lactic acid bacteria and enterobacteria isolated from fresh pork sausages packaged in different atmospheres and kept under refrigeration. *Meat Science,* 88, 368-373.

DADAKOVA, E., KRIZEK, M. & PELIKANOVA, T. 2009. Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC). *Food Chemistry,* 116, 365-370.

DE JONG, C. & BADINGS, H. T. 1990. Determination of free fatty acids in milk and cheese procedures for extraction clean up and capillary gas chromatographic analysis. *HRC Journal of High Resolution Chromatography,* 13, 94-98.

DEETAE, P., MOUNIER, J., BONNARME, P., SPINNLER, H. E., IRLINGER, F. & HELINCK, S. 2009a. Effects of *Proteus vulgaris* growth on the establishment of a cheese microbial community and on the production of volatile aroma compounds in a model cheese. *Journal of Applied Microbiology,* 107, 1404-1413.

DEETAE, P., SPINNLER, H.-E., BONNARME, P. & HELINCK, S. 2009b. 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.

DELBES-PAUS, C., POCHET, S., HELINCK, S., VEISSEIRE, P., BORD, C., LEBECQUE, A., COTON, M., DESMASURES, N., COTON, E., IRLINGER, F. & MONTEL, M.-C. 2012. Impact of Gram-negative bacteria in interaction with a complex microbial consortium on biogenic amine content and sensory characteristics of an uncooked pressed cheese. *Food Microbiology,* 30, 74-82.

DI CAGNO, R., QUINTO, M., CORSETTI, A., MINERVINI, F. & GOBBETTI, M. 2006. Assessing the proteolytic and lipolytic activities of single strains of mesophilic lactobacilli as adjunct cultures using a Caciotta cheese model system. *International Dairy Journal,* 16, 119-130.

DONATO, K. A., ZAREIE, M., JASSEM, A. N., JANDU, N., ALINGARY, N., CARUSONE, S. C., JOHNSON-HENRY, K. C. & SHERMAN, P. M. 2008. *Escherichia albertii* and *Hafnia alvei* are candidate enteric pathogens with divergent effects on intercellular tight junctions. *Microbial Pathogenesis,* 45, 377-385.

EFSA 2011. Scientific Opinion on risk based control of biogenic amine formation in fermented foods. EFSA Journal, 9.

EL SODA, M., MADKOR, S. A. & TONG, P. S. 2000. Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science,* 83, 609-619.

ERCOLINI, D., RUSSO, F., FERROCINO, I. & VILLANI, F. 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology,* 26, 228-231.

FLOREZ, A. B., HERNANDEZ-BARRANCO, A. M., MARCOS, I. & MAYO, B. 2006. Biochemical and microbiological characterization of artisan kid rennet extracts used for Cabrales cheese manufacture. *Lwt-Food Science and Technology,* 39, 605-612.

FOX, P. F. 1963. Potentiometric determination of salt in cheese. *Journal of Dairy Science,* 46, 744-745.

FOX, P. F., GUINEE, T. P., COGAN, T. M. & McSWEENEY, P. L. H. (eds.) 2000. *Fundamentals of Cheese Science*: Aspen Publishers, Gaithersburg, MD.

GARDINER, G. E., ROSS, R. P., WALLACE, J. M., SCANLAN, F. P., JAGERS, P., FITZGERALD, G. F., COLLINS, J. K. & STANTON, C. 1999. Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of Cheddar cheese. *Journal of Agricultural and Food Chemistry,* 47, 4907-4916.

GRAPPIN, R. 1986. Milk determination of nitrogen content Kjeldahl method and calculation of crude protein content. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 20A, 1-3.

HANSEN, L. T. & HUSS, H. H. 1998. Comparison of the microflora isolated from spoiled cold-smoked salmon from three smokehouses. *Food Research International,* 31, 703-711.

HORTENSIA SILLA SANTOS, M. 1998. Amino acid decarboxylase capability of microorganisms isolated in Spanish fermented meat products. *International Journal of Food Microbiology,* 39, 227-230.

HYNES, E., OGIER, J. C., SON, O. & DELACROIX-BUCHET, A. 2003. Influence of starter and adjunct lactobacilli culture on ripening of miniature washed-curd cheeses. *Lait,* 83, 17-29.

IDF 1983. Milk, cream and evaporated milk - determination of total solids content (reference method)-provisional international IDF standard 21A-1982.

IDF 1986. Cheese and processed cheese products determination of fat content gravimetric method reference method. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 5B, 1-5.

IRLINGER, F., IN YUNG, S. A. Y., SARTHOU, A.-S., DELBES-PAUS, C., MONTEL, M.-C., COTON, E., COTON, M. & HELINCK, S. 2012. Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. *International Journal of Food Microbiology,* 153, 332-8.

JUVEN, B. J., GORDIN, S., ROSENTHAL, I. & LAUFER, A. 1981. Changes in refrigerated milk caused by enterobacteriaceae. *Journal of Dairy Science,* 64, 1781-1784.

KONDYLI, E., KATSIARI, M. C., MASOURAS, T. & VOUTSINAS, L. P. 2002. Free fatty acids and volatile compounds of low-fat Feta-type cheese made with a commercial adjunct culture. *Food Chemistry,* 79, 199-205.

KUCHROO, C. N. & FOX, P. F. 1982. Fractionation of the water-soluble nitrogen from Cheddar cheese - chemical methods. *Milchwissenschaft-Milk Science International*, 37, 651-653.

KUNG, H.-F., WANG, T.-Y., HUANG, Y.-R., LIN, C.-S., WU, W.-S., LIN, C.-M. & TSAI, Y.-H. 2009. Isolation and identification of histamine-forming bacteria in tuna sandwiches. *Food Control,* 20, 1013-1017.

LINARES, D. M., MARTIN, M. C., LADERO, V., ALVAREZ, M. A. & FERNANDEZ, M. 2011. Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition,* 51, 691-703.

LORENZO, J. M., CACHALDORA, A., FONSECA, S., GOMEZ, M., FRANCO, I. & CARBALLO, J. 2010. Production of biogenic amines "in vitro" in relation to the growth phase by Enterobacteriaceae species isolated from traditional sausages. *Meat Science,* 86, 684-691.

LYNCH, C. M., McSWEENEY, P. L. H., FOX, P. F., COGAN, T. M. & DRINAN, F. D. 1996. Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. *International Dairy Journal,* 6, 851-867.

LYNCH, C. M., MUIR, D. D., BANKS, J. M., McSWEENEY, P. L. H. & FOX, P. F. 1999. Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *Journal of Dairy Science,* 82, 1618-1628.

MARINO, M., MAIFRENI, M., MORET, S. & RONDININI, G. 2000. The capacity of Enterobacteriaceae species to produce biogenic amines in cheese. *Letters in Applied Microbiology,* 31, 169-173.

MARTIN, A., BENITO, M. J., HERNANDEZ, A., PEREZ-NEVADO, F., CORDOBA, J. J. & CORDOBA, M. G. 2008. Characterisation of microbial deep spoilage in Iberian dry-cured ham. *Meat Science,* 78, 475-484.

MARTIN-PLATERO, A. M., MAQUEDA, M., VALDIVIA, E., PURSWANI, J. & MARTINEZ-BUENO, M. 2009. Polyphasic study of microbial communities of two Spanish farmhouse goats' milk cheeses from Sierra de Aracena. *Food Microbiology,* 26, 294-304.

MARTUSCELLI, M., GARDINI, F., TORRIANI, S., MASTROCOLA, D., SERIO, A., CHAVES-LÓPEZ, C., SCHIRONE, M. & SUZZI, G. 2005. Production of biogenic amines during the ripening of Pecorino Abruzzese cheese. *International Dairy Journal,* 15, 571-578.

MILESI, M. M., McSWEENEY, P. L. H. & HYNES, E. R. 2008. Viability and contribution to proteolysis of an adjunct culture of Lactobacillus plantarum in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology,* 105, 884-892.

MORALES, P., FERNANDEZ-GARCIA, E. & NUNEZ, M. 2003. Caseinolysis in cheese by Enterobacteriaceae strains of dairy origin. *Letters in Applied Microbiology,* 37, 410-414.

MOUNIER, J., MONNET, C., JACQUES, N., ANTOINETTE, A. & IRLINGER, F. 2009. Assessment of the microbial diversity at the surface of Livarot cheese using culture-dependent and independent approaches. *International Journal of Food Microbiology,* 133, 31-37.

NAJJARI, A., OUZARI, H., BOUDABOUS, A. & ZAGOREC, M. 2008. Method for reliable isolation of *Lactobacillus sakei* strains originating from Tunisian seafood and meat products. *International Journal of Food Microbiology,* 121, 342-351.

NOVEIR, M. R., DOGAN, H. B. & HALKMAN, A. K. 2000. A note on *Escherichia coli* O157 : H7 serotype in Turkish meat products. *Meat Science,* 56, 331-335.

PATTONO, D., GRASSI, M. A. & CIVERA, T. 2008. Production of biogenic amines by some enterobacteriaceae strains isolated from dairy products. *Italian Journal of Food Science*, 20, 411-417.

PHILLIPS, M., KAILASAPATHY, K. & TRAN, L. 2006. Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei*, *L. paracasei* and *L. rhamnosus*) in Cheddar cheese. *International Journal of Food Microbiology,* 108, 276-280.

PIRAINO, P., PARENTE, E. & McSWEENEY, P. L. H. 2004. Processing of chromatographic data for chemometric analysis of peptide profiles from cheese extracts: A novel approach. *Journal of Agricultural and Food Chemistry,* 52, 6904-6911.

POLYCHRONIADOU, A. 1988. A simple procedure using trinitrobenzenesulfonic acid for monitoring proteolysis in cheese. *Journal of Dairy Research*, 55, 585-596.

POVEDA, J. M., SOUSA, M. J., CABEZAS, L. & McSWEENEY, P. L. H. 2003. Preliminary observations on proteolysis in Manchego cheese made with a defined-strain starter culture and adjunct starter (*Lactobacillus plantarum*) or a commercial starter. *International Dairy Journal*, 13, 169-178.

PUCHADES, R., LEMIEUX, L. & SIMARD, R. E. 1989. Evolution of free amino-acids during the ripening of Cheddar cheese containing added lactobacilli strains. *Journal of Food Science*, 54, 885-&.

SARANTINOPOULOS, P., KALANTZOPOULOS, G. & TSAKALIDOU, E. 2002. Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. *International Journal of Food Microbiology,* 76, 93-105.

SMELA, D., PECHOVA, P., KOMPRDA, T., KLEJDUS, B. & KUBAN, V. 2004. Chromatographic determination of biogenic amines in meat products during fermentation and long-term storage. *Chemicke Listy,* 98, 432-437.

TORNADIJO, M. E., GARCIA, M. C., FRESNO, J. M. & CARBALLO, J. 2001. Study of Enterobacteriaceae during the manufacture and ripening of San Simon cheese. *Food Microbiology,* 18, 499-509.

6. Development of a symbiotic Cheddar cheese containing pre- and probiotics

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6.1. Abstract

In the present study, a low-fat Cheddar cheese was made with *Bifidobacterium animalis* subsp. *lactis*, strain BB-12®, as a probiotic adjunct culture and Hi-Maize® 260 (resistant high amylose maize starch) as a prebiotic fiber which also played the role of fat replacer. Control cheese H was made with half-fat milk, cheese H2 was made with half-fat milk and 2% w/v of resistant starch Hi-maize® 260, cheese H4 was made with half-fat milk and 4% w/v of resistant starch Hi-maize® 260, control cheese F was made with full-fat milk. *Bifidobacterium* BB-12® was added in all cheeses at level of 0.1% w/v resulting in a final inoculum of 10^8 cfu/ml. Half-fat cheese H, showed the highest level of moisture and a higher level of proteins than the full-fat cheese F. Cheeses H2 and H4 had a lower moisture content than cheese H indicating that the starch replaced a portion of water in the matrix and did not show any water binding capacity. Generally, *Bifidobacterium* BB-12 decreased by 1 log cycle after 60 d of ripening and remained steady at level of ~10^7 cfu/g during ripening. No differences in the degradation of α_{s1}-casein and β-casein were shown by urea-PAGE and capillary electrophoresis. After 180 d of ripening, no statistically significant differences ($P < 0.05$) were found in the levels of pH 4.6-soluble nitrogen as percentage of total nitrogen between cheese H, H2 and H4 in Trials A and B, but they were found to be significantly higher than cheese F, whereas in Trial C, cheese F had the highest value. Results from texture profile analysis showed that as the fat content in cheese decreased, the Young's modulus increased. The Young's modulus also increased proportionally with increasing addition of Hi-maize. Hencky strain at fracture decreased over ripening, was found to be higher in half-fat cheeses than full-fat cheese and increased with increasing in fat replacer.

6.2. Introduction

In recent years, particular attention has been focused on the production of functional foods made with the addition of probiotics, prebiotics, vitamins or minerals (Cruz et al. 2001) and fat-free or reduced fat-foods (Zalazar et al., 2002). Probiotic bacteria are defined as "living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition" (Stanton et al., 1998). Probiotic food contains probiotic microorganisms at levels of 10^6-10^7 CFU/g, which are established as therapeutic quantities. In order to maintain those numbers, probiotic microrganisms must preserve their viability and metabolic activity during manufacture and survive in the gastrointestinal tract (da Cruz et al., 2009). Due to numerous potential health claims, probiotic strains of *Lactobacillus* and *Bifidobacterium* spp. have been incorporated into functional foods such as fermented milk, yogurt, ice cream and cheese (Scheller and O'Sullivan, 2011).

Bifidobacteria are Gram-positive anaerobic bacteria with branched or irregularly shaped rods belonging to the family of Bifidobacteriaceae. They were first isolated from faeces of a breast-fed infant by Tissier in 1899 who named than *Bacillus bifidus* (Gomes and Malcata, 1999). The natural habitat of this bacterium is the intestines of humans, animal and insects, but they are also found in the oral cavity, sewage, blood and food (Ventura et al., 2007). Bifidobacteria from human origin are able to ferment glucose, galactose, lactose and usually fructose and produce acetic and lactic acids without generating CO_2. It has been also shown that they can ferment complex carbohydrates such as D-galactosamine, D-glucosamine, amylase and amylopectin

(Gomes and Malcata, 1999). *Bifidobacterium* spp. have been used as probiotic cultures in cheeses such as Cheddar (Ong et al., 2006), Gouda and Camembert (Van de Casteele et al., 2003), Cottage cheese (Obando et al., 2010), white brined cheese (Yilmaztekin et al., 2004) and Crescenza (Gobbetti et al., 1998).

Cheddar cheese may act as a good probiotic carrier: its pH may enhance the survival of the probiotic microorganisms, the matrix and high fat content may protect the bacteria during their ingestion through the gastrointestinal tract (Stanton et al., 1998). Since Cheddar cheese has a long ripening time (up to 2 years) it is important to select probiotic strains able to survive during the maturation period (Godward and Kailasapathy, 2003). In studies conducted by Ong et al. (2006) and Ong and Shah (2009), it was shown that *Bifidobacterium longum* 1941 *and Bifidobacterium animalis* subsp. *lactis* LAFTI® B94 remained viable up to ~ 10 x 10^8 CFU/g in Cheddar after 24 weeks of ripening at 4 and 8°C. Free and encapsulated strains of *Bifidobacterium infantis* CSCC1912 and *Bifidobacterium lactis* 929 survived after 24 weeks of storage at 8°C in Cheddar cheese (Godward and Kailasapathy, 2003). *Bifidobacterium lactis* BB-12 survived at numbers ≥ 10^8 CFU/g whereas *B. longum* BB536 was present at level of 10^5 CFU/g after 6 months of ripening in Cheddar cheese (McBrearty et al., 2001). *B. infantis* survived at numbers of up to 10^6 CFU/g during 84 days of ripening of Cheddar cheese at 4°C (Daigle et al., 1999).

Over the past years, the incorporation of probiotic bacteria to food and production of low-fat food, including cheese, has significantly increased all over the world. Since fat plays an important role in determining the texture, flavor and aroma of cheese, its reduction is often correlated with a negative effect on the sensory characteristics of cheese such as poor aroma, unwanted flavours, bitterness, rubbery body (over-firm and elastic). As the fat content decreases, moisture increases and protein plays a greater role in texture giving the cheese a firm texture (Mistry, 2001). In low-fat cheeses, the activity of the starter culture is affected by the higher moisture in non-fat solids (MNFS) and lower salt in moisture (S/M) contents due to the higher moisture content of cheese. Off-flavors are more detected because of a lower amount of aromatic compounds such as butanoic and hexanoic acid and methyl ketones which are formed through fat degradation. Bitterness is due to a low salt content and high moisture and it is higher in low fat cheeses because hydrophobic peptides, usually responsible for a bitter taste, are absorbed by fat (Mistry, 2001). Low cooking temperatures, high pH at milling and washing of the curd are techniques usually adopted in order to improve the quality of low-fat cheeses. The goal is to replace fat with moisture content without affecting the yield and quality of the cheese. Other methods include the use of starter culture with low proteolytic but high peptidolytic activity that are able to autolyse at low cooking temperatures and high moisture content. A slow rate of acid production is also required in order to maintain buffering capacity and to prevent the development of strong acid flavors (Mistry, 2001; Drake and Swanson, 1995).

Another technique used for improving the quality of low-fat cheeses is the use of fat replacers which are classified as fat substitutes or fat mimetics. Fat substitutes have similar physical and functional properties as fat but provide low or no calories. Typical examples are sucrose polyesters and triglycerides containing medium and long-chain fatty acids which are digested and oxidized differently to regular triglycerides (Drake and Swanson, 1995). Fat mimetics are mainly made from

natural proteins (e.g., whey, milk or egg proteins), fat (chemical alteration of fatty acids to provide less or no calories) or carbohydrate derivatives (e.g; cellulose, dextrins, maltodextrins, polydextrose, gums, fiber, modified and resistant starches) (ADA, 2005). Resistant starches (RS), are defined as "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Englyst et al., 1996). It has been shown that resistant cornstarch increases the fecal concentration and excretion of propionate and butyrate (Brown et al., 1997), it reduces the production of harmful byproducts of protein metabolism such as ammonia and phenols in the human colon (Birkett et al., 1996) and promotes regularity with a mild laxative effect (Phillips et al., 1995). For this reasons, RS can be considered a prebiotic, which is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Van Loo, 2008). RS such as high amylose corn starch (HACS) are fermented by *Bifidobacterium* spp. which possesses cell-bound starch degrading enzymes and contributes to increase faecal/caecal numbers of bifidobacteria in rats and mice (Cho and Finocchiaro, 2010).

The combination of a probiotic and a prebiotic results in a product called symbiotic which is more potent than either a probiotic or a prebiotic alone, because they act in a synergic way (Cho and Finocchiaro, 2010; Rodrigues et al., 2012). In this study, a low-fat Cheddar cheese was made with *Bifidobacterium* BB-12 as a probiotic adjunct culture and Hi-Maize® 260 (resistant high amylose maize starch) as a prebiotic fiber which also played the role of fat replacer.

6.3. Materials and methods

6.3.1. Strain

Probiotic strain of *Bifidobacterium animalis* subsp. *lactis* strain BB-12 was obtained from Chr. Hansen A/S, Hørsholm, Denmark (BB-12 is a registered trademark of Chr. Hansen).

6.3.2. Fat replacer

Resistant starch Hi-maize® 260 was obtained from National Starch and Chemicals, Manchester, England.

6.3.3. Cheesemaking

Cheesemaking was carried out in triplicate on different days. The experiment was designed as follows. Control cheese H was made with half-fat milk, cheese H2 was made with half- fat milk and 2% w/v of resistant starch Hi-maize® 260 added to the milk, cheese H4 was made with half-fat milk and 4% w/v of resistant starch Hi-maize® 260 added to the milk, control cheese F was made with full-fat milk. The fat content of milk was standardized to 4% for full-fat Cheddar cheese and 2% for half-fat Cheddar cheeses. Half-fat milk was pasteurised at 72ºC for 15 s and cooled to 30ºC, full-fat milk was pasteurized separately using a waterbath at 63ºC for 30 min. DVS starter culture (R604 Chr-Hansen, Hørsholm, Denmark) was added at a rate of level 0.03% w/v to cheesemilk and held at 30ºC for 30 min before addiction of 0.1% w/v *Bifidobacterium animalis* subsp. *lactis*, strain BB-12®, which resulted in a final inoculum of 10^8 cfu/ml. Hi-maize resistant starch was then added to the milk at level of 2% w/v in cheese H2 and 4% w/v in cheese H4 and mixed manually. Chymax-180 (Chr. Hansen, Hørshom, Denmark) was used as coagulant. Whey was drained at pH 6.2 and the curd cheddared, milled at pH 5.2 and salted to a level of 2.5 % (w/w). After pressing the curd at 150 kPa for 18 h, the cheese was vacuum packed in a vacuum bag and ripened for 180 days at 8°C.

6.3.4. Microbiological analysis

Samples were taken under aseptic conditions using a cheese trier. Ten g of cheese were homogenized in 90 ml of sterile trisodium citrate (2% w/v) in a stomacher bag (Stomacher 400, Seward Limited, Worthing, West Sussex, UK) for 5 min. Starter cells were enumerated on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30° for 3 days, NSLAB were enumerated on Rogosa medium (Merck, Darmstadt, Germany) after 5 days at 30°C. *Bifidobacterium animalis* BB-12 was enumerated on a selective medium BSM prepared as follows. MRS agar (Sigma, Dublin, Ireland) contained 0.5g/L CysHCl stock solution (Sigma, Dublin, Ireland) and 25 mg/L mupirocin (Oxoid, Dublin, Ireland). Plates were anaerobically incubated at 37° C for 3 days.

6.3.5. Compositional analysis

Protein (Grappin, 1986), fat (Gerber method; IDF 1986), moisture (oven drying at 102°C; IDF 1983), salt (Fox, 1963) and pH were determined in triplicate after 30 days of ripening. The pH was measured in cheese slurry (1:1 cheese:water) using a combined glass electrode (PHC3001-8, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standar pH Meter, Radiometer Copenhagen, Denmark).

6.3.6. Analysis of cheese

The pH 4.6 soluble and –insoluble fractions of the cheeses were prepared according to the method of Kuchroo and Fox (1982). The extraction was carried out in triplicate for each cheese. The N content of the pH 4.6 soluble fraction of the cheeses was measured by the macro-Kjeldahl method (Grappin, 1986) and expressed as a % of the total N content of the cheeses.

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was carried out on the samples after 60 d and 180 d of ripening, using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Andrews (1983) with modifications. Gels were stained directly by the method of Blakesley and Boezi (1977).

Capillary electrophoresis (CE) was performed on cheese after 60 d and 180 d using the method described for cheese in citrate dispersion by Ardö and Polychroniadou (1999). The temperature of the separation was kept at 45°C and UV detection was performed at 214 nm. Data acquisition and processing were carried out using the software HP Chem Stations, revision A.04.02 (Hewlett–Packard A/S, Birkerød, Denmark). Capillary electrophrograms were evaluated visually, and normalised areas of selected peaks were calculated by dividing the integrated peak area with the migration time for the given peak.

Individual free amino acids (IFA) were quantified at 180 d using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Welwyn Garden City, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column.

One-way analysis of variance of data from the composition of the cheeses was performed using SPSS Version 18.0 for Windows XP (SPSS Inc., Chicago, IL, USA). Significance was declared at $P \leq 0.05$. Principal component analysis (PCA) was performed using SPSS Version 18.0 for Windows XP.

6.3.7. Rheological properties by uniaxial compression

Before measurements, cylindrical samples (height 18.2 mm, diameter 15.0 mm) were prepared and conditioned for 1.5 h at 20°C in a closed Petri dish. Uniaxial compression was performed with an Instron 5564 universal testing machine (Instron Ltd, High Wycombe, UK) equipped with lubricated steel plates. The velocity at compression was constant and always 500 mm min^{-1}. From the force and displacement data, with the assumption that the sample maintained its volume and

cylindrical shape during the compression, the stress (σ) and the Hencky strain (ε_H) were calculated and plotted for each measurement. The Hencky strain is defined as $\varepsilon_H = \ln |H_t/H_0|$,

Where H_0 is the initial sample height and Ht is the sample height at time t (Steffe, 1996). The fracture point was defined as the first local maximum of the compression curve. Young's modulus (E) was calculated as the maximum slope of the $\sigma - \varepsilon_H$ curve before fracture. The Hencky strain at fracture (εf) were defined as εH at the fracture point. Eight cylindrical replicates were measured for each sample.

6.4. Results and discussion

6.4.1. pH and Compositional analysis

Results of pH and compositional analysis of the cheeses at day 30 are shown in **Table 6.1**. pH was not affected by the addition of starch. In spite of the low salt content of Hi-maize (~0.077%, results not shown), the addition of the starch to the cheese increased significantly ($P < 0.05$) the salt content in cheeses H2 and H4, probably because of the lower % of moisture in cheeses. As expected, the reduction of the fat content of cheese milk affected the casein to fat ratio and moisture content in the cheeses. Moreover, the moisture content of cheeses was inversely related to the fat and starch content. Half-fat cheese H, showed the highest level of moisture in all trials and a higher level of proteins than the full-fat cheese F. Fat reduction leads to a higher protein content and thus a higher moisture content in the cheese due to the water binding capacity of the casein matrix. Similar results were reported by other workers (Sipahioglu et al., 1999; Volikakis et al., 2004; Kucukoner and Haque, 2006). Additionally, fat and protein content decreased with increasing level of fat replacer since the starch replaced their portion in the matrix. In Trial A and B, cheese H2 containing 2% Hi-maize, showed a significantly ($P < 0.05$) higher moisture content than cheese H4 made with 4% Hi-Maize. Both cheeses had a lower moisture content than cheese H indicating that the starch replaced a portion of water in the matrix and did not show any water binding capacity. Similar results were also shown by Duggan (2008) who studied the effect of increasing resistant starch (Hi-maize 240) content on some functional properties of imitation cheese.

Table 6.1. pH and composition of Cheddar cheeses at 30 days of ripening.
Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese.

	Cheese	pH	% Salt	% Fat	% Protein	% Moisture
Trial A	**H**	4.94±0.08[a]	1.27±0.01[a]	18.33±0.58[c]	30.13±0.56[d]	46.28±0.76[c]
	H2	4.91±0.04[a]	1.40±0.01[b]	15.00±0.00[b]	24.22±0.41[c]	45.13±0.28[b]
	H4	4.86±0.02[a]	1.62±0.07[c]	12.00±0.00[a]	21.01±0.30[a]	43.40±0.50[a]
	F	4.88±0.03[a]	1.19±0.02[a]	30.00±1.00[d]	22.44±0.38[b]	43.01±1.02[a]
Trial B	**H**	4.84±0.01[a]	1.15±0.01[a]	18.67±0.58[c]	27.44±0.44[c]	48.84±0.30[c]
	H2	4.82±0.02[a]	1.49±0.01[b]	15.33±0.58[b]	22.56±0.10[b]	46.34±0.40[b]
	H4	4.83±0.03[a]	1.59±0.01[c]	12.67±0.58[a]	19.91±0.49[a]	43.69±0.57[a]
	F	4.81±0.02[b]	1.14±0.02[a]	29.00±0.00[c]	21.88±0.62[b]	44.19±1.34[a]
Trial C	**H**	4.91±0.03[a]	1.15±0.03[a]	19.00±1.00[c]	28.95±1.43[c]	46.72±0.19[b]
	H2	4.91±0.04[a]	1.38±0.03[b]	16.33±0.58[b]	25.93±0.27[b]	40.76±0.96[a]
	H4	4.92±0.06[a]	1.50±0.01[c]	12.33±0.58[a]	21.64±0.47[a]	42.05±0.54[a]
	F	4.94±0.01[a]	1.12±0.01[a]	29.33±0.58[d]	21.66±0.41[a]	41.87±0.76[a]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same column followed by the same letter are not significantly different ($P \leq 0.05$).

6.4.2. Microbiological analysis

Starter lactic acid bacteria were present at level of ~10^9 cfu/g in all cheeses in all trials at day 1 and decreased during ripening to levels of ~10^7 cfu/g in Trial A and ~10^6 cfu/g in Trial B and C (**Figure 6.1 A, B, C**). Similar results were reported by McSweeney et al. (1993), Shakeel-ur-Rehman et al. (2000) and Hickey et al. (2006), for changes in starter numbers during the ripening of Cheddar cheese.

Non-starter lactic acid bacteria started growing after 30 d of ripening in all cheeses and reached levels of ~10^5 cfu/g in all cheeses in Trial A, B and ~10^6 cfu/g in Trial C. After 180 d, counts on Rogosa agar showed numbers of ~10^7 cfu/g in Trial A, C and ~10^6 cfu/g in Trial C (**Figure 6.2 A, B, C**). These results are in agreement with those reported by Lane et al. (1997), Swearingen et al. (2001) and Broadbent et al. (2003). It is known that non-starter microbiota of Cheddar cheese increases from a low numbers (10^2-10^3 cfu/g) at the early stage of ripening to become the dominant viable microbiota (10^7-10^8 cfu/g) after 3-4 months (Fox et al., 1998).

Bifidobacterium BB-12 was present at levels of ~10^9 cfu/g in all cheeses in all trials at day 1. Counts on BSM agar showed that *Bifidobacterium* BB-12 decreased by 1 log unit after 60 d of ripening and remained steady at level of ~10^7 cfu/g during ripening. SLAB and NSLAB were previously proven to not be able to grow on BSM agar, therefore the colonies grown on this medium were considered to be *Bifidobacterium* BB-12. The presence of *Bifidobacterium* BB-12 was also confirmed by morphological examination of the cells (**Figure 6.3 A, B, C**). Ong et al. (2006) developed a probiotic Cheddar cheese containing *Lb. acidophilus* 4962, *Lb. casei* 279, *Bifidobacterium longum* 1941, *Lb. acidophilus* LAFTI® L10, *Lb. paracasei* LAFTI® L26, *B. lactis* LAFTI® B94 and showed that probiotic bacteria remained viable up to 10^7-10^8cfu/g during 6 months of ripening at 4˚C.

Ong and Shah (2009) also showed that the viability of those strains in Cheddar cheese was not affected by the ripening temperature of 4 and 8 ˚C. In a study conducted by McBrearty (2001), *Bifidobacterium* BB-12 was present at levels of 8.6 x 10^8 cfu/g of cheese after one day of manufacture and remained constant during ripening, reaching levels of 1.8 x 10^8 cfu/g of cheese after six months. Bifidobacteria remained viable at levels of 10^6 cfu/g in Festivo cheese (Ryhänen et al., 2001), 10^5-10^6 cfu/g in Canestrato Pugliese (Corbo et al., 2001), 10^6 cfu/g in Cheddar cheese (Daigle et al., 1999).

Numbers of *Bifidobacterium* BB-12 were higher in cheeses H2 and H4 containing Hi-Maize than control cheeses H and F in all trials (**Figure 6.3 A, B, C**). It is likely that the resistant starch acted as a protective agent against lysis or that *Bifidobacterium* BB-12 was able to use it as a nutrient and survived better during ripening. Resistent starch is considered to be a prebiotic substance since it is fermented by intestinal bacteria such as bifidobacteria to produce short chain fatty acids (SCFAs), particulary butyrate (Cho and Finocchiaro, 2010).

Wang et al. (1999a) showed that *Bifidobacterium* spp. LaftiTM 8B and LaftiTM 13B were able to grow in high amylose maize starch granule-containing medium. The same author also showed that starch-degrading enzymes were produced by *B. bifidum* and *B. pseudolongum* grown with amylopectin maize starch and a high-

amylose maize starch granules (Wang et al., 1999b). Crittenden et al. (2001) reported that many *Bifidobacterium* strains were able to hydrolyse Hi-Maize, but no amylolitic activity was shown by *Bifidobacterium* BB-12. On the other hand, Siew-Wai et al. (2010) showed that *Bifidobacterium* BB-12 was able to grow in a medium containing Hi-Maize instead of glucose and produced high levels of SCFAs. Since higher numbers of *Bifidobacterium* BB-12 were detected in cheeses H2 and H4, it is also likely that the resistant starch acted as a protective agent against low pH of cheese and enabled the bacteria to survive better during ripening. O'Riordan et al. (2001) suggested that *Bifidobacterium* PL1 and PL2 contain cell wall components with binding activity that enhance the attachment of bacteria cells to amylomaize starch granules. This ability allows bifidobacteria to be more selective in the ecosystem of the colon and offers protection against low pH and thus increases the numbers of viable cells. Bruno et al. (2002) reported that the growth of bifidobacteria in skim milk powder was not stimulated by the presence of Hi-Maize, but the starch was efficient in retaining the cell viability in the grown culture during storage at 4°C. However, Rosburg et al. (2010) showed that the addition of modified corn starch to yogurt did not affected the survival of *B. breve* and *B. longum*.

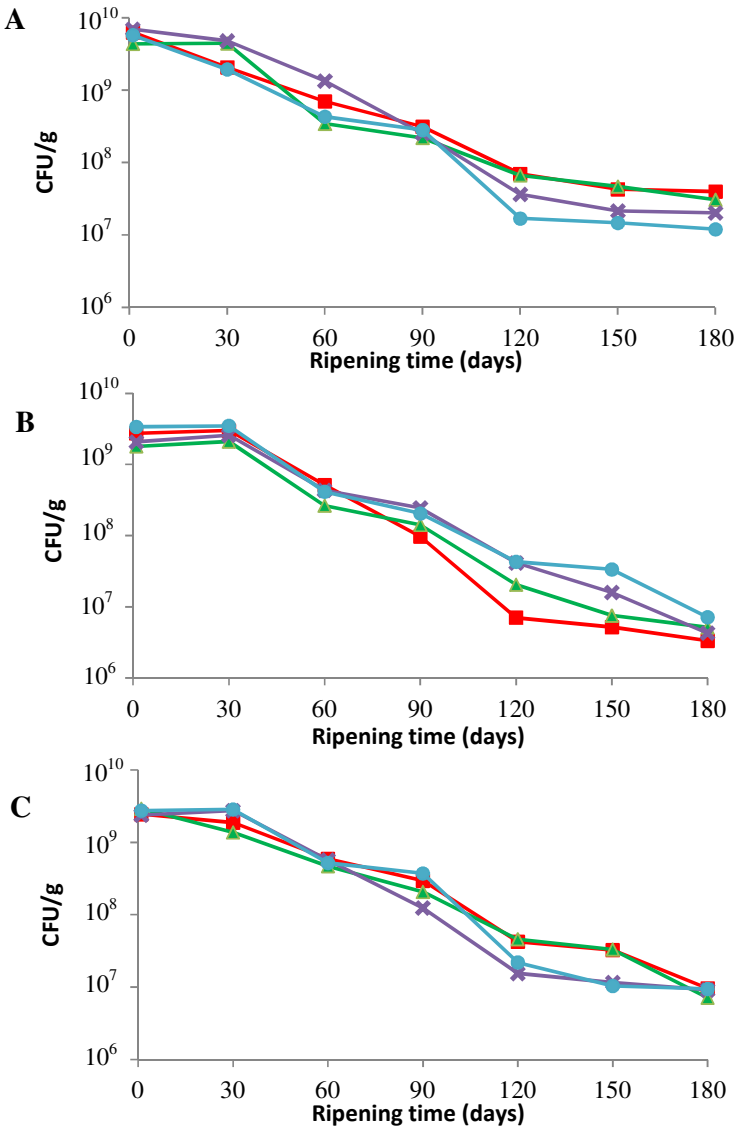

Figure 6.1. Growth of starter lactic acid bacteria (SLAB) on LM17 agar incubated 30°C for 3 days during ripening of Cheddar cheeses in Trials A, B and C. Cheese H (■), control half-fat cheese; cheese H2 (▲), half-fat cheese made with 2% resistant starch; cheese H4 (×), half-fat cheese made with 4% resistant starch; cheese F (●), control full-fat cheese.

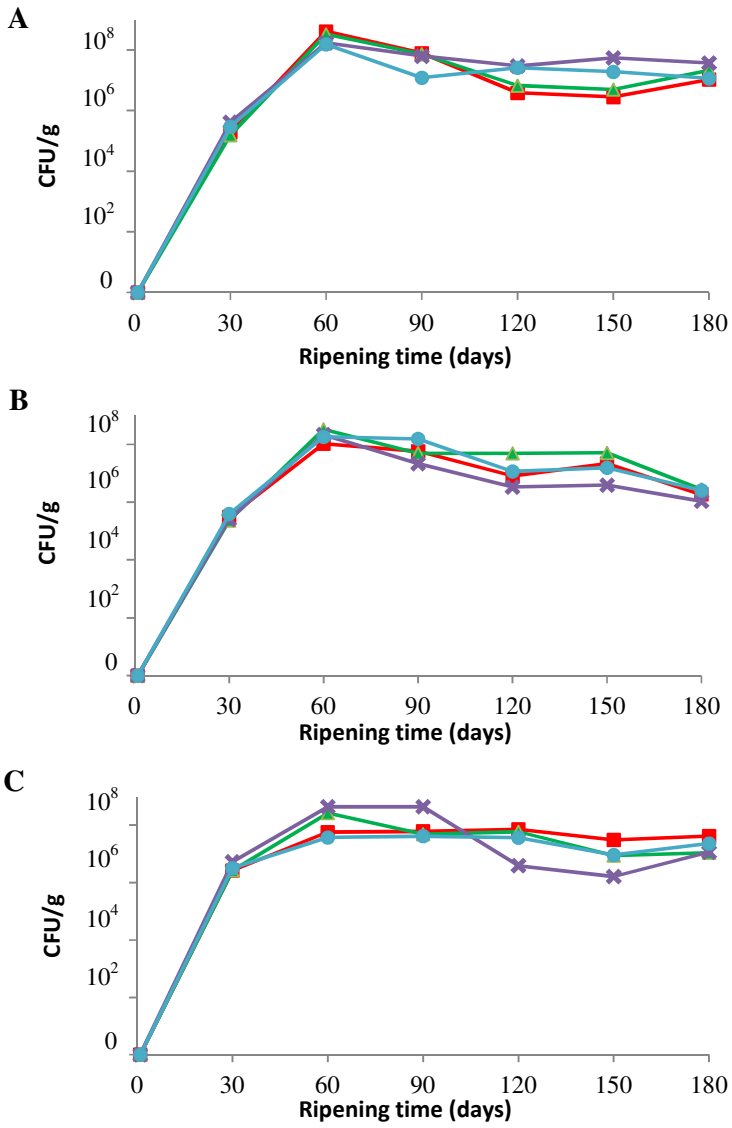

Figure 6.2. Growth of non-starter lactic acid bacteria (NSLAB) on Rogosa agar incubated at 30°C for 5 days during ripening of Cheddar cheeses in Trials A, B and C. Cheese H (■), control half-fat cheese; cheese H2 (▲), half-fat cheese made with 2% resistant starch; cheese H4 (×), half-fat cheese made with 4% resistant starch; cheese F (●), control full-fat cheese.

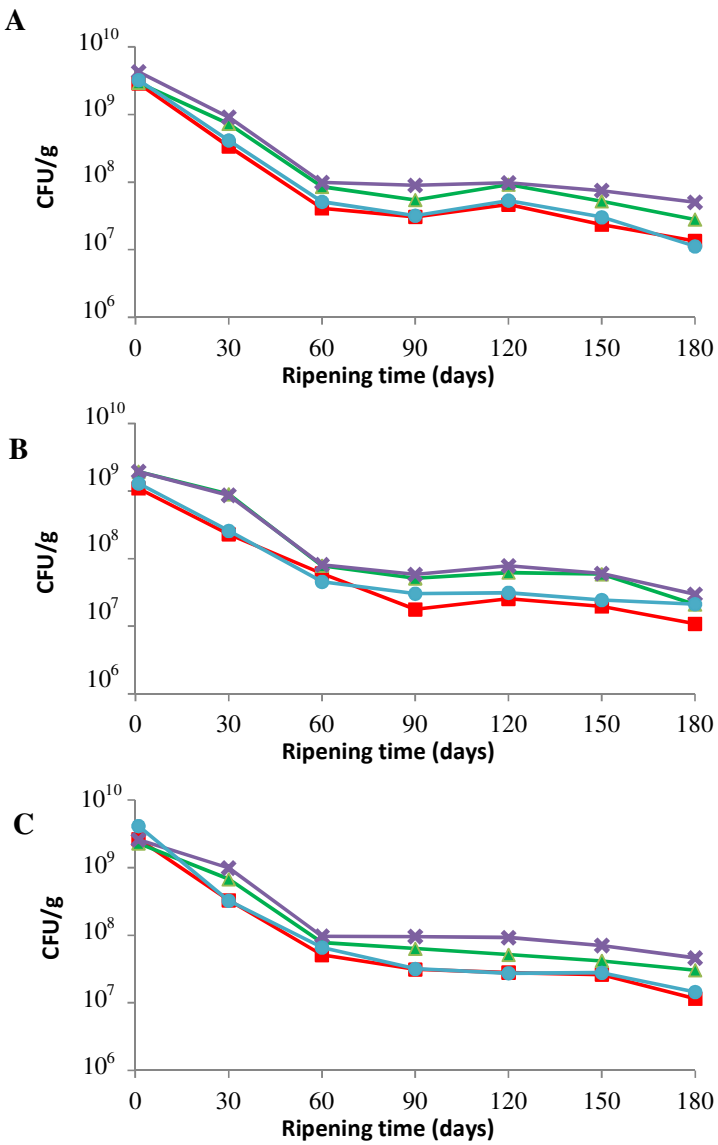

Figure 6.3. Growth of *Bifidobacterium animalis* BB-12 on BSM incubated at 37°C for 3 days during ripening of Cheddar cheeses in Trials A, B and C.
Cheese H (■), control half-fat cheese; cheese H2 (▲), half-fat cheese made with 2% resistant starch; cheese H4 (×), half-fat cheese made with 4% resistant starch; cheese F (●), control full-fat cheese.

6.4.3. Assessment of proteolysis

6.4.3.1 .Urea-PAGE

Urea-polyacrylamide gels electrophoretograms of the cheeses after 60 and 180 days of ripening are shown in **Figures 6.4** and **6.5**. The proteolysis was typical for Cheddar cheese. Both α_{s1}-casein and β-casein broke down gradually during ripening by the action of chymosin and plasmin, respectively, resulting in the accumulation of α_{s1}-CN (f24-199) and γ_1, γ_2 and γ_3-caseins. The electrophoretograms showed that the rate of α_{s1}- and β-casein degradation are similar in the experimental and control cheese. These results confirm that the proteolysis in Cheddar cheese is mainly due to the action of chymosin and plasmin (McSweeney et al., 1993; Shakeel-Ur-Rehman et al., 2000; Sousa et al., 2001; Upadhyay et al., 2004).

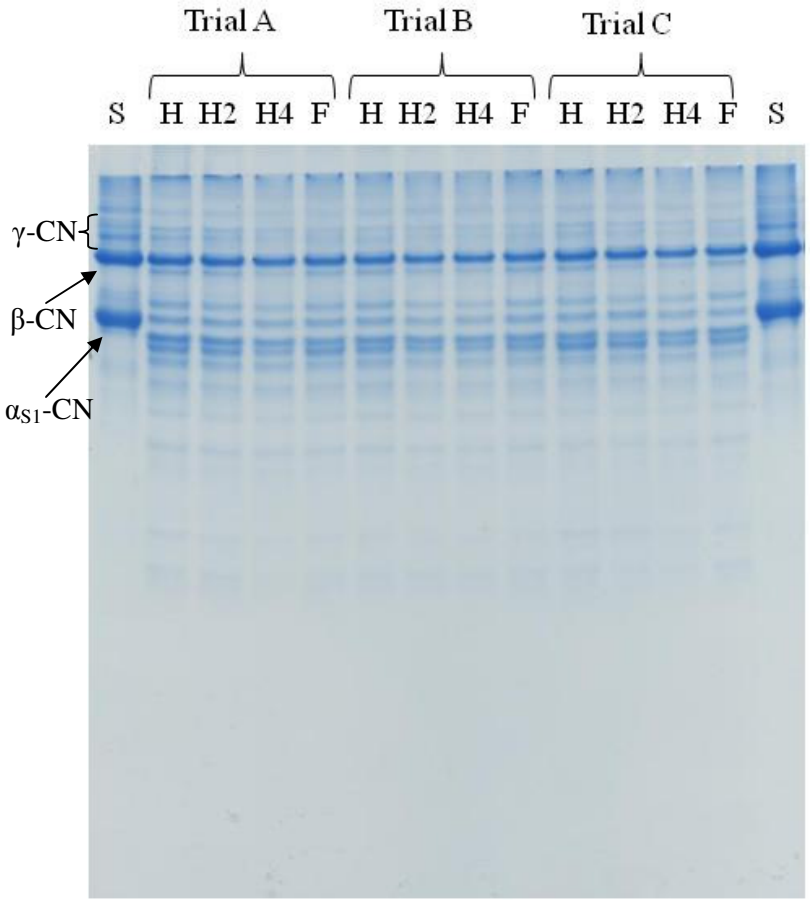

Figure 6.4. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses at 60 d of ripening in Trials A, B, C. Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese.

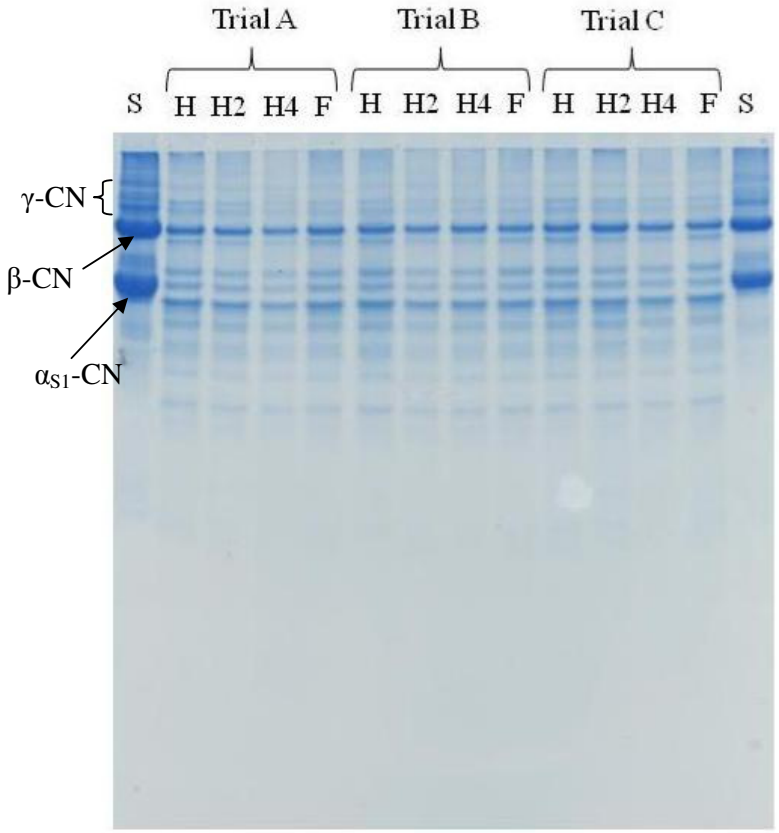

Figure 6.5. Urea-polyacrylamide gel electrophoretograms of sodium caseinate standard (S) and Cheddar cheeses at 180 d of ripening in Trials A, B, C. Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese.

6.4.3.2 . Capillary Electrophoresis

Capillary electrophoretograms at day 60 and 180 are presented in **Figure 6.6 A, B, C** and **Figure 6.7 A, B, C**. Peaks were labeled according to Rehn et al. (2011). As reported by the results from urea-PAGE, cheeses showed similar casein breakdown during ripening. Chymosin action on κ-casein resulted in the appearance of *para*-κ-casein. α_{s1}-casein in all trials disappeared before the analysis of the first time point. α_{s1}-casein f24-199 is the result of the action of chymosin on α_{s1}-casein 1-199 and during further ripening were degraded to eventually disappear completely. Similar results were also reported by other authors in studies conducted on different cheese varieties (Rehn et al., 2011; Borsting et al., 2012).

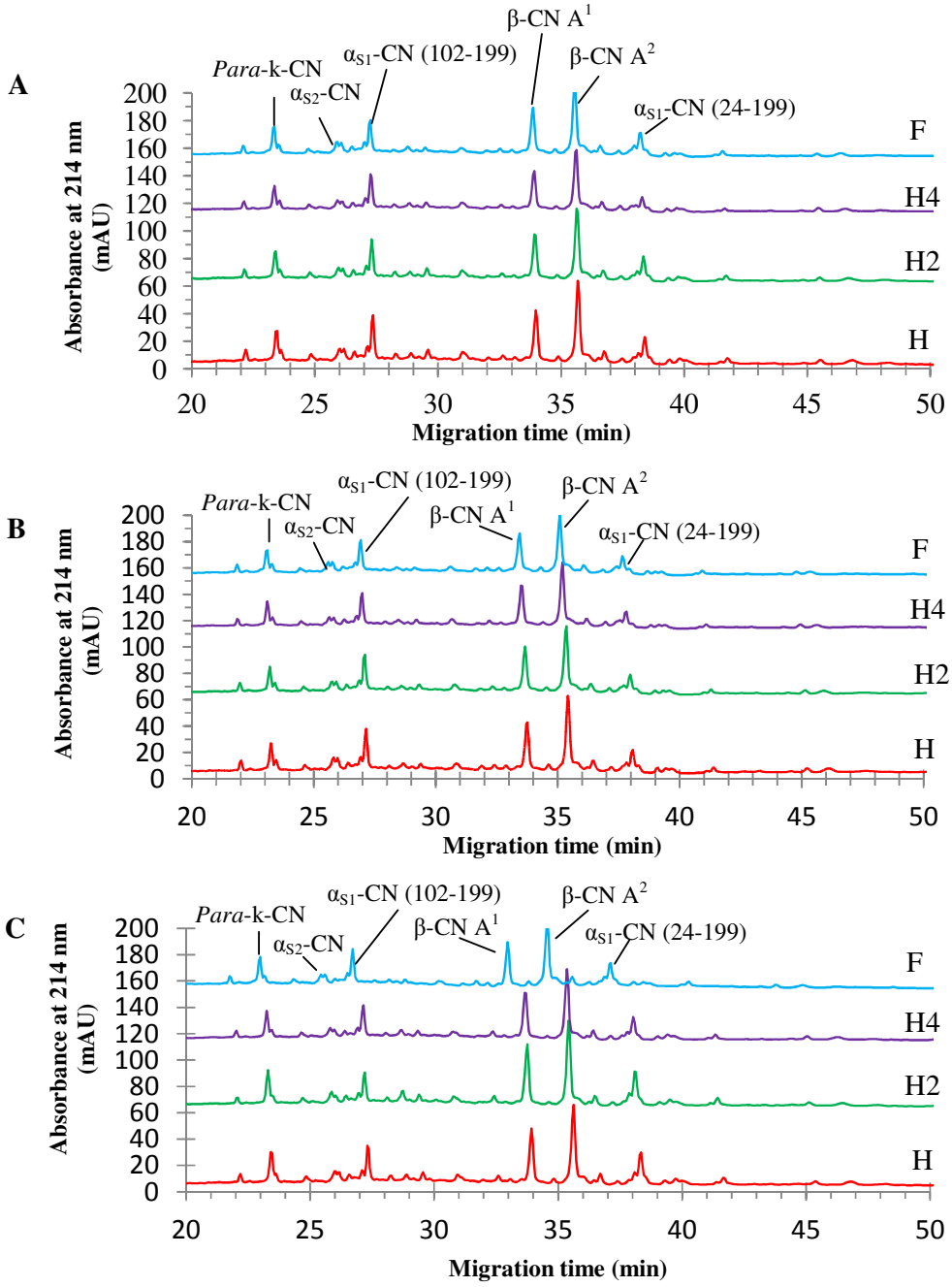

Figure 6.6. Capillary electrophoretograms of cheeses at 60 d of ripening in Trials A, B, C. Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese. β-CN A1 and A2 refers to the two genetic variants of β-casein.

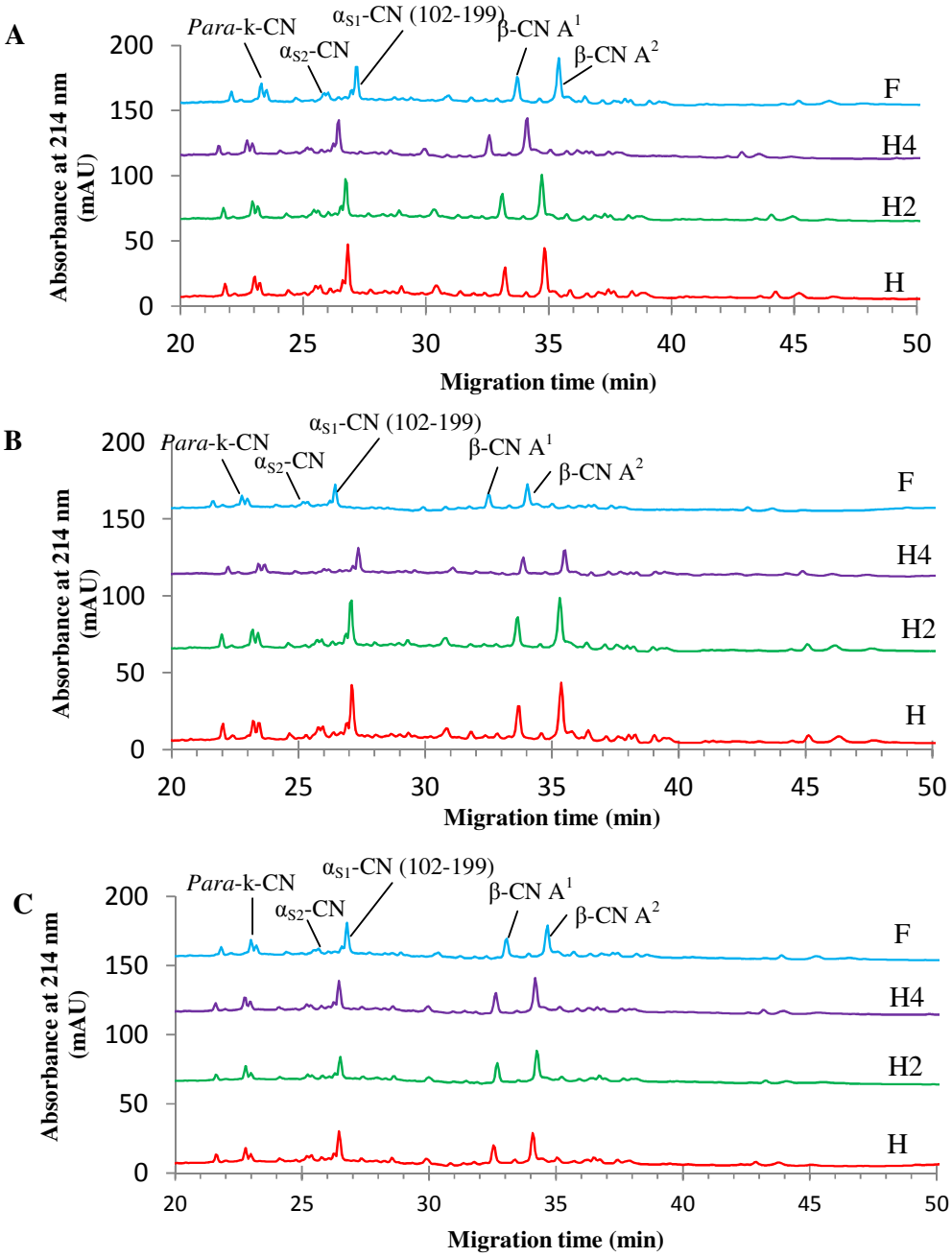

Figure 6.7. Capillary electrophoretograms of cheeses at 180 d of ripening in Trials A, B, C. Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese. β-CN A1 and A2 refers to the two genetic variants of β-casein.

6.4.3.3 . pH 4.6-soluble nitrogen

Mean levels of pH 4.6-soluble nitrogen as percentage of total nitrogen at 30 d were found to be significantly higher ($P < 0.05$) in cheeses H2, H4, F than cheese H in Trials A and B. In particular, cheese H, had the lowest value (**Table 6.2**). In agreement with the results of urea-PAGE, the addition of Hi-maize had no effect on levels of pH 4.6-soluble nitrogen since no significant differences ($P < 0.05$) were found between control cheese H and experimental cheeses H2 and H4. In Trial C, after 30 d of ripening, cheese H4 and F showed significantly higher ($P < 0.05$) levels of pH 4.6-soluble nitrogen than cheeses H and H2. After 180 d of ripening, no statistically significant differences ($P < 0.05$) were found between cheese H, H2 and H4 in Trials A and B, but they were found to be significantly higher than cheese F, probably because of the higher level of protein in low fat cheeses (**Table 6.2**).

These results are in agreement with those reported by Küçüköner and Haque (2006) who showed that the level of total soluble nitrogen in low fat Cheddar cheese was higher than the full-fat Cheddar cheese. Trial C showed a contrasting result: cheese H2 developed the lowest value, whereas cheese F showed the highest. No statistical difference ($P < 0.05$) was found between cheeses H and H4. Results from Trial C are in agreement with those reported by Fenelon et al. (2000a), Awad et al. (2005) and Guinee et al. (2000b) who showed that full-fat Cheddar had a higher level of pH 4.6-soluble nitrogen than reduced-fat Cheddar cheese.

Table 6.2. Mean levels of pH 4.6 soluble nitrogen as percentage of total nitrogen in Cheddar cheese at 60 and 180 days of ripening.
Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese.

pH 4.6-SN	Cheese	Ripening time (days)	
		30	180
Trial A	H	12.80±0.92[a]	25.23±0.30[b]
	H2	15.41±0.18[b]	25.49±0.26[b]
	H4	16.58±0.15[bc]	25.89±0.23[b]
	F	17.34±0.36[c]	22.73±0.61[a]
Trial B	H	12.68±0.19[a]	26.22±0.09[b]
	H2	17.75±1.01[b]	25.93±0.52[b]
	H4	16.98±1.65[b]	26.60±0.47[b]
	F	18.39±0.38[b]	24.70±0.25[a]
Trial C	H	16.87±0.73[a]	27.93±0.45[b]
	H2	16.17±0.32[a]	24.55±0.37[a]
	H4	20.48±1.18[b]	28.16±0.11[b]
	F	19.38±0.33[b]	30.52±0.32[c]

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.
[a] Values in the same column followed by the same letter are not significantly different ($p \leq 0.05$).

6.4.3.4 Individual free amino acids

Results of levels of individual free amino acids (IFA) in cheeses from Trials A, B and C are shown in **Table 6.3.** In Trial A, levels of Thr, Ala and GABA and Lys in control cheese H and cheese H2 were significantly higher ($P < 0.05$) than cheese H4 and control cheese F. Levels of Ser, Gly, Val, Ile, Leu and Phe in control cheese H, cheese H2 made with 2% Hi-Maize and control cheese F were significantly higher ($P < 0.05$) than cheese H4 made with 4% Hi-maize. Only experimental cheeses H2 and H4 contained significantly higher ($P < 0.05$) levels of Glu and Arg, whereas levels of Met, Tyr and His in cheeses H, H2 and H4 were significantly higher ($P < 0.05$) than control cheese F. Only control cheese H showed significantly higher ($P < 0.05$) levels of Asp than all the other cheeses. No significant differences ($P < 0.05$) were detected in levels of Pro between all cheeses.

In Trial B, control cheese H and experimental cheeses H2 and H4 had all amino acids present at significantly higher ($P < 0.05$) levels than control cheese F, except for Ser that was present in significantly higher ($P < 0.05$) levels in cheeses H, H2, F than cheese H4 and Phe that was present in significantly higher ($P < 0.05$) levels only in cheese H and H2 than the other cheeses. Also in Trial B, no significant differences ($P < 0.05$) were detected for Pro levels between all cheeses.

In Trial C, levels of Asp, Thr, Glu, Met, His and Lys were significantly higher ($P < 0.05$) in control cheese H, cheese H2 and H4 than control cheese F. Only control cheese H and experimental cheese H2 had significantly higher ($P < 0.05$) levels of Gly, Ala, Val, Ile and GABA than cheese C4 and control cheese F. Levels of Leu and Phe were significantly higher ($P < 0.05$) in control cheese H, experimental cheeses H2 and H4 than control cheese F, whereas levels of Arg were significantly higher ($P < 0.05$) in experimental cheeses H2, H4 and control cheese F than in control cheese H. As for the others trials, no significant differences ($P < 0.05$) were observed for Pro levels between all cheeses.

The presence of resistant starch in experimental cheeses H2 and H4 did not seem to affect the IFA concentrations in all trials. These results suggested that generally, the liberation of amino acids is greater in half-fat cheese than full-fat cheese. The reduction in the fat level had an effect on the concentration of protein and therefore on amino acids released following the proteolysis process during ripening. Similar results were also reported by Romeih et al. (2002) and Guinee et al. (2000) who showed that liberation of amino acids was affected by the fat content and that it was higher in low-fat cheese than full-fat cheese. Also Fenelon et al. (2000b) reported that the concentration of all individual FAA decreased numerically in the order half-fat › reduced-fat › full-fat.

Figure 6.8 (A, B) shows the score plot and loadings and for the first and second principal components of principal component analysis (PCA) for individual free amino acid data at 6 months of ripening in Trials A, B and C. Results suggest that there was relatively little trial effect between all the control cheeses H in all trials as all grouped in the upper right quadrant of the score plot, whereas a big trial effect was observed for cheeses H2 and F. Cheeses F in all trials had negative scores on PC1. All cheeses H4 grouped close to the origin and cheese H4 from trial C contained higher levels of Glu than cheese H4 in Trial A and B.

Table 6.3. Levels of individual free amino acids in Cheddar cheeses at 180 days of ripening.

Cheese H, control half-fat cheese; cheese H2, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese.

IFA mg/100g Cheese	***Trial A***			
	H	H2	H4	F
Asp	15.45±0.31^{b}	12.00±0.07^{a}	11.29±0.35^{a}	11.65±0.47^{a}
Thr	11.38±0.56^{c}	8.12±0.21^{b}	6.95±0.23^{a}	7.37±0.32^{ab}
Ser	20.95±0.39^{c}	12.95±0.45^{b}	8.67±0.20^{a}	13.08±0.09^{b}
Glu	34.28±1.26^{a}	37.10±0.42^{b}	40.63±1.17^{c}	32.82±0.87^{a}
Gly	14.00±0.26^{c}	10.74±0.14^{b}	9.76±0.15^{a}	10.74±0.06^{b}
Ala	26.10±0.47^{c}	17.38±0.35^{b}	14.78±0.18^{a}	15.56±0.31^{a}
Val	75.38±0.27^{d}	58.99±0.22^{c}	51.15±0.92^{a}	55.89±1.81^{b}
Met	23.81±0.08^{d}	17.40±0.43^{c}	16.21±0.12^{b}	14.31±0.17^{a}
Ile	10.04±0.16^{d}	7.13±0.08^{c}	5.79±0.09^{a}	6.39±0.14^{b}
Leu	220.82±0.36^{d}	176.09±2.27^{c}	156.44±0.46^{a}	166.57±1.29^{b}
Tyr	12.76±0.17^{c}	8.81±0.47^{b}	11.58±1.08^{c}	6.17±0.19^{a}
Phe	129.09±0.68^{d}	119.93±0.82^{c}	105.14±1.62^{a}	109.09±0.88^{b}
GABA	52.95±2.19^{c}	36.59±0.58^{b}	30.55±0.73^{a}	31.69±0.68^{a}
His	44.32±0.77^{d}	33.18±0.12^{c}	31.13±0.76^{b}	26.44±0.46^{a}
Lys	41.21±1.23^{c}	30.01±0.40^{b}	25.38±0.36^{a}	24.14±0.29^{a}
Arg	9.26±0.46^{a}	12.43±0.14^{b}	15.24±0.41^{c}	9.63±0.30^{a}
Pro	14.02±0.41^{a}	11.87±1.25^{a}	11.97±1.71^{a}	10.26±2.45^{a}

IFA mg/100g Cheese	***Trial B***			
	H	H2	H4	F
Asp	13.89±0.16^{bc}	15.39±1.32^{c}	13.18±0.24^{b}	10.36±0.33^{a}
Thr	9.61±0.77^{c}	8.69±0.80^{c}	6.81±0.38^{b}	5.02±0.37^{a}
Ser	16.98±0.10^{c}	8.44±0.66^{b}	6.18±0.13^{a}	7.56±0.31^{b}
Glu	22.93±0.79^{b}	43.24±1.07^{d}	38.79±0.19^{c}	20.54±0.60^{a}
Gly	11.84±0.27^{c}	13.15±0.25^{d}	11.10±0.13^{b}	9.06±0.26^{a}
Ala	22.58±0.10^{d}	16.69±0.67^{c}	14.93±0.24^{b}	13.40±0.68^{a}
Val	68.36±0.10^{d}	62.28±0.77^{c}	53.82±2.28^{b}	50.43±0.38^{a}
Met	20.72±0.27^{c}	16.43±0.48^{b}	15.960.04^{b}	12.00±0.63^{a}
Ile	7.55±0.12^{b}	8.75±0.38^{c}	6.87±0.35^{b}	4.92±0.23^{a}
Leu	214.48±0.12^{d}	198.97±5.43^{c}	174.46±1.48^{b}	162.58±1.37^{a}
Tyr	9.27±0.71^{b}	17.78±1.03^{c}	9.57±0.46^{b}	5.72±0.97^{a}
Phe	177.39±1.23^{c}	124.92±3.20^{b}	115.07±1.85^{a}	112.17±1.77^{a}
GABA	53.09±0.74^{d}	40.75±0.84^{c}	34.20±0.10^{b}	31.70±0.26^{a}
His	41.19±0.49^{d}	32.56±0.68^{c}	30.82±0.53^{b}	27.28±0.48^{a}
Lys	33.85±0.04^{d}	31.80±0.57^{c}	27.50±0.25^{b}	18.74±0.20^{a}
Arg	9.50±0.32^{b}	18.34±0.27^{d}	11.84±0.44^{c}	6.92±0.53^{a}
Pro	16.09±3.36^{a}	13.75±5.00^{a}	13.18±3.37^{a}	13.03±3.98^{a}

IFA mg/100g Cheese	***Trial C***			
	H	H2	H4	F
Asp	13.16±0.15^{b}	16.13±0.39^{d}	13.22±0.17^{b}	10.13±0.47^{a}
Thr	9.94±0.74^{c}	8.31±0.10^{b}	7.42±0.07^{b}	6.32±0.17^{a}
Ser	5.22±0.31^{a}	8.22±0.41^{c}	7.22±0.35^{b}	6.39±0.34^{b}
Glu	45.09±1.54^{b}	43.28±0.70^{b}	46.76±0.53^{c}	33.94±0.63^{a}
Gly	12.62±0.14^{b}	12.28±0.38^{b}	11.30±0.13^{a}	11.00±0.33^{a}
Ala	25.60±0.59^{c}	18.65±0.25^{b}	16.63±0.16^{a}	17.12±0.43^{a}
Val	67.54±0.44^{c}	60.62±2.02^{b}	55.44±1.21^{a}	56.27±1.34^{a}
Met	18.03±0.35^{c}	16.58±0.85^{bc}	16.37±0.85^{b}	11.88±0.18^{a}
Ile	8.14±0.64^{b}	8.16±0.43^{b}	7.09±0.47^{ab}	6.09±0.13^{a}
Leu	211.20±1.34^{c}	179.36±3.29^{b}	169.87±3.60^{a}	184.35±2.33^{b}
Tyr	6.58±0.41^{b}	4.15±0.86^{a}	10.33±0.40^{c}	6.90±0.64^{b}
Phe	156.69±0.81^{c}	117.40±0.75^{b}	109.15±2.26^{a}	117.34±0.43^{b}
GABA	40.29±0.59^{c}	35.13±0.34^{b}	31.40±0.17^{a}	31.25±0.51^{a}
His	34.33±0.55^{c}	28.46±0.50^{b}	29.63±0.51^{b}	26.11±0.41^{a}
Lys	34.03±0.43^{d}	31.23±0.50^{c}	27.41±0.26^{b}	21.75±0.23^{a}
Arg	9.07±0.11^{a}	11.27±0.27^{b}	11.60±0.62^{b}	14.62±0.40^{c}
Pro	14.85±3.77^{a}	10.36±4.87^{a}	11.01±1.72^{a}	14.37±1.89^{a}

The results are shown as an average of three replicates for each trial (A, B, C) ± standard deviation.

[a] Values in the same row followed by the same letter are not significantly different ($p \leq 0.05$)

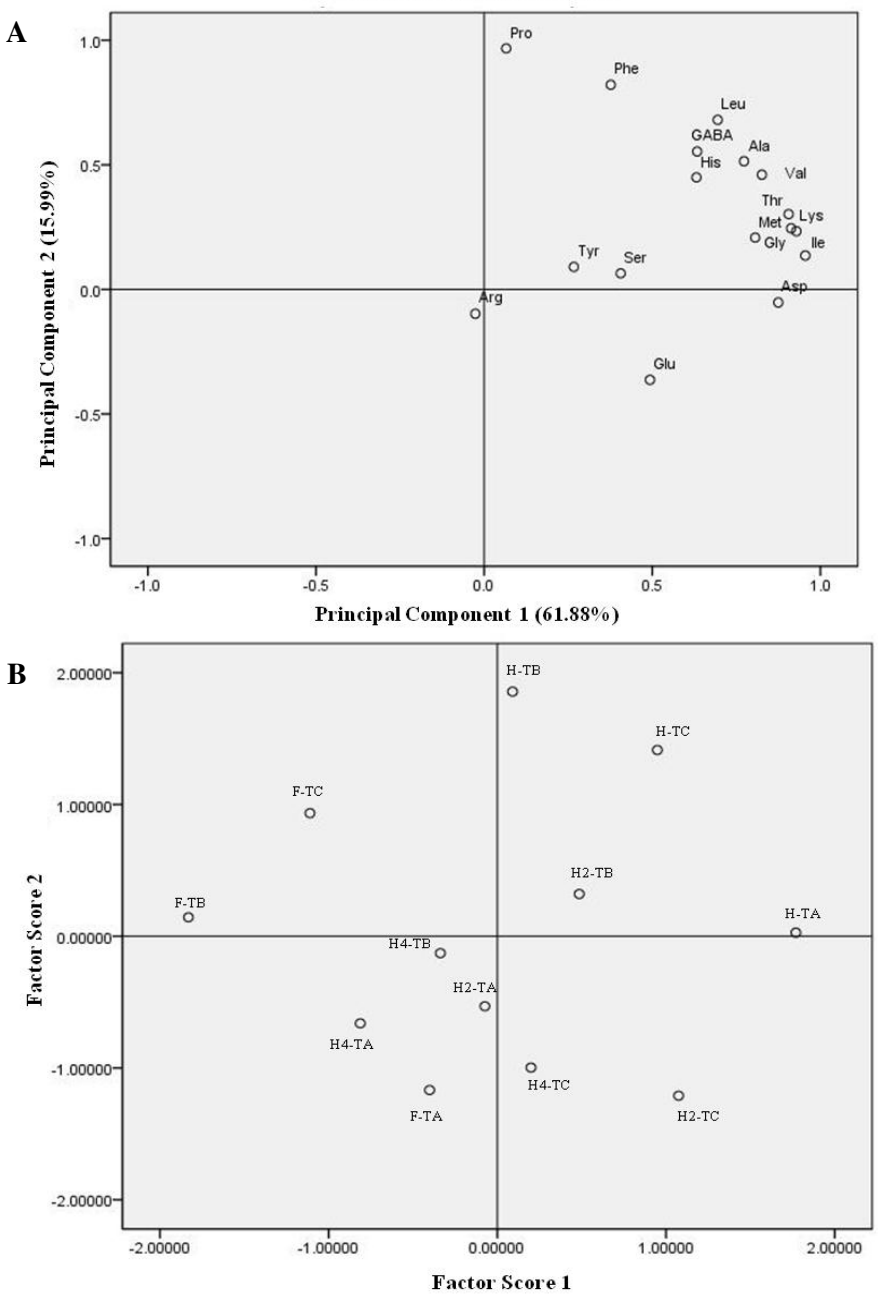

Figure 6.8. Plot of loadings (A) and scores (B) for the first and second principal components following principal component analysis (PCA) for individual free amino acids at 180 d of ripening in Trial A, B, C. Cheese H, control half-fat cheese; cheese H, half-fat cheese made with 2% resistant starch; cheese H4, half-fat cheese made with 4% resistant starch; cheese F, control full-fat cheese.

6.4.4. Texture (Uniaxial compression)

Young's modulus (*E*) is a measurement of the hardness of cheese and generally decreases during ripening as shown in **Figure 6.9**. Results confirm that Young's modulus decreased over ripening as also reported by Creamer and Olson (1982). During ripening of Cheddar cheese, proteolysis and changes in pH are responsible for textural changing since causes the network to become shorter in texture (Lucey et al., 2005; O'Mahony et al., 2005).

Generally during ageing of Cheddar cheese, the content of intact casein decreases and so does the hardness and fracture strain. It is also known that the reduction of fat increases the hardness and fracture strain of cheese because of the increase in the level of intact casein in the cheese which play a greater role in the texture development (Fenelon and Guinee, 2000; Guinee et al., 2000a; Mistry, 2001). Moreover, the softening of cheese texture during the early stages of ripening is also due to the solubilization of some of the residual colloidal calcium phosphate (CCP) associated with the *para*-casein matrix of the cheese (O'Mahony et al., 2005). In the low fat cheese, there is an inadequate breakdown of casein which result in a cheese with a firmer texture (Mistry, 2001).

Results from texture profile analysis showed that as the fat content in cheese decreased, the Young's modulus increased. The Young's modulus also increased proportionally with increasing Hi-maize. Indeed, cheese H4, containing 4% Hi-maize, was found to be the hardest one in all trials, followed by cheeses H2, H and F. As expected, the full-fat cheese F was the softest. The addition of starch into the cheeses increased the overall hardness of the products as also showed by Mounsey and O'Riordan (2001), Montesinos-Herrero et al. (2006), Noronha et al. (2007), Duggan (2008) and Gampala and Brennan (2008). According to Mounsey and O'Riordan (2001) the starch is distributed in the cheese as granules or particles. The more oval and lenticular the granules are, the harder the cheese. This is probably due to a better filler effect created by elongated or flat than spheres particles.

Hencky strain at fracture decreased over ripening (**Figure 6.10**) showing that the cheese become more brittle, as also confirmed by Creamer and Olson (1982), Fenelon and Guinee (2000) and Hort and Le Grys (2001). Hencky strain was found to be higher in half-fat cheeses than full-fat cheese and increased with increasing in fat replacer. Cheese H4 had the highest value, followed by cheeses H2, H and F.

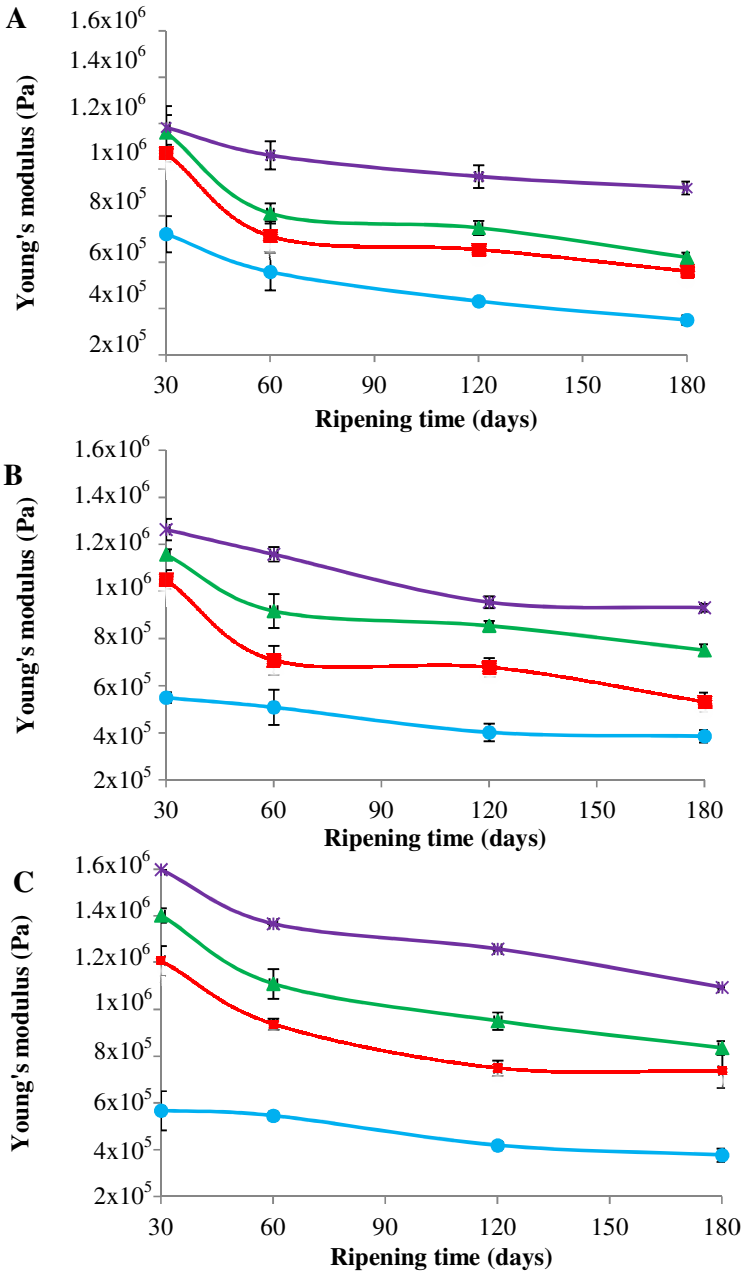

Figure 6.9. Values of Young's modulus calculated from curves of uniaxial compression for Cheddar cheeses during ripening in Trial A, B and C. Cheese H (■), control half-fat cheese; cheese H2 (▲), half-fat cheese made with 2% resistant starch; cheese H4 (×), half-fat cheese made with 4% resistant starch; cheese F (●), control full-fat cheese.

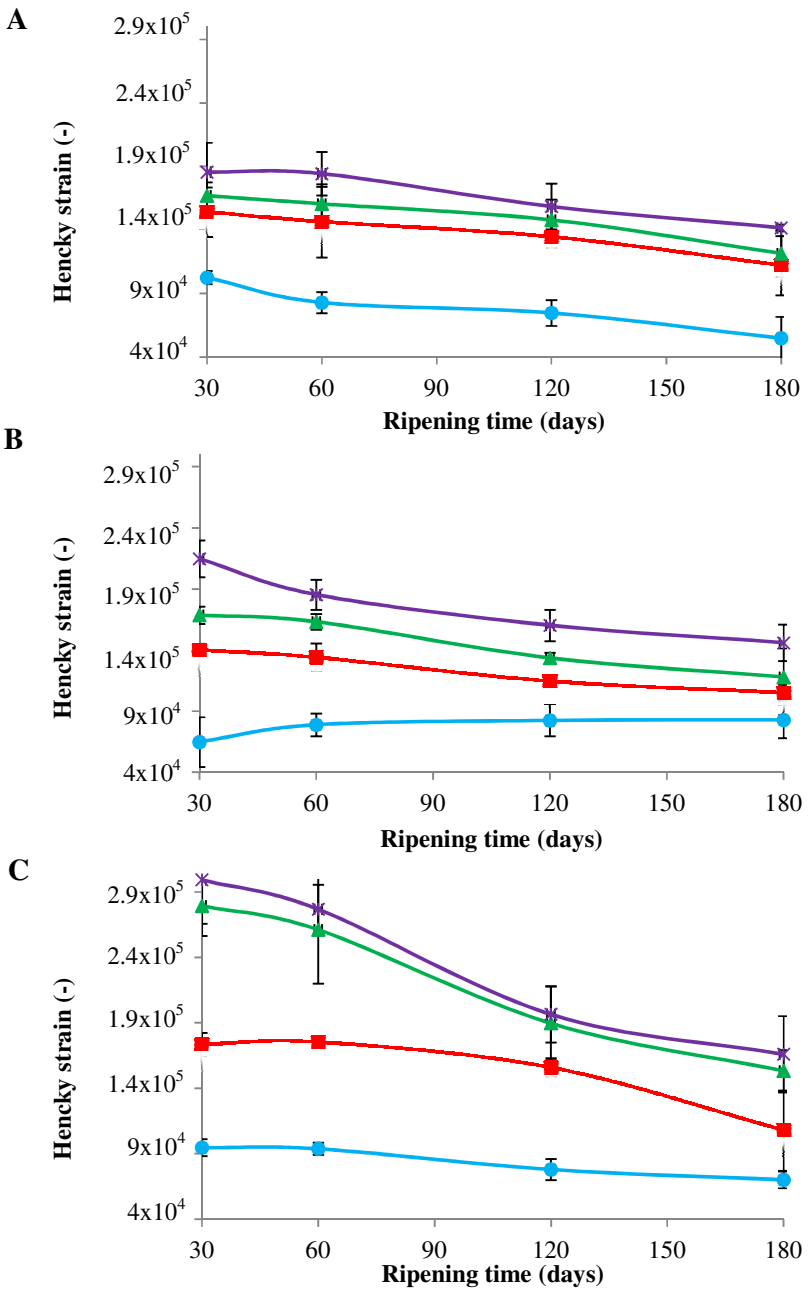

Figure 6.10. Hencky strain at fracture from uniaxial compression data for Cheddar cheeses during ripening in Trial A, B and C. Cheese H (■), control half-fat cheese; cheese H2 (▲), half-fat cheese made with 2% resistant starch; cheese H4 (×), half-fat cheese made with 4% resistant starch; cheese F (●), control full-fat cheese.

6.5. Conclusions

Bifidobacterium BB-12 was able to survive successfully during ripening of Cheddar cheese and remained viable up to ~10^7 cfu/g after 180 days of ripening. The addition of Hi-maize® 260 significantly affected the composition of half-fat cheeses: the resistant starch replaced part of the main components of the cheese matrix resulting in a decrease of moisture, protein and fat content with increasing in resistant starch. Young's modulus decreased during ripening and increased with increasing level of resistant starch. Cheese H4 containing 4% of Hi-maize® 260 was the hardest, followed by cheeses H2, H and F. The same trend was obtained for the strain at fracture. It can be concluded that the addition of Hi-maize® 260 did not improve the texture of half-fat Cheddar cheese since it made the experimental cheeses harder than the control. In spite of this, the starch seemed to act as a protective agent and/or nutrient for *Bifidobacterium* BB-12 since the cheeses H2 and H4 showed higher numbers of probiotic bacteria than the control cheese H and F made without resistant starch.

6.6. References

ADA 2005. Position of the American Dietetic Association: Fat replacers. *Journal of the American Dietetic Association*, 105, 266-275.

ANDREWS, A. T. 1983. Proteinases in normal bovine-milk and their action on caseins. *Journal of Dairy Research,* 50, 45-55.

ARDO, Y., & POLYCHRONIADOU, A. (Eds.). (1999). *Laboratory manual for chemical analysis of cheese.* Luxemburg: European Communities, office for official publications of the European Communities, 92-828-6599-1.

AWAD, S., HASSAN, A. & HALAWEISH, F. 2005. Application of exopolysaccharide-producing cultures in making reduced fat Cheddar cheese. Composition and proteolysis. *Journal of Animal Science*, 83, 269-270.

BIRKETT, A., MUIR, J., PHILLIPS, J., JONES, G. & ODEA, K. 1996. Resistant starch lowers fecal concentrations of ammonia and phenols in humans. *American Journal of Clinical Nutrition*, 63, 766-772.

BLAKESLEY, R. W. & BOEZI, J. A. 1977. New staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry*, 82, 580-582.

BORSTING, M. W., QVIST, K. B., RASMUSSEN, M., VINDELOV, J., VOGENSEN, F. K. & ARDO, Y. 2012. Impact of selected coagulants and starters on primary proteolysis and amino acid release related to bitterness and structure of reduced-fat Cheddar cheese. *Dairy Science & Technology*, 92, 593-612.

BROADBENT, J. R., HOUCK, K., JOHNSON, M. E. & 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.

BROWN, I., WARHURST, M., ARCOT, J., PLAYNE, M., ILLMAN, R. J. & TOPPING, D. L. 1997. Fecal numbers of bifidobacteria are higher in pigs fed *Bifidobacterium longum* with a high amylose cornstarch than with a low amylose cornstarch. *Journal of Nutrition*, 127, 1822-1827.

BRUNO, F. A., LANKAPUTHRA, W. E. V. & SHAH, N. P. 2002. Growth, viability and activity of *Bifidobacterium* spp. in skim milk containing prebiotics. *Journal of Food Science*, 67, 2740-2744.

CHO, S. S. & FINOCCHIARO, E. T. 2010. Natural resistant starches as prebiotics and synbiotics. *Handbook of prebiotics and probiotics ingredients: health benefits and food applications*. Edited by S. S. Cho and E. T. Finocchiaro. CRC Press, Taylor and Francis Group, Boca Raton, London, 124-138.

CORBO, M. R., ALBENZIO, M., DE ANGELIS, M., SEVI, A. & GOBBETTI, M. 2001. Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. *Journal of Dairy Science*, 84, 551-561.

CREAMER, L. K. & OLSON, N. F. 1982. Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science*, 47, 631-&.

CRITTENDEN, R. G., MORRIS, L. F., HARVEY, M. L., TRAN, L. T., MITCHELL, H. L. & PLAYNE, M. J. 2001. Selection of a *Bifidobacterium* strain to complement resistant starch in a synbiotic yoghurt. *Journal of Applied Microbiology*, 90, 268-278.

CRUZ, A. G., CADENA, R. S., WALTER, E. H. M., MORTAZAVIAN, A. M., GRANATO, D., FARIA, J. A. F. & BOLINI, H. M. A. 2010. Sensory analysis: relevance for prebiotic, probiotic, and synbiotic product development. *Comprehensive Reviews in Food Science and Food Safety*, 9, 358-373.

DA CRUZ, A. G., ALONSO BURITI, F. C., BATISTA DE SOUZA, C. H., FONSECA FARIA, J. A. & ISAY SAAD, S. M. 2009. Probiotic cheese: health benefits, technological and stability aspects. *Trends in Food Science & Technology*, 20, 344-354.

DAIGLE, A., ROY, D., BELANGER, G. & VUILLEMARD, J. C. 1999. Production of probiotic cheese (Cheddar-like cheese) using enriched cream fermented by *Bifidobacterium infantis*. *Journal of Dairy Science*, 82, 1081-1091.

DRAKE, M. A. & SWANSON, B. G. 1995. Reduced-fat and low-fat cheese technology : a review. *Trends in Food Science & Technology*, 6, 366-369.

DUGGAN, E., NORONHA, N., O'RIORDAN, E. D. & O'SULLIVAN, M. 2008. Effect of resistant starch on the water binding properties of imitation cheese. *Journal of Food Engineering*, 84, 108-115.

ENGLYST, H. N., KINGMAN, S. M., HUDSON, G. J. & CUMMINGS, J. H. 1996. Measurement of resistant starch *in vitro* and *in vivo*. *British Journal of Nutrition*, 75, 749-755.

FENELON, M. A. & GUINEE, T. P. 2000. Primary proteolysis and textural changes during ripening in Cheddar cheeses manufactured to different fat contents. *International Dairy Journal*, 10, 151-158.

FENELON, M. A., GUINEE, T. P., DELAHUNTY, C., MURRAY, J. & CROWE, F. 2000a. Composition and sensory attributes of retail Cheddar cheese with different fat contents. *Journal of Food Composition and Analysis*, 13, 13-26.

FENELON, M. A., O'CONNOR, P. & GUINEE, T. P. 2000b. The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. *Journal of Dairy Science*, 83, 2173-2183.

FOX, P. F. 1963. Potentiometric determination of salt in cheese. *Journal of Dairy Science*, 46, 744-745.

FOX, P. F., McSWEENEY, P. L. H. & LYNCH, C. M. 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. *Australian Journal of Dairy Technology*, 53, 83-89.

GAMPALA, P. & BRENNAN, C. S. 2008. Potential starch utilisation in a model processed cheese system. *Starch-Starke*, 60, 685-689.

GOBBETTI, M., CORSETTI, A., SMACCHI, E., ZOCCHETTI, A. & DE ANGELIS, M. 1998. Production of Crescenza cheese by incorporation of bifidobacteria. *Journal of Dairy Science*, 81, 37-47.

GODWARD, G. & KAILASAPATHY, K. 2003. Viability and survival of free and encapsulated probiotic bacteria in Cheddar cheese. *Milchwissenschaft-Milk Science International*, 58, 624-627.

GOMES, A. M. P. & MALCATA, F. X. 1999. *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends in Food Science & Technology*, 10, 139-157.

GRAPPIN, R. 1986. Milk determination of nitrogen content Kjeldahl method and calculation of crude protein content. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 20A, 1-3.

GUINEE, T. P., AUTY, M. A. E. & FENELON, M. A. 2000a. The effect of fat content on the rheology, microstructure and heat-induced functional characteristics of Cheddar cheese. *International Dairy Journal*, 10, 277-288.

GUINEE, T. P., AUTY, M. A. E., MULLINS, C., CORCORAN, M. O. & MULHOLLAND, E. O. 2000b. Preliminary observations on effects of fat content and degree of fat emulsification on the structure-functional relationship of Cheddar-type cheese. *Journal of Texture Studies*, 31, 645-663.

HICKEY, D. K., KILCAWLEY, K. N., BERESFORD, T. P. & WILKINSON, M. G. 2006. Starter bacteria are the prime agents of lipolysis in Cheddar cheese. *Journal of Agricultural and Food Chemistry*, 54, 8229-8235.

HORT, J. & LE GRYS, G. 2001. Developments in the textural and rheological properties of UK Cheddar cheese during ripening. *International Dairy Journal*, 11, 475-481.

IDF 1983. Milk, cream and evaporated milk - determination of total solids content (reference method)-provisional international IDF standard 21A-1982.

IDF 1986. Cheese and processed cheese products determination of fat content gravimetric method reference method. *FIL-IDF (Federation Internationale de Laiterie International Dairy Federation) Standards*, 1-5.

KUCUKONER, E. & HAQUE, Z. U. 2006. Physicochemical properties of low-fat and full-fat Cheddar cheeses. *International Journal of Dairy Technology*, 59, 166-170.

LANE, C. N., FOX, P. F., WALSH, E. M., FOLKERTSMA, B. & McSWEENEY, P. L. H. 1997. Effect of compositional and environmental factors on the growth of indigenous non-starter lactic acid bacteria in Cheddar cheese. *Lait*, 77, 561-573.

LUCEY, J. A., MISHRA, R., HASSAN, A. & JOHNSON, M. E. 2005. Rheological and calcium equilibrium changes during the ripening of Cheddar cheese. *International Dairy Journal*, 15, 645-653.

McBREARTY, S., ROSS, R. P., FITZGERALD, G. F., COLLINS, J. K., WALLACE, J. M. & STANTON, C. 2001. Influence of two commercially available bifidobacteria cultures on Cheddar cheese quality. *International Dairy Journal*, 11, 599-610.

McSWEENEY, P. L. H., FOX, P. F., LUCEY, J. A., JORDAN, K. N. & COGAN, T. M. 1993. Contribution of the indigenous microflora to the maturation of Cheddar cheese. *International Dairy Journal*, 3, 613-634.

MISTRY, V. V. 2001. Low fat cheese technology. *International Dairy Journal*, 11, 413-422.

MONTESINOS-HERRERO, C., COTTELL, D. C., O'RIORDANA, E. D. & O'SULLIVAN, M. 2006. Partial replacement of fat by functional fibre in imitation cheese: Effects on rheology and microstructure. *International Dairy Journal*, 16, 910-919.

MOUNSEY, J. S. & O'RIORDAN, E. D. 2001. Characteristics of imitation cheese containing native starches. *Journal of Food Science*, 66, 586-591.

NORONHA, N., O'RIORDAN, E. D. & O'SULLIVAN, M. 2007. Replacement of fat with functional fibre in imitation cheese. *International Dairy Journal*, 17, 1073-1082.

O'MAHONY, J. A., LUCEY, J. A. & McSWEENEY, P. L. H. 2005. Chymosin-mediated proteolysis, calciums, and texture development during the ripening of Cheddar cheese. *Journal of Dairy Science*, 88, 3101-3114.

OBANDO, M., BRITO, C. S., SCHOBITZ, R. P., BAEZ, L. A. & HORZELLA, M. Y. 2010. Viability of the probiotic microorganisms *Lactobacillus casei* 01, *Lactobacillus acidophilus* La-5. *Bifidobacterium* BB12 during Cottage cheese shelf life. *Revista De La Facultad De Quimica Farmaceutica*, 17, 141-148.

ONG, L. & SHAH, N. P. 2009. Probiotic Cheddar cheese: Influence of ripening temperatures on survival of probiotic microorganisms, cheese composition and organic acid profiles. *Lebensmittel-Wissenschaft & Technologie*, 42, 1260-1268.

ONG, L., HENRIKSSON, A. & SHAH, N. P. 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal*, 16, 446-456.

O'RIORDAN, K., MULJADI, N. & CONWAY, P. 2001. Characterization of factors affecting attachment of *Bifidobacterium* species to amylomaize starch granules. *Journal of Applied Microbiology*, 90, 749-754.

PHILLIPS, J., MUIR, J. G., BIRKETT, A., LU, Z. X., JONES, G. P., ODEA, K. & YOUNG, G. P. 1995. Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *American Journal of Clinical Nutrition*, 62, 121-130.

REHN, U., VOGENSEN, F. K., PERSSON, S. E., SAEDEN, K. H., NILSSON, B. F. & ARDO, Y. 2011. Influence of microflora on texture and contents of amino acids, organic acids, and volatiles in semi-hard cheese made with DL-starter and propionibacteria. *Journal of Dairy Science*, 94, 1098-1111.

RODRIGUES, D., ROCHA-SANTOS, T. A. P., GOMES, A. M., GOODFELLOW, B. J. & FREITAS, A. C. 2012. Lipolysis in probiotic and synbiotic cheese: The influence of probiotic bacteria, prebiotic compounds and ripening time on free fatty acid profiles. *Food Chemistry*, 131, 1414-1421.

ROMEIH, E. A., MICHAELIDOU, A., BILIADERIS, C. G. & ZERFIRIDIS, G. K. 2002. Low-fat white-brined cheese made from bovine milk and two commercial fat mimetics: chemical, physical and sensory attributes. *International Dairy Journal*, 12, 525-540.

ROSBURG, V., BOYLSTON, T. & WHITE, P. 2010. Viability of bifidobacteria strains in yogurt with added oat beta-glucan and corn starch during cold storage. *Journal of Food Science*, 75, C439-C444.

RYHÄNEN, E.-L., PIHLANTO-LEPPÄLÄ, A. & PAHKALA, E. 2001. A new type of ripened, low-fat cheese with bioactive properties. *International Dairy Journal*, 11, 441-447.

SCHELLER, M. & O'SULLIVAN, D. J. 2011. Comparative analysis of an intestinal strain of *Bifidobacterium longum* and a strain of *Bifidobacterium animalis* subspecies lactis in Cheddar cheese. *Journal of Dairy Science*, 94, 1122-1131.

SHAKEEL-UR-REHMAN, FOX, P. F. & McSWEENEY, P. L. H. 2000. Methods used to study non-starter microorganisms in cheese: a review. *International Journal of Dairy Technology*, 53, 113-119.

SHAKEEL-UR-REHMAN, McSWEENEY, P. L. H., BANKS, J. M., BRECHANY, E. Y., MUIR, D. D. & FOX, P. F. 2000. Ripening of Cheddar cheese made from blends of raw and pasteurised milk. *International Dairy Journal*, 10, 33-44.

SIEW-WAI, L., ZI-NI, T., KARIM, A. A., HANI, N. M. & ROSMA, A. 2010. Fermentation of Metroxylon sagu Resistant Starch Type III by *Lactobacillus* sp. and *Bifidobacterium bifidum*. *Journal of Agricultural and Food Chemistry*, 58, 2274-2278.

SIPAHIOGLU, O., ALVAREZ, V. B. & SOLANOL-LOPEZ, C. 1999. Structure, physico-chemical and sensory properties of Feta cheese made with tapioca starch and lecithin as fat mimetics. *International Dairy Journal*, 9, 783-789.

SOUSA, M. J., ARDÖ, Y. & McSWEENEY, P. L. H. 2001. Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, 11, 327-345.

STANTON, C., GARDINER, G., LYNCH, P. B., COLLINS, J. K., FITZGERALD, G. & ROSS, R. P. 1998. Probiotic cheese. *International Dairy Journal*, 8, 491-496.

STEFFE, J. F. 1996. *Rheological methods in food process engineering*, Second edition. Freeman Press, East Lansing, MI, USA.

SWEARINGEN, P. A., O'SULLIVAN, D. J. & WARTHESEN, J. J. 2001. Isolation, characterization, and influence of native, nonstarter lactic acid bacteria on Cheddar cheese quality. *Journal of Dairy Science*, 84, 50-59.

UPADHYAY, V. K., SOUSA, M. J., RAVN, P., ISRAELSEN, H., KELLY, A. L. & McSWEENEY, P. L. H. 2004. Use of exogenous streptokinase to accelerate proteolysis in Cheddar cheese during ripening. *Lait*, 84, 527-538.

VAN DE CASTEELE, S., RUYSSEN, T., VANHEUVERZWIJN, T. & VAN ASSCHE, P. 2003. Production of Gouda cheese and Camembert with probiotic cultures: The suitability of some commercial probiotic cultures to be implemented in cheese. *Communications in Agricultural and Applied Biological Sciences*, 68, 539-542.

VAN LOO, J. 2006. Inulin-type fructans as prebiotics. *Prebiotics: Development & Application*, edited by G. R. Gibson and R. A. Rastall, M. B., John Wiley & Sons Ltd, England, 57-100.

VENTURA, M., CANCHAYA, C., TAUCH, A., CHANDRA, G., FITZGERALD, G. F., CHATER, K. F. & VAN SINDEREN, D. 2007. Genomics of Actinobacteria: Tracing the evolutionary history of an ancient phylum. *Microbiology and Molecular Biology Reviews*, 71, 495-548.

VOLIKAKIS, P., BILIADERIS, C. G., VAMVAKAS, C. & ZERFIRIDIS, G. K. 2004. Effects of a commercial oat beta-glucan concentrate on the chemical, physico-chemical and sensory attributes of a low-fat white-brined cheese product. *Food Research International*, 37, 83-94.

WANG, X., BROWN, I. L., EVANS, A. J. & CONWAY, P. L. 1999a. The protective effects of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. *Journal of Applied Microbiology*, 87, 631-639.

WANG, X., CONWAY, P. L., BROWN, I. L. & EVANS, A. J. 1999b. *In vitro* utilization of amylopectin and high-amylose maize (amylomaize) starch granules by human colonic bacteria. *Applied and Environmental Microbiology*, 65, 4848-4854.

YILMAZTEKIN, M., OZER, B. H. & ATASOY, F. 2004. Survival of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB-02 in white-brined cheese. International *Journal of Food Sciences and Nutrition*, 55, 53-60.

ZALAZAR, C. A., ZALAZAR, C. S., BERNAL, S., BERTOLA, N., BEVILACQUA, A. & ZARITZKY, N. 2002. Effect of moisture level and fat replacer on physicochemical, rheological and sensory properties of low fat soft cheeses. *International Dairy Journal*, 12, 45-50.

7. Aminopeptidase, aminotransferase and esterase activities of *Lactobacillus danicus*

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7.1. Abstract

In this study, a cheese-based medium (CBM) was developed with the purpose of mimicking the cheese environment early in ripening stage. The aminotransferase, aminopeptidase and esterase activities in cell free extracts (CFEs) of *Lactobacillus danicus* 9M3 and 13M1 were investigated after growth to early stationary phase in CMB or in MRS broth. The strains showed aminopeptidase activity only against Gly-, Arg-, Pro- and Phe-*p*-nitroanalide in CBM, whereas, when grown in MRS they were active against all the substrates tested. Both *Lb. danicus* strains grown in MRS and in CBM had the highest aminotransferase activity towards aromatic amino acids (Phe and Trp). Also branched-chain amino acids (Leu and Val) were degraded by the two strains grown in both MRS and CBM, but at much lower rates. Generally, the strains grown in CBM showed an activity 10-fold lower than when grown in MRS. However, aminotransferase activity was broader in the strains grown in CBM. Esterase activity was expressed against *p*-nitrophenyl-acetate (C2), *p*-nitrophenyl-butyrate (C4) and *p*-nitrophenyl-palmitate (C16) and was significantly higher in CBM than in MRS. The results of this study showed that *Lb. danicus* had good aminotransferase, aminopeptidase and esterase activities.

7.2. Introduction

Starter lactic acid bacteria (SLAB) are added to cheesemilk in order to produce acid during manufacture and to contribute to ripening process. SLAB are usually members of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* (Beresford et al., 2001). Non-starter lactic acid bacteria (NSLAB) belong to a secondary microbiota which is not involved in acid production since many strains do not grow well in milk. NSLAB can grow internally or externally the cheese and usually are facultatively heterofermentative (mesophilic) lactobacilli (Beresford et al., 2001). Starter lactococci and mesophilic lactobacilli are involved in the development of flavour or off-flavours in Cheddar cheese during ripening (Williams et al., 2001).

NSLAB in Cheddar cheese are present at levels of 10^3-10^4 cfu/g after the first 10 d of ripening and then grow up to ~10^8 cfu/g within few weeks (Peterson and Marshall, 1990; Fox et al., 1998). NSLAB are able to grow in cheese during ripening even though the main carbohydrates from milk (lactose, glucose and galactose) have been used up by starter bacteria (Beresford et al., 2001; Adamberg et al., 2003). It is still not clear which energy sources NSLAB use to grow in cheese; potential sources include amino acids, organic and fatty acids, glycerol or carbohydrates released from the glycomacropeptide of κ-casein (galactose, *N*-acetylgalactosamine, *N*-acetylneuraminic acid), from glycoproteins and glycolipids in the milk-fat globule membrane (galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid) or from lysed cells of starter bacteria (ribose, deoxyribose, *N*-acetylglucosamine, *N*-acetylmuramic acid) (Adamberg et al., 2003).

Some studies have also reported that *Lb. casei*, *Lb. plantarum*, and *Lb. brevis* were capable of using citrate as a source of energy in the absence of carbohydrate (Campbell and Gunsalus, 1944; Fryer, 1970). Similar results were also reported by Prashant et al. (2009) and Nanda et al. (2011) in a study conducted on lactobacilli

isolated from Churpi cheese made from yak milk and from camel cheese, respectively.

Adjunct cultures are used for the manufacture of cheese in order to develop and control the flavor, color, and texture of the cheese (Rattray and Eppert, 2011) and usually consist of carefully selected strains of NSLAB, *Brevibacterium linens*, *Propionibacterium* spp., yeasts or molds. Traditional adjunct cultures play an important role for the development of the typical flavor of cheeses such as Roquefort, Emmental, Camembert, and Limburger (El Soda et al., 2000). In some studies, organisms such as *Lactobacillus casei*, *Lactobacillus paracasei, Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus curvatus, Lactobacillus rhamnosus* or *Bifidobacterium* sp. have been added to cheese in order to evaluate their impact on ripening and/or aroma development (Puchades et al., 1989; Drake et al., 1996; Lane and Fox, 1996; Thage et al., 2005; Phillips et al., 2006; Ong et al., 2007; Milesi et al., 2008).

Thage et al. (2005) showed that some strains of *Lb. paracasei* enhanced the formation of volatile flavour compounds from branched amino acids and Asp in semi-hard reduced-fat cheese. Drake et al. (1996) reported that proteolysis and consumer acceptance scores were higher in Cheddar cheese made with *Lb. helveticus* as adjunct than the control cheese, while Milesi et al. (2008) showed that *Lb. plantarum* I91 accelerated the ripening of Cheddar cheese by modifying its peptide profile and increasing the level of some individual amino acids as well the level of total free amino acids.

Many studies showed that certain lactobacilli exhibit good proteinase, peptidase and esterase activities. Williams et al. (2001) showed that some LAB isolated from 9 mature Cheddar cheeses were able to catabolise protein hydrolysates and individual amino acids; all the isolates had aminotransferase activity against Leu, Phe, Met and amongst the isolates, five strains of *Lactobacillus* and two *Lc. lactis* also showed deaminase activity. Oliszewski et al. (2007) showed that all the indigenous lactic acid bacteria isolated from goats' milk and cheese had esterase activity on α-naphthyl derivatives of 2–6 carbon fatty acids. In a study conducted by Bintsis et al. (2003), four strains of *Lb. paracasei* subsp. *paracasei* had proteolytic activity against casein; dipeptidase, aminopeptidase, dipeptidyl aminopeptidase, endopeptidase, carboxypeptidase and esterase activities were also observed against a number of substrates.

Jensen and Ardo (2010) reported that six strains of *Lb. helveticus*, grown in skim milk or MRS broth, had peptidase activity against all 10 substrates tested, in particular, the highest activity was recorded against the substrates Arg- and Lys-*p*-nitroanaline (*p*NA); in most cases, the activities were higher after growth in skim milk than after growth in MRS. In the same study, the authors also showed that all six strains had aminotransferase activity against the aromatic amino acids Phe, Tyr and Trp, as well as the branched-chain amino acids Leu, Ile and Val. Similar results were obtained by Thage et al. (2004) who showed that strains of *Lb. helveticus* had high specificity for aromatic amino acids, while strains of *Lb. paracasei* subsp. *paracasei* degraded branched-chain amino acids and Asp; strains of *Lb. danicus* had

up to about 20 times higher aminotransferase activity against Leu, Asp and aromatic amino acids than *Lb. paracasei* and *Lb. helveticus*.

Lb. danicus is a newly discovered *Lactobacillus* species isolated from Danish and Estonian cheese and from several traditional Danish and dairy starter cultures (Kask et al., 2003). Some strains of *Lb. danicus* do not grow well in milk, have an optimal growth temperature at 30°C, do not tolerate NaCl concentrations higher than 2%, and are able to ferment ribose, D-xylose, galactose, D-glucose, *N*-acetyl-glucosamine, maltose and lactose (Kask et al., 2003). Adamberg et al. (2003) showed that ribose and *N*-acetyl-glucosamine were the preferred substrates for *Lb. danicus* since they were fermented faster than glucose.

In order to identify potential pathways for citrate catabolism by *Lactobacillus casei* ATCC334 under conditions similar to ripening cheese, Diaz-Muniz et al. (2006) studied the growth of the strain in a modified chemically defined media (mCDM) containing a source of citrate, limiting and excess of carbohydrate and in a Cheddar cheese extract (CCE). The results showed that under conditions similar to those present in ripening cheese, citric acid was converted to acetic acid, L/D-lactic acid, acetoin, diacetyl, ethanol, and formic acid. In a similar study conducted by Diaz-Muniz and Steele (2006), the authors investigated the conditions required for citrate utilization during growth of *Lactobacillus casei* ATCC334 in a chemically defined medium (mCDM) and Cheddar cheese extract (CCE). The results obtained with mCDM suggested that *Lb. casei* ATCC334 used citrate as an energy source only in the presence of limiting galactose. Citrate utilization of *Lactobacillus casei* ATCC334 in CCE was inhibited by an excess of galactose and began when the cultures reached late-logarithmic phase and more than 70% of the initial lactose concentration was depleted. Cheese extracts represent a complex environment and it is a more accurate model for studying the growth of microorganisms under conditions similar to those present during ripening of cheese than conventional cultivation in broth.

In this study, a cheese-based medium (CBM) was developed in order to explore the potential role of aminopeptidase, aminotransferase and esterase activities of two strains of *Lactobacillus danicus* in cheese flavour formation. The activity was investigated after growth in both MRS and a newly developed CBM, which simulated the substrates available in a young cheese.

7.3. Materials and methods

7.3.1. Experimental design

Two *Lactobacillus danicus* strains were grown in duplicate to early stationary phase in MRS broth (Merck, Darmstadt, Germany) and a newly developed cheese-based medium (CBM). Cells were harvested and disrupted and cell free extracts (CFEs) were prepared and analyzed in duplicate for aminopeptidase, aminotransferase and esterase activities. Specific activities of the aminopeptidases were investigated by using ten selected chromogenic substrates. Aminotransferase activity was determined against 20 amino acids. Esterase activity was tested against four nitrophenyl ester substrates.

7.3.2. Bacterial strains

Lb. danicus 9M3 and 13M1 where obtained from the culture collection of Department of Food Science, University of Copenhagen and were originally isolated from a Danish semi-hard cheeses of different varieties (Adamberg et al., 2005; Christiansen et al., 2005).

7.3.3. Preparation of cheese-based medium (CBM)

A cheese-based medium (CBM) was developed with the purpose of mimicking the cheese environment at an early ripening stage, at which the heterofermentative organisms from the starter are likely to be active. CBM was obtained from a young Danbo cheese; the cheese was produced at a Danish dairy plant, using a standard cheese recipe and made with a traditional DL-starter. After the final cooling step the cheese was vacuum-packed and ripened for two weeks at 16 °C. The unsalted cheese was cut in to small cubes (1 x 1 cm) and stored at -45 °C until lyophilisation. The freeze-dried cheese was ground to a fine powder using a blender, vacuum-packed and stored at -20 °C until extraction of CBM. The lyophilized cheese powder was extracted with sterile double deionised water in the proportion 1:2. It was mixed for 30 min at room temperature and then centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was adjusted to an NaCl concentration of 0.9 % (w/v) and a citrate concentration of 0.2 % (w/v). The mixture was autoclaved at 100 °C for 15 min and centrifuged at 5000 g for 15 min at 4 °C. Finally the lactose content was adjusted to 1 % using a sterile lactose solution. The CBM was tested for sterility by incubation at 30 °C for three days followed by plating on MRS plates which where incubated anaerobically at 30 °C for 72 h.

7.3.4. Growth of bacteria

Strains were maintained as stock cultures frozen at -80 °C in reconstituted skim milk with 10% (v/v) glycerol. Stock cultures were revived on MRS plates, for 24 h at 30 °C followed by two transfers in MRS broth for 16 h before inoculation into MRS broth or CBM. Strains were grown in duplicate. The cells were grown to early stationary phase in MRS and CBM and harvested by centrifugation at 7,000 g for 10 min at 4 °C. The OD (600 nm) value associated with this growth stage was determined in preliminary experiments. *Lb. danicus* 9M3 cells were harvested after 50 h incubation at 30°C in MRS and after 72 h at 30°C in CBM. *Lb. danicus* 13M1

cells were harvested after 40 h incubation at 30°C in MRS and after 50 h at 30°C in CBM. The supernatant was discarded and the extract was washed three times with 100 ml 0.9 % NaCl and finally dispersed in 50 mM sodium phosphate buffer (pH 7.0). The volume of the buffer was adjusted to give a 10 fold concentration of the cells. The number of colony forming units (CFU) was found by plating the cells on MRS agar incubated anaerobically at 30 °C for 72 h.

7.3.5. Preparation of crude cell free extracts (CFE)

The 10-fold concentrated cell suspensions were passed three times through a one shot model cell disruptor (Constant Systems, Daventry, UK) at 250 MPa. Cell debris was removed by centrifugation at 10,000 g for 20 min at 4 °C and the supernatants were filtered (0.2 µm). The resulting CFEs were stored at -18 °C in aliquots of 1 ml. A new portion (1 ml) of CFE was taken up from the freezer immediately before use. To evaluate the efficiency of the cell disruption, cell counts were determined before and after cell disruption by plating appropriated dilutions on MRS agar (pH 6.2), incubated anaerobically at 30 °C for 72 h. Enzyme assay were carried out in duplicate from each CFE.

7.3.6. Protein determination

Protein content of the CFEs was determined using the Bradford assay (Bradford, 1976). The assay was carried out in triplicate in microtiter plates (Nunc, Roskilde, Denmark).Bovine serum albumin was used to prepare a standard curve (0–2 mg ml^{1}) and was included each time the assay was performed.

7.3.7. Determination of aminopeptidase activities

Aminopeptidase activity was investigated using the chromogenic substrates Lys-, Arg-, Leu-, Val-, Met-, Phe-, Gly-, Pro-*p*-nitroanilide (*p*NA) and X-prolyl dipeptidyl aminopeptidase activity using Gly-Pro- and Arg-Pro-*p*NA, by measuring initial rates in microtiter plates (Exterkate, 1975; Ardo & Jonsson, 1994; Jensen & Ardo, 2010). All substrates were purchased from Sigma-Aldrich (St. Louis, MO, USA) except for Arg-Pro-*p*NA which was purchased from Bachem (Weil am Rhein, Germany). Stock solutions (1 mM) of the *p*NA derivatives were prepared in 50 mM sodium phosphate buffer, pH 7.0. The substrates Met-, Leu-, Gly- and Phe-*p*NA, required 30 min in an ultrasonic bath and in the case of Phe-*p*NA another 15 min in a 70 °C waterbath to dissolve, while the other substrates were easily dissolved. The assay mixture contained 50 µL of the substrate and 50 µL of CFE and the reaction was carried out in duplicate in microtiter plates. Enzyme activity was determined at 30 °C by continuously measuring the release of nitroaniline at 405 nm using a microtiter plate scanner spectrophotometer (PowerWave 200, Bio-Tek Instruments, Winooski, VT, USA). The amount of released *p*-nitroaniline was quantified with the use of a standard curve. The standard curve was included each time an experiment was done. Activity was expressed as mmol nitroaniline released per min per kg protein. The assay was carried out in duplicate for each strain.

7.3.8. Determination of aminotransferase activities

Aminotransferase activity was measured against 20 amino acids as described by Thage et al. (2004) with adaption of the temperature used to suit *Lb. danicus*. CFE (50 µL) was incubated for 15 min at 30 °C in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7), 0.05 mM PLP, 6 mM α-ketoglutaric acid (αKGA) and 1 mM of one of each of the specific amino acids (Phe, Tyr, Trp, Leu, Ile, Val, Met, Asn, Asp, Lys, Gln, His, Pro, Orn, Cit, Ala, Arg, Gly, Ser or Thr; Sigma-Aldrich, St. Louis, MO, USA) in a total volume of 250 µL. The enzymatic reaction was stopped by heating at 80 °C for 15 min. The amount of Glu produced was measured using an L-Glu assay kit (Boehringer Mannheim, Darmstadt, Germany) based on enzymatic reactions converting NAD^+ to NADH and downscaled to a volume of 250 µL for use in microtiter plates. Glu content was determined by end-point measuring the absorbance at 492 nm in 45 min at 30 °C. The amount of Glu produced during the 15 min incubation time was calculated by subtracting the amount of Glu present in the reaction mixture after 0 min of incubation from the amount of Glu present in the reaction mixture after 15 min of incubation. A control sample was included in the experiment containing only the reaction mixture in a total volume of 250 µL. Glu content was quantified by including samples for a standard curve of Glu on the microtiter plate in each run. One unit of aminotransferase activity was defined as 1 mmol Glu produced per min per kg protein. The assay was carried out in duplicate for each strain.

7.3.9. Determination of esterase activities

The esterase activity was measured as described by Casaburi et al. (2006) with some modifications. Five *p*-nitrophenyl (*p*NP) esters (*p*NP-acetate, *p*NP-butyrate, *p*NP-caprylate, *p*NP-laurate and *p*NP-palminate; Sigma, St. Louis, MO) were used as substrates. Solutions of 2 mM *p*NP substrates were prepared in 96 % ethanol. The reaction mixture consisted of 50 µl *p*NP substrate solution, 100 µL 50 mM phosphate buffer (pH 7) and 50 µL CFE. Enzyme activity was determined at 30 °C by continuously measuring the release of *p*-nitrophenol in the supernatant at 410 nm using a microtiter plate scanner spectrophotometer (PowerWave 200; Bio-Tek Instruments, Winooski, VT, USA). The extent of release of *p*-nitrophenol was quantified by including a standard curve of *p*-nitrophenol on the microtiter plate each time the experiment was done. One unit of esterase was expressed as 1 mmol *p*-nitrophenol released per min per kg protein. The assay was carried out in duplicate for each strain.

7.3.10. Statistical analysis

Mean values of enzyme activities for each strain and substrate were calculated from results of duplicate analysis of CFEs from each of two inoculations and preparations made at two separate occasions. Student's t-test for independent samples was used to compare the two groups and Student's t-test for paired samples was used for comparison within the same group. Statistical analysis was performed using SPSS Version 18.0 for Windows XP (SPSS Inc., Chicago, IL, USA). Significance was declared at $P \leq 0.05$.

7.4. Results and discussion

7.4.1. Growth in MRS or CBM and production of cell free extracts

Before inoculation, the cheese-based medium (CBM) was found to be sterile after incubation at 30 °C for three days (results not shown). OD, CFU, pH and protein content at harvest are shown in **Table 7.1**. *Lb. danicus* 9M3 cells were harvested after 50 h incubation at 30°C in MRS and after 72 h at 30°C in CBM. *Lb. danicus* 13M1 cells were harvested after 40 h incubation at 30°C in MRS and after 50 h at 30°C in CBM (results not shown). Both strains needed a longer incubation time in CBM to reach the stationary phase than in MRS broth. Interestingly, cell numbers in CBM and MRS at harvesting were both in the order of ~10^7 cfu/ml. In particular, after growth in MRS, the two strains had a higher OD value, but lower cell number than after growth in CMB. A higher cell number would be expected after growth in MRS since the medium is more complex and richer in nutrients than the CBM. pH at harvest and protein content after growth in MRS and CBM were similar for both strains. The cell disruption led to a 95-98 % decrease in viable cell numbers for both strains tested in CBM and MRS (results not shown). In the study of Kask et al. (2003), strains of *Lb. danicus* reached ~10^9 cfu/ml only after 24 h of incubation in MRS at 30°C and pH decreased to levels ranging from 4.54 to 4.92. Similar pH values were also obtained by Thage et al. (2004) after growth of *Lb. danicus* under the same conditions.

Table 7.1. Numbers of *Lactobacillus danicus* 9M3 and 13M1 grown in MRS broth or cheese-based medium (CBM).

Strain	OD at harvest		CFU at harvest		pH at harvest		Protein content (mg/ml)	
	MRS	CBM	MRS	CBM	MRS*	CBM	MRS	CBM
9M3	1.6	1.2	$1x10^7$	$2x10^7$	-	4.96	1.21	1.26
13M1	1.5	2.0	$3x10^7$	$6x10^7$	-	5.00	1.08	1.17

The values are average values from the two independent growth cycles.
* pH was not measured after growth in MRS.

7.4.2. Aminopeptidase activity (AP)

The proteolytic activity of dairy LAB is essential for bacterial growth in milk and it is involved in the development of organoleptic properties in fermented milk products (Ayad et al., 2004). Enzymatic activity of *Lb. danicus* 9M3 and *Lb. danicus* 13M1 against aminopeptidase substrates in is shown in **Table 7.2 and Figure 7.1**.

Both strains of *Lb. danicus* had activity on all the substrates tested after growth in MRS broth, whereas activity after growth in CBM was detected only against Arg-, Phe-, Gly- and Pro-*p*Na. Strain 9M3 showed a significantly higher ($P \leq 0.05$) activity on Arg-, Gly- and Arg-Pro-*p*NA than strain 13M1 after growth in MRS, and a significantly higher ($P \leq 0.05$) activity against Phe- and Pro-*p*NA than strain 13M1 after growth in CBM. Also, strain 9M3 had a significantly higher ($P \leq 0.05$) activity against Arg-, Phe- and Pro-*p*NA when grown in MRS broth than in CMB. Same results were obtained for strain 13M1 when tested against Phe- and Pro-*p*NA. No significant differences ($P \leq 0.05$) where detected between the two strains grown in MRS and CBM against all the other substrates. CFE of *Lb. danicus* grown in CBM had activity on Arg-*p*NA but not on Lys-*p*NA, which are two activities common for aminopeptidases of lactic acid bacteria with broad substrate specificity, such as PepN or PepC that degrade both Arg and Lys-containing substrates (Christensen et al., 1999).

In this study, *Lb. danicus* 9M3 and 13M1 had AP activity toward Pro-*p*NA when grown in both MRS and CBM; contrasting results were reported by Perez et al. (2003) who found that CFE of lactobacilli isolated from Tenerife cheese and grown in MRS had no activity against this substrate. However, the authors reported AP activity against Ala-, Lys-, Leu- and Arg-Pro-*p*NA. In agreement to this study, Di Cagno et al. (2012) found that *Lb. casei* LC01, *Lb. plantarum* CC3M8, and *Lb. paracasei* CC3M35 used as adjunct cultures or attenuated adjunct cultures had AP activity against Leu- and Pro-*p*NA, whereas Ayad et al. (2004) reported that stains of lactobacilli had AP activity against Leu-*p*NA and Giraffa et al. (2004) showed that *Lactobacillus delbrueckii* subsp. *lactis* had activity against Arg-, Lys-, and Phe-Pro-*p*NA.

In a study by Gatti et al. (2004), strains of *Lb. helveticus*, *Lb. delbrueckii* subsp. *lactis*, *Lb*. *delbrueckii* subsp. *bulgaricus* were isolated from whey starters for Grana Padano and Provolone cheese and their peptidase activity in whole and disrupted was evaluated after growth in MRS broth. In whole cells, the most commonly and highly expressed enzymatic activity among the strains was for Phe-Pro-β-nitroaniline (βNA) as substrate, whereas moderate and variable activities were found for Arg-βNA, Lys-βNA and Leu-βNA as substrates. The authors showed that all the strains had a very low activity towards Pro-βNA. In disrupted cells, the highest enzymatic activities expressed were those against Phe-Pro-βNA, Lys-βNA and Arg-βNA whereas a lower and strain-specific activity was detected against Leu-βNA. Also in disrupted cells the lowest activity was detected towards Pro-βNA. These results are in agreement to those reported in the present study, where the highest peptidase activity in CBM of both *Lb. danicus* strains was found on substrates Arg- and Lys-*p*NA, whereas activity expressed against Pro-*p*NA was one of the lowest.

Similar results are also reported by Pan and Tanokura (2004) who showed that *Lb. helveticus* JCM 1004 had AP activity against Lys–, Leu–, Ala–, Arg– and Phe–*p*NA and towards a number of dipeptides with relatively hydrophobic amino acids at their N-termini but did not hydrolyze dipeptides containing proline at their N-termini or C-termini. González et al. (2010) reported that strains of *Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides, Lb. paracasei, Lb. plantarum* and *Enterococcus faecalis* showed different levels of AP activity against Ala-, Lys-, Pro-, Leu-*p*NA; in particular *Lb. paracasei* exhibited the highest level of AP activity. In general, the dipeptidase activity of the strains tested was higher towards Leu-Leu, Tyr-Leu and Phe-Ala substrates. Both strains of *Lb. danicus* exhibited X-prolyl aminopeptidase activity against Arg-Pro-*p*NA and Gly-Pro-*p*NA only when grown in MRS broth.

Bintsis et al. (2003) reported that *Lb. paracasei* subsp. *paracasei* had aminopeptidase, dipeptidase, dipeptidyl aminopeptidase, endopeptidase and carboxypeptidase activities against a wide range of substrates; dipeptidyl aminopeptidase activities on Gly-Arg-*p*NA and Arg-Pro-*p*NA were very low compared with either aminopeptidase or dipeptidase specific activities, while Gly-Pro-*p*NA was degraded to some extent. In the study of Donkor et al. (2007), *Lactobacillus acidophilus*, *Bifidobacterium* spp., *Lb. casei*, *Streptococcus thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* had activity towards Gly-Pro-*p*NA, whereas, in contrast to the results of this study, Tan et al. (1995) observed very low or no intracellular dipeptidase activity towards Pro-containing dipeptides in *Lb. helveticus* SET 2171.

High X-prolyl aminopeptidase is of technological interest, as the enzyme cleaves the peptide bond after proline in the penultimate position allowing the degradation of the proline-rich peptides that are abundant in β-casein (Fortina et al., 1998). The AP activity of *Lb. danicus* strains tested in this study was highly dependent on the growth medium, which is believed to reflect differences in substrate availability between the two growth media. As AP play an important role in the hydrolysis of bitter peptides and in amino acid liberation, the use of lactobacilli with high AP activity as adjunct cultures can increase proteolysis and therefore improve the flavour of cheese (Perez et al., 2003).

Table 7.2. Aminopeptidase activity against 10 chromogenic *p*-nitroanaline (*p*NA) derivates of amino acids by cell free extract (CFE) of *Lactobacillus danicus* 9M3 and 13M1 grown in MRS broth or cheese-based medium (CBM).

Substrate	Growth medium	*Lactobacillus danicus*	
		9 M3	13 M1
Arg-*p*NA	MRS	11.14[a*]	8.86[b]
	CBM	3.05[a]	3.72[a]
Lys-*p*NA	MRS	13.51[a]	15.03[a]
	CBM	n.d.	n.d.
Leu-*p*NA	MRS	9.13[a]	9.45[a]
	CBM	n.d.	n.d.
Met-*p*NA	MRS	3.75[a]	4.00[a]
	CBM	n.d.	n.d.
Phe-*p*NA	MRS	2.54[a*]	2.25[a*]
	CBM	0.70[a]	0.29[b]
Val-*p*NA	MRS	2.24[a]	2.54[a]
	CBM	n.d.	n.d.
Gly-*p*NA	MRS	0.12[a]	0.09[b]
	CBM	0.12[a]	0.12[a]
Pro-*p*NA	MRS	0.72[a*]	0.70[a*]
	CBM	0.15[a]	0.07[b]
Arg-Pro-*p*NA	MRS	1.53[a]	1.26[b]
	CBM	n.d.	n.d.
Gly-Pro-*p*NA	MRS	1.65[a]	1.39[a]
	CBM	n.d.	n.d.

Values are means of duplicate analyses of CFE prepared on two separate occasions. Activity is significantly different (*, $P < 0.05$) when comparing a strain after growth in CBM and MRS. Different superscript letters within a row indicate significant differences ($P < 0.05$) among the strains 9M3 and 13M1. Activities are expressed as mmol *p*-nitroaniline released per kg^{-1} protein min^{-1}.
n.d., activity not detected.

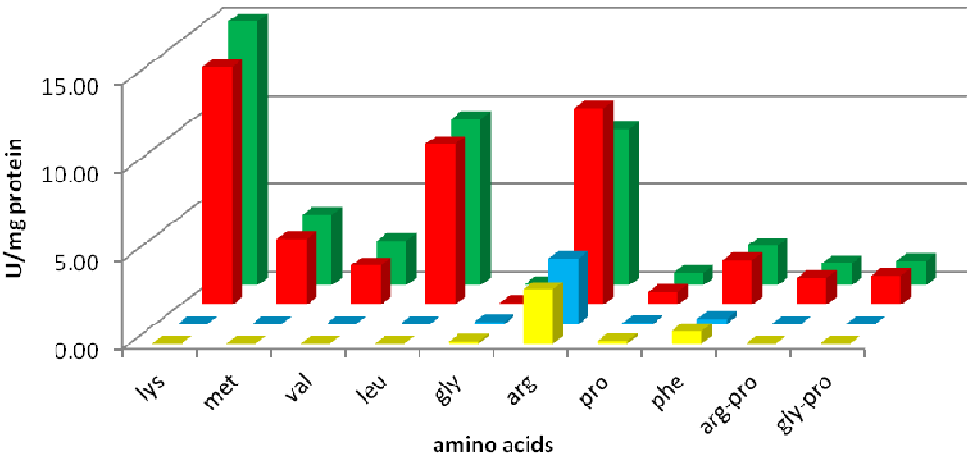

Figure 7.1. Aminopeptidase activity against 10 chromogenic *p*-nitroanaline derivates amino acids and dipeptides by cell free extract (CFE) of *Lactobacillus danicus* 9M3 and 13M1. *Lactobacillus danicus* 9M3 grown in a cheese-based medium (CBM) (■) or MRS broth (■) and *Lb. danicus* 13 M1 grown in CBM (■) or MRS broth (■).

7.4.3. Aminotransferase activity (AT)

Enzymatic activity against aminotransferase substrates in *Lb. danicus* 9M3 and *Lb. danicus* 13M1 is shown in **Table 7.3** and **Figure 7.2**. AT activity in MRS against Phe, Tyr, Trp, Leu, Val, Asp, Ala and Thr was significantly higher ($P \leq 0.05$) in strain 9M3 than in 13M1, whereas AT activity in MRS against Met, Arg and Cit was significantly higher ($P \leq 0.05$) in 13M1 than in 9M3. AT activity against all substrates in CBM was significantly higher ($P \leq 0.05$) in strain 13M1 than 9M3, except for AT on Thr that was significantly higher ($P \leq 0.05$) in 9M3 than 13M1. Generally, AT activity was around 10-fold higher in the strains grown in MRS broth than in CBM: AT activity against all substrates was significantly higher ($P \leq 0.05$) in 9M3 after growth in MRS than in CBM except for Ile, His, Lys (activity was not detected in cells grown in MRS), Cit, Pro, Gly (no significant differences found) and Thr (activity significantly higher in CBM).
Also, AT activity in strain 13M1 against all substrates was significantly higher ($P \leq 0.05$) after growth in MRS than in CBM, except for Ile, His, Lys, Pro (activity was not detected in cells grown in MRS), Cit, Gly and Ser (no significant differences found) and Asn, Orn and Thr (activity significantly higher in CBM). Interestingly, the AT activity expressed in cells grown in CBM was broader than in MRS. In fact, both *Lb. danicus* strains showed AT against Ile, His, Lys and Pro, whereas no activity against these amino acids was detected in cells grown in MRS broth.

Jensen & Ardö (2010) showed dependency on growth medium of AT activity when comparing CFE of *Lb. helveticus* after growth in MRS broth and milk. They reported a higher AT activity on the aromatic amino acids after growth in MRS broth, which is in agreement with our results for *Lb. danicus*. In a study conducted by Thage et al. (2004), AT activity of *Lb. helveticus*, *Lb. paracasei* and *Lb. danicus* was studied. The authors showed that the degradation of Leu, Phe, Trp, Tyr, Asp and Met by *Lb.*

danicus isolates was significantly higher the other two lactobacilli. These authors studied two strains of *Lb. danicus*, including strain 13M1.

Comparing the results obtained in this study to those obtained by Thage et al. (2004), we can observe that the authors found that strain 13M1 had activity against Ile (1.2 U/OD) whereas no activity was detected in the present study. On the other hand, the authors did not report activity against Ala, Gln, Gly, whereas activity towards these amino acids was detected in this study. In agreement with the findings of Thage et al. (2004), strain 13M1 had no activity against His, Lys and Pro. The different pattern of amino acid degradation for some amino acids could be due to the growth stage at harvest and method of cell disruption, which differed from the methods used in this study. The differences in AT activity of lactobacilli strains has an impact on the flavor development in cheese during ripening.

Degradation of branched-chain (BcAA) and aromatic amino acids (ArAA) by lactococci is essential for the formation of cheesy, fruity, malty or floral notes (Yvon and Rijnen, 2001a; Thage et al., 2004). Also activity on Met is important for the cheese flavor since its degradation leads to the formation of volatile compounds such as methanethiol, methional, dimethyl sulphide, dimethyl trisulphide or hydrogen sulphide that contribute to the typical Cheddar and Gouda cheese flavor (Dias and Weimer, 1998;, Amarita et al., 2001; Yvon and Rijnen, 2001a,b). One of the mechanisms that produces methanethiol from Met is the deamination reaction to form α-keto-γ-methyl thiobutyrate that can be catalyzed by various aminotransferases or amino acid oxidases present in many bacteria. Both strains tested in the present study had AT activity against Met; in particular strain 13M1 showed a higher activity than strain 9M3 after growth in both MRS and in CBM.

In a study by Dias and Weimer (1998), *Lb. helveticus* CNRZ32 lost less than half of the activity against Met when assayed under cheese-like conditions in a chemically defined medium (pH 5.2, 5% of NaCl) compared to the control medium (pH 7.2, 0% NaCl), whereas all the other bacteria tested (lactococci and brevibacteria) completely lost their activity towards Met. In this study, the strains did not lose their ability to degrade Met under cheese-like conditions, but the activity toward this amino acid in CBM was nearly 10-fold lower than when the cells were grown in MRS.

Kieronczyk et al. (2004) evaluated the ability of two *Lactobacillus* strains to produce aroma compounds from amino acids in a cheese model produced commercially from young Gouda cheese. The two strains had AT activity against Phe, Asp, Leu and a much lower activity against Ile and Val. The authors also showed that no carboxylic acids derived from aromatic amino acids and Met were detected in any cheese. On the other hand, the two strains were able to produce 3-methylbutanoic acid from Leu. When the strains were co-inoculated with *L. lactis*, the production of 2-methylpropanoic acid from Val and the production of alcohols derived from branched-chain (Leu, Ile and Val) significantly decreased.

Table 7.3. Aminotransferase activity against 20 amino acids in cell free extract (CFE) of *Lactobacillus danicus* 9M3 and 13M1 grown in MRS broth or in a cheese-based medium (CBM).

Amino acid	Growth medium	*Lactobacillus danicus*	
		9M3	13M1
Phe	MRS	96.04a^{a*}	68.74^{b*}
	CBM	2.83^{b}	6.73^{a}
Tyr	MRS	62.42^{a*}	48.88^{b*}
	CBM	4.57^{b}	9.12^{a}
Trp	MRS	113.30^{a*}	85.82^{b*}
	CBM	8.61^{b}	18.35^{a}
Leu	MRS	14.33^{a*}	6.26^{b*}
	CBM	1.14^{b}	2.67^{a}
Ile	MRS	n.d.	n.d.
	CBM	0.92^{a}	1.01^{a}
Val	MRS	14.19^{a*}	11.76^{b*}
	CBM	1.88^{a}	1.71^{a}
Met	MRS	11.41^{b*}	38.00^{a*}
	CBM	1.00^{b}	4.20^{a}
Asn	MRS	3.40^{a*}	2.82^{a}
	CBM	0.44^{b}	3.47^{a*}
Asp	MRS	46.80^{a*}	31.35^{b*}
	CBM	8.74^{b}	12.58^{a}
Gln	MRS	6.79^{a*}	6.76^{a*}
	CBM	1.32^{a}	1.00^{a}
His	MRS	n.d.	n.d.
	CBM	1.50^{a}	1.77^{a}
Ala	MRS	7.65^{a*}	5.85^{b*}
	CBM	0.16^{b}	0.43^{a}
Lys	MRS	n.d.	n.d.
	CBM	1.02^{b}	1.60^{a}
Arg	MRS	4.77^{b*}	6.12^{a*}
	CBM	2.12^{a}	1.98^{a}
Cit	MRS	0.78^{b}	0.97^{a}
	CBM	0.84^{b}	1.16^{a}
Orn	MRS	0.91^{a}	0.91^{a}
	CBM	2.03^{a*}	2.84^{a*}
Pro	MRS	n.d.	n.d.
	CBM	1.45^{b}	2.49^{a}
Gly	MRS	1.61^{a}	1.22^{a}
	CBM	0.92^{b}	1.44^{a}
Ser	MRS	0.75^{a*}	0.87^{a}
	CBM	0.48^{b}	1.12^{a}
Thr	MRS	1.70^{a}	0.62^{b}
	CBM	4.30^{a*}	3.42^{b*}

Values are means of duplicate analyses of CFE prepared on two separate occasions. Activity is significantly different (*, $P < 0.05$) when comparing a strain after growth in CBM and MRS. Different superscript letters within a row indicate significant differences ($P < 0.05$) among the strains 9M3 and 13M1. Activities are expressed as mmol Glu produced kg^{-1} protein min^{-1} during the 15 min incubation period.
n.d., not detected.

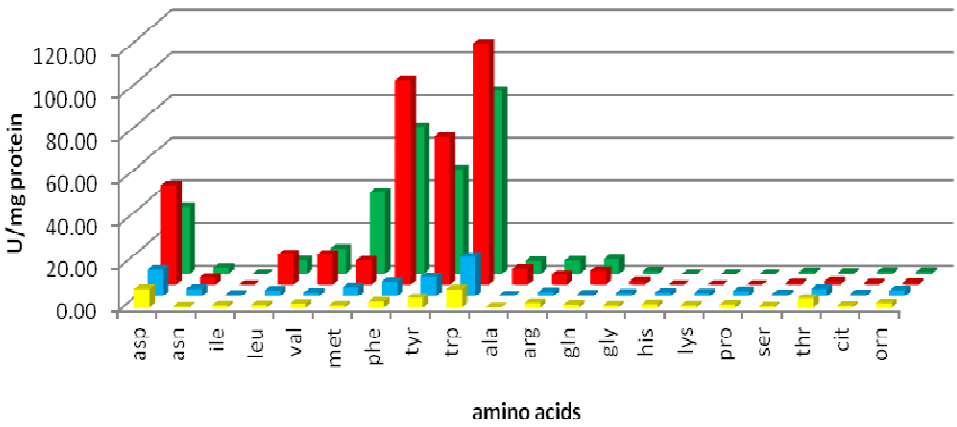

Figure 7.2. Aminotransferase activity against 10 chromogenic *p*-nitroanalide derivates of amino acids in cell free extract (CFE) of *Lactobacillus danicus* 9M3 and 13M1. *Lactobacillus danicus* 9M3 grown in a cheese-based medium (CBM) (■) or MRS broth (■). *Lb. danicus* 13 M1 grown in CBM (■) or MRS (■).

7.4.4. Esterase activity

The esterase activity of *Lb. danicus* 9M3 and 13M1 after growth in MRS or CBM was tested against *p*NP-acetate (C2), *p*NP-butyrate (C4), *p*NP-caprylate (C8), *p*NP-laurate (C12), *p*NP-palmitate (C16) and the results are shown in **Table 7.4** and **Figure 7.3**.

Esterase activity was detected only against C2, C4 and C16. No significant differences ($P \leq 0.05$) were found against C4 between the two strains grown in CBM or MRS. After growth in MRS, esterase activity against C2 was significantly higher ($P \leq 0.05$) in strain 13M1 than in strain 9M3, whereas esterase activity against C16 was significantly higher ($P \leq 0.05$) in 9M3 than in 13M1.

After growth in CBM, esterase activity toward C2 was significantly higher ($P \leq 0.05$) in 9M3 than in 13M1 and no significant differences ($P \leq 0.05$) were found against the other substrates. In both strains, esterase activity was significantly higher ($P \leq 0.05$) after growth in CBM than in MRS, which differs from the results for AP and AT activity, which were more expressed after growth in MRS broth than in CBM.

Usually, esterase activity of lactic acid bacteria is greater on short-chain fatty acids (Williams and Banks, 1997). In this study, both strains of *Lb. danicus* tested did not show any esterase activity against C8 and C12, but they showed esterase activity against a long-chain fatty acids such as *p*NP-palmitate (C16). However, the flavor impact of the long chain fatty acids (>12 carbon atoms) is considered less important (Collins et al., 2003).

González et al. (2010), reported that *E. faecalis* had activity against β-naphthyl butyrate (C4), β-naphthyl caprylate (C8) and β-naphthyl myristate (C14), whereas *Lb. paracasei* had esterase activity against C8, C14 and *Lb. plantarum* had activity

only on C8. Herreros et al. (2004) studied the esterase activity of the cell-free extract of lactic acid bacteria isolated from Armada cheese and showed that strains of *Lb. plantarum* preferentially hydrolyzed β-naphthyl C8 and C14 substrates; the strains of *Lb. brevis* had higher esterase activity on C4 and C8 fatty acids than those of *Lb. plantarum*, whereas the esterase activity of *Lb. casei* subsp. *casei* against the substrates tested was the lowest.

In the study of Vafopoulou-Mastrojiannaki et al. (1996) the authors reported that *Lb. buchneri*, *Lb. brevis* and *Lb. hilgardii* degrade preferentially substrates esterified to low molecular weight fatty acids. The strains tested showed higher esterase activity against α- and β-naphthyl-acetate (C2) and -propionate (C3) than -butyrate (C4); -caproate (C6); -caprylate (C8) and -caprate (C10). Similar results were also reported by Katz et al. (2002) who showed that *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Enterococcus* isolated from ewes' milk and cheeses had intracellular esterase activities which preferentially degraded the α- and β-naphthyl derivatives of 2 to 6 carbon fatty acids.

In the study of Perez et al. (2003), strains of *Lb. plantarum*, *Lb. paracasei* ssp. *paracasei Lb. curvatus* ssp. *curvatus*, isolated from Tenerife cheese, had esterase activity against *p*NP-butyrate (C4) and *p*NP-caproate (C6). On the other hand, Bintsis et al. (2003) reported that strains of *Lb. paracasei* subsp. *paracasei* had esterase activity against α- and β-naphthyl-acetate (C2), -propionate (C3), -butyrate (C4), -caproate (C6), -caprylate (C8) and -caprate (C10). Some strains of *Lb. plantarum*, *Lb. pentosus*, *Lc. mesenteroides* and *Lb. brevis*, isolated from Tunisian refrigerated raw milk, had esterase activity against C4 and C8, whereas no activity was detected against C14 using API-ZYM system (Moussa et al., 2008). Similar results were also reported by Buffa et al. (2006) in a study conducted on lactic acid bacteria isolated from raw milk of Guirra ewes' milk cheese using API-ZYM system.

Microbial esterases and lipases may improve quality or accelerate the maturation of cheeses, since these enzymes are responsible for the formation of flavour compounds such as volatile free fatty acids or esters from lipid degradation. The results of this study showed that *Lb. danicus* 9M3 and 13M1 had esterase activity against C2, C4 and C16 and higher activity was detected after growth in CBM than in MRS broth. This could indicate that there is higher concentration of fatty acids in CBM than in MRS. The artificial *p*-nitrophenyl ester substrates used in this project could be hydrolysed by other enzymes, e.g. peptidases which are present in the cell free extract. The esterase activities were, however, higher after growth in CBM while aminopeptidase activities were higher after growth in MRS broth, which clearly showed that the activity of different enzymes are measured using the different artificial substrates. To verify the results experiments with alternative substrates could be performed.

Table 7.4. Esterase activity against 5 chromogenic *p*-nitrophenyl (*p*NP) derivates of fatty acids in cell free extract (CFE) of *Lactobacillus danicus* 9M3 and 13M1 grown in MRS broth or in a cheese-based medium (CBM).

Fatty acid	Growth medium	*Lactobacillus danicus*	
		9 M31	13M1
C2	MRS	0.77^{b}	0.92^{a}
	CBM	2.34^{a*}	1.84^{b*}
C4	MRS	0.66^{a}	0.65^{a}
	CBM	2.03^{a*}	2.16^{a*}
C8	MRS	n.d.	n.d.
	CBM	n.d.	n.d.
C12	MRS	n.d.	n.d.
	CBM	n.d.	n.d.
C16	MRS	0.88^{a}	0.73^{b}
	CBM	2.10^{a*}	1.95^{a*}

Values are means of duplicate analyses of CFE prepared on two separate occasions. Activity is significantly different (*, $P < 0.05$) when comparing a strain after growth in CBM and MRS. Different superscript letters within a row indicate significant differences ($P < 0.05$) among the strains 9M3 and 13M1. Activities are expressed as mmol nitrophenyl released kg^{-1} protein min^{-1}.
n.d., not detected.

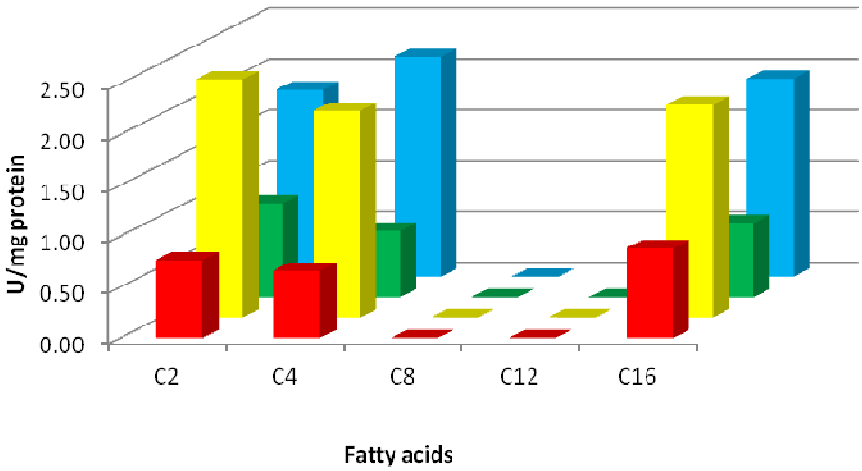

Figure 7.3. Esterase activity against five chromogenic *p*-nitrophenyl substrates in cell free extract (CFE) of *Lactobacillus danicus* 9M3 and 13M1.
Lactobacillus danicus 9M3 grown in a cheese-based medium (CBM) (■) or in MRS broth (■). *Lb. danicus* 13 M1 grown in CBM (■) or in MRS broth (■).

7.5. Conclusions

The results of this study showed interesting different metabolic activities for *Lb. danicus* 9M3 and 13M1 grown in MRS broth or in CBM. After growth in CBM, the strains showed AP activity only against Arg-, Phe-, Gly- and Pro-*p*NA, whereas in MRS they were active against all the substrates tested. Both *Lb. danicus* strains grown in MRS had the highest AT activity towards ArAA (Phe and Trp).

AT activity against ArAA was detected also in *Lb. danicus* strains grown in CBM, especially in strain 13M1 that showed the highest activity against Trp. Also branched-chain amino acids (Leu and Val) were degraded by the two strains grown in both MRS or CBM, but at lower rate than ArAA. Generally, the strains grown in CBM had an activity 10-fold lower than the strains grown in MRS. However, AT specificity was broader in the strains grown in CBM, in fact both *Lb. danicus* 9M3 and 13M1 showed AT against Ile, His, Lys and Pro, whereas no activity against these amino acids was detected after growth in MRS.

The strains also showed activity on Met possibly suggesting that they can be used as adjunct culture for improving the flavor of cheese. Esterase activity was expressed only against *p*NP-acetate (C2), *p*NP-butyrate (C4) and *p*NP-palmitate (C16) and was significantly higher in CBM which was probably richer in free fatty acids than in MRS. The growth medium had some influence on the substrate specificity on the enzymes expressed.

The CBM is more likely to reflect a cheese environment in terms of substrate availability, specificity and concentration of substrates compound, in contrast to MRS where there is excess substrate availability. The results of this study showed that *Lb. danicus* had good AP, AT and esterase activity and therefore can be used as adjunct culture for the production of cheese in order to accelerate proteolysis and improve flavor.

7.6. References

ADAMBERG, K., ANTONSSON, M., VOGENSEN, F. K., NIELSEN, E. W., KASK, S., MOLLER, P. L. & ARDO, Y. 2005. Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semi-hard Danish cheese. *International Dairy Journal*, 15, 873-882.

ADAMBERG, K., KASK, S., LAHT, T. M. & PAALME, T. 2003. The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. *International Journal of Food Microbiology*, 85, 171-183.

AMARITA, F., REQUENA, T., TABORDA, G., AMIGO, L. & PELAEZ, C. 2001. *Lactobacillus casei* and *Lactobacillus plantarum* initiate catabolism of methionine by transamination. *Journal of Applied Microbiology*, 90, 971-978.

ARDO, Y., & JONSSON, L. 1994. Chromatographic profiling of aminopeptidolytic activities in lactobacilli as a tool for strain identification. *Journal of Dairy Research*, 61(4), 573-579.

AYAD, E. H. E., NASHAT, S., EL-SADEK, N., METWALY, H. & EL-SODA, M. 2004. Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. *Food Microbiology*, 21, 715-725.

BERESFORD, T. P., FITZSIMONS, N. A., BRENNAN, N. L. & COGAN, T. M. 2001. Recent advances in cheese microbiology. *International Dairy Journal*, 11, 259-274.

BINTSIS, T., VAFOPOULOU-MASTROJIANNAKI, A., LITOPOULOU-TZANETAKI, E. & ROBINSON, R. K. 2003. Protease, peptidase and esterase activities by lactobacilli and yeast isolates from Feta cheese brine. *Journal of Applied Microbiology*, 95, 68-77.

BRADFORD, M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.

BUFFA, M., MORAIS, J., JIMENEZ-BELENGUER, A., HERNANDEZ-GIMENEZ, E. & GUAMIS, B. 2006. Technological characterisation of lactic acid bacteria isolated from raw ewes' milk for cheese making. *Milchwissenschaft*, 61, 404-407.

CAMPBELL, J. J. & GUNSALUS, I. C. 1944. Citric acid fermentation by streptococci and lactobacilli. *Journal of Bacteriology*, 48, 71-6.

CASABURI, A., VILLANI, F., TOLDRA, F., & SANZ, Y. 2006. Protease and esterase activity of staphylococci. *International Journal of Food Microbiology*, 112 (3), 223-229.

CHRISTENSEN, J. E., DUDLEY, E. G., PEDERSON, J. A. & STEELE, J. L. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek*, 76, 217-246.

CHRISTIANSEN, P., PETERSEN, M. H., KASK, S., MOLLER, P. L., PETERSEN, M., NIELSEN, E. W., VOGENSEN, F. K. & ARDO, Y. 2005. Anticlostridial activity of *Lactobacillus* isolated from semi-hard cheeses. International Dairy Journal, 15, 901-909.

COLLINS, Y. F., McSWEENEY, P. L. H. & WILKINSON, M. G. 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *International Dairy Journal*, 13, 841-866.

DI CAGNO, R., DE PASQUALE, I., DE ANGELIS, M. & GOBBETTI, M. 2012. Accelerated ripening of Caciocavallo Pugliese cheese with attenuated adjuncts of selected nonstarter lactobacilli. *Journal of Dairy Science*, 95, 4784-4795.

DIAS, B. & WEIMER, B. 1998. Conversion of methionine to thiols by lactococci, lactobacilli, and brevibacteria. *Applied and Environmental Microbiology*, 64, 3320-3326.

DIAZ-MUNIZ, I. & STEELE, J. L. 2006. Conditions required for citrate utilization during growth of *Lactobacillus casei* ATCC334 in chemically defined medium and Cheddar Cheese extract. *Antonie Van Leeuwenhoek*, 90, 233-243.

DIAZ-MUNIZ, I., BANAVARA, D. S., BUDINICH, M. F., RANKIN, S. A., DUDLEY, E. G. & STEELE, J. L. 2006. *Lactobacillus casei* metabolic potential to utilize citrate as an energy source in ripening cheese: a bioinformatics approach. *Journal of Applied Microbiology*, 101, 872-882.

DONKOR, O. N., HENRIKSSON, A., VASILJEVIC, T. & SHAH, N. P. 2007. Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of growth and *in vitro* angiotensin-converting enzyme inhibitory activity in fermented milk. *Lait,* 87, 21-38.

DRAKE, M. A., BOYLSTON, T. D., SPENCE, K. D. & SWANSON, B. G. 1996. Chemical and sensory effects of a *Lactobacillus* adjunct in Cheddar cheese. *Food Research International*, 29, 381-387.

EL SODA, M., MADKOR, S. A. & TONG, P. S. 2000. Adjunct cultures: Recent developments and potential significance to the cheese industry. *Journal of Dairy Science*, 83, 609-619.

EXTERKATE, F. 1975. Introductory study of proteolytic system of *Streptococcus cremoris* strain hp. *Netherlands Milk and Dairy Journal*, 29(4), 303-318.

FORTINA, M. G., NICASTRO, G., CARMINATI, D., NEVIANI, E. & MANACHINI, P. L. 1998. *Lactobacillus helveticus* heterogeneity in natural cheese starters: the diversity in phenotypic characteristics. *Journal of Applied Microbiology*, 84, 72-80.

FOX, P. F., McSWEENEY, P. L. H. & LYNCH, C. M. 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. *Australian Journal of Dairy Technology*, 53, 83-89.

FRYER, T. F. 1970. Utilization of citrate by lactobacilli isolated from dairy products. *Journal of Dairy Research*, 37, 9-&.

GATTI, M., FORNASARI, M. E., LAZZI, C., MUCCHETTI, G. & NEVIANI, E. 2004. Peptidase activity in various species of dairy thermophilic lactobacilli. *Journal of Applied Microbiology*, 96, 223-229.

GIRAFFA, G., ANDRIGHETTO, C., ANTONELLO, C., GATTI, M., LAZZI, C., MARCAZZAN, G., LOMBARDI, A. & NEVIANI, E. 2004. Genotypic and phenotypic diversity of *Lactobacillus delbrueckii* subsp. *lactis* strains of dairy origin. International *Journal of Food Microbiology*, 91, 129-139.

GONZÁLEZ, L., SACRISTÁN, N., ARENAS, R., FRESNO, J. M. & EUGENIA TORNADIJO, M. 2010. Enzymatic activity of lactic acid bacteria (with antimicrobial properties) isolated from a traditional Spanish cheese. *Food Microbiology*, 27, 592-597.

HERREROS, M. A., FRESNO, J. M., SANDOVAL, H., CASTRO, J. M. & TORNADIJO, M. E. 2004. Esterolytic activity of lactic acid bacteria isolated

from Armada cheese (a Spanish goat milk cheese). *Milchwissenschaft*, 59, 526-529.

JENSEN, M. P. & ARDO, Y. 2010. Variation in aminopeptidase and aminotransferase activities of six cheese related *Lactobacillus helveticus* strains. *International Dairy Journal*, 20, 149-155.

KASK, S., ADAMBERG, K., ORLOWSKI, A., VOGENSEN, F. K., MOLLER, P. L., ARDO, Y. & PAALME, T. 2003. Physiological properties of *Lactobacillus paracasei, L. danicus* and *L. curvatus* strains isolated from Estonian semi-hard cheese. *Food Research International*, 36, 1037-1046.

KATZ, M., MEDINA, R., GONZALEZ, S. & OLIVER, G. 2002. Esterolytic and lipolytic activities of lactic acid bacteria isolated from ewe's milk and cheese. *Journal of Food Protection*, 65, 1997-2001.

KIERONCZYK, A., SKEIE, S., LANGSRUD, T., LE BARS, D. & YVON, M. 2004. The nature of aroma compounds produced in a cheese model by glutamate dehydrogenase positive *Lactobacillus* INF15D depends on its relative aminotransferase activities towards the different amino acids. *International Dairy Journal*, 14, 227-235.

LANE, C. N. & FOX, P. F. 1996. Contribution of starter and adjunct lactobacilli to proteolysis in Cheddar cheese during ripening. *International Dairy Journal*, 6, 715-728.

MILESI, M. M., McSWEENEY, P. L. H. & HYNES, E. R. 2008. Viability and contribution to proteolysis of an adjunct culture of *Lactobacillus plantarum* in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology*, 105, 884-892.

MOUSSA, O. B., MANKAI, M., SETTI, K., BOULARES, M., MAHER, M. & HASSOUNA, M. 2008. Characterisation and technological properties of psychotropic lactic acid bacteria strains isolated from Tunisian raw milk. *Annals of Microbiology*, 58, 461-469.

NANDA, D. K., TOMAR, S. K., SINGH, R., MAL, G., SINGH, P., ARORA, D. K., JOSHI, B. K., CHAUDHARY, R. & KUMAR, D. 2011. Phenotypic and genotypic characterisation of lactobacilli isolated from camel cheese produced in India. *International Journal of Dairy Technology*, 64, 437-443.

OLISZEWSKI, R., MEDINA, R. B., GONZALEZ, S. N. & PEREZ CHAIA, A. B. 2007. Esterase activities of indigenous lactic acid bacteria from Argentinean goats' milk and cheeses. *Food Chemistry*, 101, 1446-1450.

ONG, L., HENRIKSSON, A. & SHAH, N. P. 2007. Chemical analysis and sensory evaluation of Cheddar cheese produced with Lactobacillus acidophilus, *Lb. casei, Lb. paracasei* or *Bifidobacterium* sp. *International Dairy Journal*, 17, 937-945.

PAN, D. D. & TANOKURA, M. 2004. Purification and characterization of an aminopeptidase from *Lactobacillus helveticus* JCM 1004. *Food Chemistry*, 88, 511-516.

PEREZ, G., CARDELL, E. & ZARATE, V. 2003. Technological characterization of lactic acid bacteria from Tenerife cheese. *International Journal of Food Science and Technology*, 38, 537-546.

PETERSON, S. D. & MARSHALL, R. T. 1990. Nonstarter lactobacilli in Cheddar cheese - a review. *Journal of Dairy Science*, 73, 1395-1410.

PHILLIPS, M., KAILASAPATHY, K. & TRAN, L. 2006. Viability of commercial probiotic cultures (*L. acidophilus, Bifidobacterium* sp., *L. casei, L. paracasei*

and *L. rhamnosus*) in Cheddar cheese. *International Journal of Food Microbiology*, 108, 276-280.

PRASHANT, TOMAR, S. K., SINGH, R., GUPTA, S. C., ARORA, D. K., JOSHI, B. K. & KUMAR, D. 2009. Phenotypic and genotypic characterization of lactobacilli from Churpi cheese. *Dairy Science and& Technology*, 89, 531-540.

PUCHADES, R., LEMIEUX, L. & SIMARD, R. E. 1989. Evolution of free amino-acids during the ripening of Cheddar cheese containing added lactobacilli strains. *Journal of Food Science*, 54, 885-&.

RATTRAY, F. P. & EPPERT, I. 2011. Cheese-Secondary Cultures. In: J.W. Fuquay, P.F. Fox and P.L.H. McSweeney. (eds.) Encyclopedia of Dairy Sciences (Second Edition). San Diego: Academic Press, 567-573.

TAN, P. S. T., SASAKI, M., BOSMAN, B. W. & IWASAKI, T. 1995. Purification and characterization of a dipeptidase from *Lactobacillus helveticus* sbt-2171. *Applied and Environmental Microbiology*, 61, 3430-3435.

THAGE, B. V., BROE, M. L., PETERSEN, M. H., PETERSEN, M. A., BENNEDSEN, M. & ARDO, Y. 2005. Aroma development in semi-hard reduced-fat cheese inoculated with *Lactobacillus paracasei* strains with different aminotransferase profiles. *International Dairy Journal*, 15, 795-805.

THAGE, B. V., HOULBERG, U. & ARDO, Y. 2004. Amino acid transamination in permeabilised cells of *Lactobacillus helveticus*, *Lb. paracasei* and *Lb. danicus*. *Journal of Dairy Research*, 71, 461-470.

VAFOPOULOUMASTROJIANNAKI, A., LITOPOULOUTZANETAKI, E. & TZANETAKIS, N. 1996. Esterase activities of cell-free extracts from 'wild' strains of leuconostocs and heterofermentative lactobacilli isolated from traditional Greek cheese. *Letters in Applied Microbiology*, 23, 367-370.

WILLIAMS, A. G. & 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.

WILLIAMS, A. G., NOBLE, J. & BANKS, J. M. 2001. Catabolism of amino acids by lactic acid bacteria isolated from Cheddar cheese. *International Dairy Journal*, 11, 203-215.

YVON, M. & RIJNEN, L. 2001. Cheese flavour formation by amino acid catabolism. *International Dairy Journal*, 11, 185-201.

8. Conclusions and recommendations

8. Conclusions and recommendations

From our experiments on the addition of a bacteriocin-producing strain of *Lactobacillus paracasei* DPC 4715 to Cheddar cheese milk for controlling the growth of non-starter lactic acid bacteria (Chapter 2) we can conclude that:

1. The strain was able to express its antimicrobial activity against some indicator strains only when tested *in vitro* by a well-diffusion assay.
2. The antimicrobial activity was not detected by a well-diffusion assay against the indicator strain *Lactobacillus casei* 2048^{CM} in cheese and cheese extract when the bacteriocin-producing strain was used as an adjunct culture in Cheddar cheese.
3. pH lower than 5.5 and the temperature used for ripening affected the production of bacteriocin in cheese.
4. The bacteriocin was sensitive to chymosin and cathepsin D and it may have been cleaved by the rennet used for the cheese manufactured or by indigenous milk proteases.

Based on the experiments carried out, the following recommendations are suggested:

1. Since bacteriocins are anti-microbial proteinaceous molecules it is recommended that they should be screened for sensitivity to protease activity when a bacteriocin producing strains is used as adjunct culture for cheesemaking. The interaction between the bacteriocin and cheese ingredients such as rennet used for coagulation might compromise its efficiency.
2. Bacteriocin producing strains used for cheesemaking should be previously tested for their ability to produce the bacteriocin under cheese ripening condition, e.g. pH and low ripening temperature.

From our experiments on the use of *Microbacterium casei* DPC 5281, *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 as adjunct cultures at level of 10^9 cfu/ml in Cheddar cheese milk (Chapter 3) we can conclude that:

1. Coryneform bacteria were present in experimental cheeses at level of ~10^8 cfu/g in all trials at day one and decreased by ~2 log cycles during the first week of ripening. At the end of ripening, coryneforms were present at level of ~10^5 cfu/g in all cheeses.
2. The strains did not affect the composition of cheeses.
3. The strains affected the proteolysis of cheese by increasing the level of pH 4.6 -soluble nitrogen as percentage of total nitrogen and total free amino acids after 60 and 180 days of ripening and the levels of some individual free amino acids.
4. Cheese made with *Corynebacterium variabile* DPC 5305 had the highest level of total free fatty acids in all trials whereas no significant difference was

observed in levels of free fatty acids between the control and experimental cheeses.
5. in Trial A, almost all strains appeared to be good producers of phenylethylamine, putrescine, tyramine, spermidine and spermine, whereas in Trial B and C *Corynebacterium casei* DPC 5293 and *Corynebacterium variabile* DPC 5305 significantly increased only the level of phenylethylamine, putrescine and spermidine.

Generally, coryneform strains accelerated the ripening of Cheddar cheese, but they also contributed to the production of some biogenic amines.

From our experiments on the use of microfluidized strains of *Yarrowia lipolytica* DPC 6266, *Yarrowia lipolytica* DPC 6268 and *Candida intermedia* DPC 6271 to accelerate the ripening of Cheddar cheese (Chapter 4), we can conclude that:

1. After four passes through the microfluidizer, the number of the viable yeast cells decreased from 10^7 log cfu/ml to ~10^3 log cfu/ml.
2. After freeze-drying the yeasts extracts retained their aminopeptidase, dipeptidyl aminopeptidase, 5'phosphodiesterase and esterase activities.
3. The addition of the yeast extracts al level of 2% w/w to the cheese curd during milling did not affect composition, but affected proteolysis, increasing the level of pH 4.6-soluble nitrogen as percentage of total nitrogen and total free amino acids after 60 and 180 days of ripening.
4. Cheese made with *Y. lipolytica* DPC 6266 had significantly higher level of Gly, Phe, GABA, Lys, Arg and Pro than the control cheese; cheese made with *Y. lipolytica* DPC 6268 had significantly higher amount of Tyr and His than the control cheese; the cheese made with *C. intermedia* DPC 6271 exhibited significantly higher concentration of Glu, Val, Leu, Phe, Lys, Arg and Pro than the control cheese.
5. Cheeses made with *Y. lipolytica* DPC 6268 and *C. intermedia* DPC 6271 had the highest levels of lipolysis.
6. After 180 d of ripening, all the experimental cheeses had significantly higher levels of aminopeptidase, dipeptidase and esterase activity than the control cheese. 5'phospohodiesterase activity was detected only in the experimental cheeses.
7. Cheese made with yeasts extracts had higher levels of phenylethylamine tyramine and spermine than control cheese.

Microfluidization and freeze freeze-drying did not affected the enzyme activity of yeasts despite the cells being completely disrupted. The yeast extract showed to positively affect proteolysis in Cheddar cheese.

From our experiments on the use of *Hafia alvei* as an adjunct culture at level of 10^7 and 10^8 cfu/ml in the manufacture of Cheddar cheese (Chapter 5) we can conclude that:

1. Numbers of *H. alvei* in Cheddar cheese dramatically reduced after the first days of ripening and then remained constant at level of ~10^3 cfu/g until the end of ripening (180 d).
2. The strain significantly increased the level of pH 4.6-soluble nitrogen, total free amino-acids after 120 and 180 days, and some individual free amino-acids such as Thr, Ser, Glu, Ala, Val, Gaba, Lys and Asp, whereas no differences in the urea-polyacrylamide gel electrophoresis (urea-PAGE) of the cheeses were detected.
3. *H. alvei* significantly affected the total lipolysis in cheeses only in Trial A.
4. Tyramine and cadaverine were produced at low levels in experimental cheeses, whereas phenylethylamine was present at high level only in cheese C3 in Trial A. Tryptamine was not produced in any of the cheeses and histamine was produced only in cheese C3 in all trials at very low levels.

Generally, *H. alvei* accelerated the ripening of Cheddar cheese, increasing the level of pH 4.6-soluble nitrogen, total free amino acids and some individual free amino acids.

From our experiments on the use of *Bifidobacterium animalis* subsp. *lactis*, strain BB-12®, as a probiotic adjunct culture and Hi-Maize® 260 (resistant high amylose maize starch) as a prebiotic fiber at level of 2 and 4% w/v for the manufacture of low-fat Cheddar cheese (Chapter 6) we can conclude that:

1. Low fat experimental cheeses made with 2 or 4% w/v of Hi-Maize® 260 had a lower moisture content than low fat control cheese, indicating that the starch replaced a portion of water in the matrix and did not show any water-binding capacity.
2. *Bifidobacterium animalis* subsp. *lactis* strain BB-12® acted as a potentially good probiotic culture since it remained viable up to ~10^7 cfu/g during ripening.
3. Numbers of *Bifidobacterium* BB-12 were higher in low-fat experimental cheeses containing Hi-Maize than low-fat and full-fat control cheeses made without starch. This was probably due to the resistant starch acting as a protective agent against lysis or as a nutrient for *Bifidobacterium* BB-12.
4. No significant differences were found in proteolysis by urea-PAGE and capillary electrophoresis analysis after 60 and 180 days of ripening.
5. Hi-Maize® 260 did not affect the level of pH 4.6 soluble nitrogen as percentage of total nitrogen in Cheddar cheese at 60 and 180 days of ripening.
6. Results from texture profile analysis showed that Young's modulus increased proportionally with increasing Hi-maize.
7. Hencky strain at fracture decreased over ripening, was found to be higher in half-fat cheeses than full-fat cheese and increased with increasing in fat replacer.

Hi-maize® 260 did not improve the texture of half-fat Cheddar cheese since it made the experimental cheeses harder than the control, but it increased the viability of *Bifidobacterium* BB-12 during ripening.

Based on the experiments carried out, the following recommendations are suggested:

1. How Hi-Maize® 260 affects the taste and quality of Cheddar cheese should be investigated by sensory analysis.
2. Using lower amount of starch in order to avoid the cheese becoming too hard and brittle.
3. Investigating the effect of other fat replacers such as fructo-oligosaccharide, short chain fructo-oligosaccharide, galacto-oligosaccharide soybean-, isomalto- and xylo-oligosaccharides on texture, sensory properties and the survival of probiotics in cheese.

From our experiments on the aminopeptidase, aminotransferase and esterase activities of *Lactobacillus danicus* 9M3 and 13M1 after growth in cheese-based medium (CBM) or MRS broth (Chapter 7) we can conclude that:

1. Both strains showed aminopeptidase activity against all the substrates tested when grown in MRS, whereas they were active only against Gly-, Arg-, Pro- and Phe-*p*-nitroanalide when grown in CBM.
2. Both strains had the highest aminotransferase activity towards aromatic amino acids (Phe and Trp); branched-chain amino acids (Leu and Val) were degraded at much lower rates.
3. Esterase activity was expressed against *p*-nitrophenyl-acetate, *p*-nitrophenyl-butyrate and *p*-nitrophenyl-palmitate and was significantly higher in CBM than in MRS.

Appendix