YEASTS AS ADJUNCT STARTER CULTURES IN CHEESE MAKING

MEHLOMAKULU N. N.

YEASTS AS ADJUNCT STARTER CULTURES IN CHEESE MAKING

By

Ngwekazi Nwabisa Mehlomakulu

Submitted in fulfilment of the requirements for the degree of

MAGISTER SCIENTIAE

In the

Faculty of Natural and Agricultural Sciences

Department of Microbial, Biochemical and Food Biotechnology

University of the Free State

Bloemfontein

January 2011

Supervisor: Prof. B.C. Viljoen

DECLARATION

I Mehlomakulu N.N. declare that the dissertation hereby submitted by me for the Magister Scientiae degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I further more cede copyright of the dissertation in favour of the University of the Free State.

“I have strength for everything through him who gives me power”

Philipians 4:13

Dedicated to the Bucwa and Tobeka Mehlomakulu family

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions for their contribution to the successful completion of this study:

Prof. B. C. Viljoen for his advice, time, guidance, and patience during this study.

Dairy Belle, Bloemfontein for providing the facilities and the help during cheese making.

Mai Nguyen, Department of Microbial, Biochemical and Food Biotechnology for her contribution during cheese making and sensory analysis.

Prof. Judy Narvhus, Norway, for the assistance with the free amino acid and organic acid analysis.

The **National Research Fund (NRF)** for funding.

The **Food Biotechnology Group,** Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, for their encouragement and support during this study.

To **Tata** and **Mama**, **Nolisindiso**, **Sakhile** and **Simphiwe** thank you for the love, support and encouragement.

To **friends** thank you for the support and encouragement.

To the **team at Biosafety South Africa**, thank you for the amazing support throughout the completion of this study.

To **mighty God**, with whom I had the strength, patience, wisdom and the will pursue this study.

LIST OF ABBREVIATIONS

LAB	Lactic Acid Bacteria
NSLAB	Non-lactic acid bacteria
cfu/g	Colony Forming Units/ gram
a_w	Water activity
KMTBA	α-hydroxy-γ-methylthiobutyrate
HMTBA	α-keto-γ-methylthiobutyrate
VSCs	Volatile sulfur compounds
MTA	S- methyl thioacetate
MTB	S- methyl thiobutyrate
UHT	Ultra High Temperature
FFA	Free fatty acids
ArAAs	Aromatic amino acids
BcAAs	Branched chain amino acids
FAA	Free amino acids
LPL	Lipoprotein lipase
HTST	High temperature short time
SCT	Short chain triglycerides
MCT	Medium chain triglycerides
LCT	Long chain triglycerides
EA	Ethylacetate
MTL	Methanethiol

WSF	Water soluble fraction
AACE	Amino acid converting enzymes
DMS	Dimethylsulfide
DMDS	Dimethyldisulfide
DMTS	Dimethyltrisulfide
HPLC	High Performance Liquid Chromatography
GC/MS	Gas Chromatography/Mass Spectrophometry
PTA	Phosphotungstic acid
VFA	Volatile Fatty Acids
MCA	Milk Clotting Activity
ca	Approximately
FAA	Free Amino Acids
CdNR	Cadmium- Ninhydrin
CdNR-N	Cadium Ninhydrin Nitrogen
YE	Yeast Extract
SP	Secreted Protein
CWAP	Cell wall-associated proteinase
Prt	Proteinase
GDL	Glucono – δ – lactone
nd	Not detected
GABA	γ-amino-butyric acid

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX

CHAPTER 2

Appendix 1: Sensory analysis in single inoculated cheeses at 4 months

CHAPTER 3

Appendix 2: Sensory analysis in co-inoculated cheeses at 4 months

CHAPTER 1

Literature Review

1.1. INTRODUCTION

Milk from several species may be used in the production of cheese, but cow's milk is the most usual milk source, although goat and sheep milk are fairly common for producing specialty cheeses in many countries. Basically the production of cheese is based on three fundamental processes.

- A concentration of the milk constituents.
- A preservation of the milk constituents.
- A biological/enzymatic modification of the milk constituents (McSweeney and Sousa, 2000; Singh *et al*., 2003).

Cheese making involves a number of steps (Fig. 1), of which the coagulation of the protein and acidification of the milk play a major role in the appearance, aroma and flavour of the final product. The primary constituent of cheese is milk, consisting of water, fat, carbohydrates, proteins and trace amounts of vitamins, minerals as well as organic acids. During the acidification of the milk, starter cultures are added to produce lactic acid and to bring about change in texture and flavour of the cheese during the curing and ripening stages. The main milk protein, casein, is degraded to begin the process of proteolysis which results from the breakdown of the protein network. In addition the a_w decreases through water binding by liberated carboxyl and amino groups, and the pH increases.

Secondary catabolic changes also occur such as deamination, decarboxylation, transamination, desulfuration and catabolism of aromatic compounds including phenylalanine, tyrosine, tryptophan and reactions of amino acids with other compounds which aid in texture development. Proteolysis contributes to the taste of cheese by the breakdown of proteins into peptides (small-, medium- and large peptides) and amino acids, and the sapid flavour components generally partition into the soluble fraction on extraction of cheese with water. Large peptides (water insoluble) do not contribute directly to cheese flavours, but are important for the development of the correct texture.

The second dominant component of milk is the lipid fraction, also known as milk fat (or butter fat), which has as a very complicated composition and structure. Milk fat is relatively rich in low molecular weight fatty acids including: butyric, caproic and capric. These fatty acids are released on hydrolysis and contribute to the cheese flavour due to their volatile nature. Milk fat is composed primarily of triglycerides (triacyglycerides), which account for 98 % of the total milk fat with small amounts of other milk lipids constituting the remaining 2 %. Fat hydrolysis (lipolysis) during cheese ripening results in low molecular weight molecules such as ketones, secondary alcohols, lactones and esters. Lactose is the dominant carbohydrate present in milk, also known as milk sugar and is dispersed throughout the milk serum. The principal products of lactose metabolism are L- or D- lactate or a racemic mixture of both. Lactate contributes to the flavour of acid curd cheeses and probably also contributes to the flavour of ripened cheese varieties, particularly in early maturation (Fig. 2) (Singh *et al*., 2003).

1.2. MICROORGANISMS ASSOCIATED WITH DAIRY PRODUCTS

Microorganisms are an essential component of all natural cheese varieties and play an important role during both cheese manufacture and cheese ripening. They can be divided into both starters and secondary microflora. Starter cultures are homofermentative lactic acid bacteria which are added to bring about a fermentation process, and consisting of one or more species of bacteria (Beresford *et al.*, 2000; Welthagen and Viljoen, 1996). Starter cultures may be either in blends of defined strains or, as in the case of many cheeses manufactured by traditional methods, composed of undefined mixtures of strains which are either added at the beginning of manufacture or are naturally present in the cheese milk. During cheese ripening, the starter culture and the secondary flora promote a complex series of biochemical reactions that are vital for proper development of both flavour and texture (Beresford *et al.,* 2000). Yeasts as adjunct starters in cheese making increase the pH during ripening and thereby support the growth of LAB (lactic acid bacteria) which contribute to the texturisation and coagulation of the curd (Table 1).

In the modern cheese industry, microbial starter cultures are prerequisites for the production of safe products of uniform quality. The primary function of starters is to produce acid at a reliable and predictable rate. Adjunct starters and non-starter lactic acid bacteria (NSLAB) may originate in the milk or in the cheese making environment or may be intentionally added e.g. yeasts as adjunct starters (Fox *et al*., 1996).

The role of starters in the dairy industry is as follows:

- The production of lactic acid as a result of lactose fermentation, the lactic acid imparts a distinctive and fresh, acidic flavour during manufacture of fermented milks and in cheese making, lactic acid is important during the coagulation and texturisation of the curd.
- The production of volatile compounds (e.g. diacetyl and acetaldehyde) which contribute towards the flavour of the dairy products.
- The starter cultures may possess a proteolytic and lipolytic activity, which may be desirable, especially during the maturation of some types of cheeses.
- Compounds such as alcohol may be produced, which are essential during manufacture of products such as Kefir and Kumiss.
- The acidic condition of the products due to the activity of the starters prevents the growth of pathogens as well as spoilage organisms.

Primary starter cultures are mesophilic lactic acid cultures and thermophilic lactic acid cultures. The mesophilic lactic acid starter cultures (optimum temperature 20 - 30 °C) are homofermentative producing lactic acid only and are widely used in the cheese industry. Mesophilic starters include but are not limited to *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Thermophilic lactic acid starter cultures (optimum temperature 37 - 45 °C) are heterofermentative producing lactic acid; carbon dioxide; aroma compounds (e.g. ethanol and acetic acid) from glucose rapidly at high temperatures and are used in the manufacture of yoghurt, acidophilus milk and high scalded cheese (e.g. Swiss varieties). Thermophilic starters include *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*. The combined activity of mesophilic and thermophilic lactic acid cultures and yeasts yields a lactic acid/ alcohol fermentation in milk. Ethyl

alcohol is mainly produced, and the level can reach as high as 1.5 %; the flavour components are due to acetaldehyde, diacetyl and lactic acid (Tamime, 2002).

The contributions of yeasts to cheese flavour development during ripening are generally underestimated because their occurrence is not widely appreciated, and their roles are generally not well established. The significance of the presence of yeasts depends on the particular type of cheese. They develop at the early stages of ripening, when they participate in the deacidification of the curd through lactate/lactose consumption and they could also be involved in flavour compound biosynthesis. The presence of yeasts during ripening is essential, since the rise in pH enables the acid-sensitive bacteria that are necessary for the typical cheese to develop at the cheese surface. The occurrence of yeasts in cheese is not unexpected because of the low pH, low moisture content, elevated salt concentration and refrigerated storage of these products. In some cheeses, yeasts contribute to spoilage or make a positive contribution to flavour development during maturation.

The predominance and growth of yeasts in dairy products is due to the following:

- Fermentation or assimilation of lactose.
- Production of extracellular proteolytic enzymes.
- Assimilation of lactic acid.
- Assimilation of citric acid.
- Growth at low temperatures.
- Tolerance of elevated salt concentration (Fleet, 1990; Welthagen and Viljoen, 1998).

Yeasts contribute positively to the fermentation and maturation process of cheeses by inhibiting undesired microorganisms present, supporting the function of the starter culture and by metabolizing lactic acid leading to an increase in pH. In addition, the formation of alkaline metabolism products, such as ammonia from amino acid deamination, aid in the deacidification of the cheese and promotes the growth of bacteria. Yeasts also have proteolytic and lipolytic activity, excrete growth factors, like B-vitamins, pantothenic acid, niacin, riboflavin and biotin, which promote the growth of

lactic acid bacteria and produce gas that leads to curd openness (Viljoen and Greyling, 1995).

The smear microorganisms that develop on the surface of the cheese play a role in the ripening process, both through the action of proteolytic and lipolytic enzymes, and the formation of many alkaline products that penetrate the body of the cheese. The yeasts are involved both directly and indirectly in the ripening process. Assimilation of lactate, formation of alkaline metabolites, liberation of bacterial growth factors, fermentation of lactose, lipolysis, proteolysis and formation of aroma compounds are some of the yeast activities that are considered important for the typical characteristics of the smear surface-ripened cheeses. The flavouring activity is usually considered, as one of the most important in supporting the use of some yeasts as starter cultures for cheese production and some yeasts appear to induce particular flavour, especially when associated with bacteria. Some surface yeasts are involved in the interactions with bacterial flora thereby supporting the function of surface-growing bacteria, which, through their proteolyic and lipolytic activities, are also essential for cheese ripening. The metabolism of lactate and the formation of alkaline metabolites, such as ammonia from amino acid deamination, lead to the deacidification of the cheese surface, enabling the growth of less acid-tolerant, but more proteolytic, salt-tolerant microorganisms such as micrococci, *Brevibacterium lines*, *Arthrobacter* and *Corynebacterium* spp (Corsetti *et al*., 2001).

1.2.1. Yeasts frequently isolated in dairy products

Although not dominant within the microorganisms of the surface-ripened cheeses, yeasts of the genera *Candida*, *Cryptococcus*, *Debaryomyces*, the yeast-like *Geotrichum candidum* and its perfect form *Galactomyces geotrichum*, *Hansenula*, *Kluyveromyces, Pichia*, *Rhodotorula*, *Saccharomyces*, *Yarrowia* and *Zygosaccharomyces* have been found. The most important species of yeasts isolated from Camembert and Tilsiter include those that can be considered as participating in the ripening process of other surface-ripened cheeses; they are *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Debaryomyces hansenii*, and their imperfect forms, *Saccharomyces cerevisiae*, *Galactomyces candidum*, *Candida catenulata* and

Yarrowia lipolytica. Studies on the evolution of yeast population on the surface of cheeses such as Tilsiter, Reblochon and Limburger have shown that they reach the highest number of 10^8-10^9 cfu/g of smear after about seven days of ripening (Corsetti *et al*., 2001).

Debaryomyces hansenii

Debaryomyces hansenii strains have varying abilities to produce proteolytic and lipolytic enzymes and also have the ability to utilise lactic acid, citric acid, glucose and galactose. The utilisation of organic acids results in an increase in the pH; rendering the environment more favourable for other microorganisms, (*D. hansenii* inhibits the growth of *Clostridium tyrobutyricum* and *Clostridium butyricum* in cheese brines) (Fatichenti *et al*., 1983). *Debaryomyces hansenii* the perfect form of *Candida famata*, predominated in most studies of yeasts associated with dairy products. The reason for the high numbers of *D. hansenii* in cheeses is due to the species ability to grow at low temperatures, high salt concentrations, and low a_w values and also to their proteolytic and lipolytic activity. A synergistic effect between LAB and *D. hansenii,* has been reported resulting with a long survival in cheese of the lactic acid bacteria.

The incorporation of *D. hansenii* as an adjunct starter in matured Cheddar cheese was studied in co-inoculation with *Y. lipolytica* and LAB, and also as the sole inoculum. When *D. hansenii* was applied as the sole co-inoculum, the yeast numbers decreased gradually during the ripening period to a minimum value of 4.25 X 10^2 cfu/g after six months of maturation. The cheese developed a fruity flavour after two months of ripening in addition to an advanced development of the desired Cheddar flavour compared to the control cheese. After six months, the cheese developed a bitter taste. Co-inoculation of *D. hansenii* and *Y. lipolytica* together as starters was investigated with the number of yeasts in the cheese remaining significantly higher >2 - 3 log units compared to the cheeses with individual inoculated yeasts during the initial two months of maturation. *Debaryomyces hansenii* predominated *Y. lipolytica* species during the maturation stage in the cheese exhibiting counts of 3 log units higher. During the final three months of ripening only *D. hansenii* was found. With the addition of both yeast species to the starter culture, the cheese developed a good,

slightly sweet Cheddar taste after two months of ripening, and after four mouths, there was a stronger Cheddar taste development compared to the control cheese. At the end of the ripening period, the development of a mature taste was more significant compared to that of the control cheese. After nine months, the cheese had a clean, slightly sweet, pleasant taste and retained its good, strong flavour, while the control cheese developed a bitter and slightly impure taste at the end (Ferreira and Viljoen, 2003).

Growth of *D. hansenii* in a co-culture with *K. marxianus* and *Y. lipolytica* as well as sole cultures in a cheese ecosystem was also investigated in another study. Development of *K. marxianus* was after a 24 h lag phase during which the initial population remained unchanged ca. 2.104 cfu mL^{-1} and this population steadily increased between 30 h and 62 h of culture, reaching around 3.108 cfu mL^{-1} until the end of the experiment. *Debaryomyces hansenii* and *Y lipolytica* displayed a continuous and rapid development in the first hours of the cultivation, increasing from an initial population of almost 2.104 cfu mL^{-1} to between 4.108 cfu mL^{-1} and 8.107 cfu mL^{-1} at 38 h, respectively. Thereafter, *D. hansenii* showed a stationary phase until 80 h, whereas the *Y. lipolytica* population stabilized at 108 cfu mL^{-1} after 58 h of cultivation. When the strains were cultivated in co-culture the development of *Y. lipolytica* did not differ significantly, the maximal populations were significantly decreased (< 1 to 1.5 log units) for *D. hansenii* and *K. marxianus* compared to those of pure cultures. However, the initial growth of all yeasts was accelerated when cultivated in co-culture and this was clear with *K. marxianus*, for which no lag phase was observed when it was cultivated in co-culture with *D. hansenii* and *Y. lipolytica*. The initial growth acceleration observed in co-culture, compared to that in pure cultures, suggested that there is a competition for growth among the yeasts as reported by the authors (Cholet *et al*., 2007).

Yarrowia lipolytica

Yarrowia lipolytica can be isolated from cheese, yoghurts, kefir, shoyu, meat or shrimp salads and similar materials. Since it is aerobic, it is easy to eliminate from dairy products, and its maximum growth temperature is below 32 – 34 °C thus it is not

considered a possible human pathogen (Spencer *et al*., 2002). *Yarrowia lipolytica* occurs frequently in milk products; the species has the ability to predominate over naturally occurring yeasts and is recognised as the species having the strongest lipolytic activity. Its compatibility and stimulating action when co-inoculated have been indicated. The species is known for its strong proteolytic and lipolytic activity, and it was possible to accelerate cheese ripening and to improve the quality of cheese by the addition of this yeast species during cheese manufacture. The use of *Y. lipolytica* as the sole co-inoculum in matured Cheddar cheese resulted in the number of yeasts gradually decreasing and ceasing to survive after four months of the ripening period. After two months of ripening, an enhanced Cheddar flavour was obtained for the cheese. The cheese retained its strong Cheddar flavour and the fruity taste after six months of maturation (Ferreira and Viljoen, 2003).

The ability of *Y. lipolytica* to grow and compete with other naturally occurring microbial groups or yeasts was also evaluated when *Y. lipolytica* RO21 strain was inoculated in association with the lactic starter culture. Analysis of the composition of the yeast population showed that *Y. lipolytica* tended to become the dominant yeast species present in the inoculated samples, at least over the period considered. *Yarrowia lipolytica* was able to grow up to 8 log cfu/g in the rind, while its levels did not exceed 7 log cfu/g in the cheese centre. The co-inoculum with *Y. lipolytica* did not show any inhibition of both inoculated and naturally occurring lactic acid bacteria; on the contrary, the strain RO21 seemed to stimulate the proliferation with respect to the control inoculated milk culture at least in the rind and in the centre of the cheese. The highest score for flavour, body and texture was attributed to the cheese using *Y. lipolytica* as a starter without milk culture. These samples were characterised by a significant intensification of flavour and an accelerated textural development. *Yarrowia lipolytica* was shown to possess some of the essential attributes for use as a cheese starter, (i) the ability to grow and compete with naturally occurring yeasts; (ii) the compatibility with LAB and possible stimulating action when co-inoculated and (iii) the remarkable proteolytic activity on both α_{s1} -and β- caseins (Guerzoni *et al*., 1996).

Kluyveromyces lactis

According to literature *K. lactis* is a strong producer of aroma compounds responsible for the fruity flavours such as alcohols (isoamyl alcohol, isobutyl alcohol and 2-phenyethanol), aldehydes (2-phenyacetaldehyde), esters (ethylacetate and 2-phenyacetate) as well as monoterpenes. These compounds play a major role in the final development of the cheese flavour and aroma (Law, 2001). Large numbers (log 7.47cfu/g) of *K. lactis* were found in Feta cheese, but were not found in 22 other cheese types when yeasts were isolated from different cheeses (Welthagen and Viljoen, 1996). *Kluyveromyces lactis* metabolizes lactose as well as glucose, and mixtures of glucose and galactose can be used as carbon sources, though glucose is metabolized first. The yeast produces a ß-galactosidase that hydrolyzes lactose, allowing utilization of the sugars by the yeast (Spencer *et al.,* 2002).

Saccharomyces cerevisiae

In a study by Welthagen and Viljoen (1996), on the presence of yeasts in different cheese types, *S. cerevisiae* was only isolated from Cheddar cheese and Pecorina Topico. The infrequent isolation of *S. cerevisiae* with high-salt cheese is related to its weak ability or inability to tolerate NaCl at concentrations exceeding 5 % (w/w). However, *S. cerevisiae* is capable of growth in cheese with a low salt concentration and utilise lactic acid as a possible growth substrate.

Kluyveromyces marxianus

The presence of this yeast in dairy products is common, due to its ability to ferment and assimilate lactose, lactic acid and citric acid. In addition, the production of proteases and lipases that could hydrolyse milk casein and fat, favour its growth in dairy products (Fleet and Mian, 1987). The main primary and secondary products of lactose fermentation by *K. marxianus* are ethanol, glycerol, lactic acid, acetic acid and propionic acid (Roostita and Fleet, 1996). Although, *K. marxianus* may proliferate in the interior of the cheese due to its ability to grow at low oxygen levels, it is well known for its ability to grow on the surface of cheeses (Martin *et al*., 1999). The yeast strain *K. marxianus* $44_{(8)}$ initially *K. lactis* $44_{(8)}$ was used as a model organism in a cheese

ecosystem, and its development started after a 24-h lag phase during which the initial population remained unchanged (approximately 2 X 10^4 cfu/ mL^{-1}). The *K. marxianus* population steadily increased between 30 h and 62 h of culture, reaching around 3 X 10^8 cfu/ mL^{-1} until the end. When co-cultured with *D. hansenii* and *Y. lipolytica* the initial growth of all yeasts was accelerated. This was particularly clear with *K. marxianus*, for which no lag phase was observed. Substrate consumption and metabolite production resulted in almost over 92 % of lactose to be consumed between 30 h and 62 h with *K. marxianus* cultures, while lactate was hardly consumed during the remaining time from an initial lactose concentration of 18.3 $\pm$ 0.3 g/ $liter^{-1}$ and lactate concentration of 22.9 $\pm$ 0.1 g/ $liter^{-1}$. During the stationary phase, 16 % of the lactate was consumed, and lactose was completely exhausted after 72 h (Cholet *et al*., 2007) which shows the ability of the yeast to ferment or assimilate lactose.

Geotrichum candidum

Geotrichum candidum is a fungus that colonizes nearly all fungal surface-ripened cheeses during the early stages of ripening. On some cheeses, like St. Marcellin, it is responsible for the appearance of the cheese, imparting a uniform, white, velvety coat to the surface. On soft cheeses, such as Camembert, and semi-hard cheeses, such as St. Nectaire and Reblochon, the biochemical attributes of *G. candidum* impact the course of cheese ripening. Lipases and proteases of *G. candidum* release fatty acids and peptides that can be metabolized by ensuing microbial populations that contribute to the development of distinctive flavours and other qualities. *Geotrichum candidum* neutralizes the curd by catabolizing lactic acid produced by lactic acid bacteria and by releasing ammonia during the metabolism of amino acids. The latter activity prepares the cheese surface for colonization by acid-sensitive bacteria such as *Brevibacterium* species. Metabolites produced by *G. candidum* can also inhibit *Listeria monocytogenes* (Marcellino *et al*., 2001).

1.2.2. Microbial interactions between yeasts and lactic acid bacteria

The types of interactions found in mixed populations of microorganisms are classified on the basis of effects, as direct or indirect interactions. Indirect interactions refer to competition, commensalism, mutualism, ammensalism or neutralism, and direct

interactions to predation and parasitism. Dairy products develop their nutritional and organoleptic qualities as a result of the metabolic activity of a succession of different microorganisms and it is unlikely that the interactions will separate into these discrete groups since more than one type of interaction may occur simultaneously. The yeasts as part of the interactions, either contribute to the fermentation by supporting the starter culture, inhibiting undesired microorganisms causing quality defects or adding to the final product by means of desirable biochemical changes like the production of aromatic compounds, and with proteolytic and lipolytic activities. The interactions may also be detrimental causing spoilage, by inhibiting the growth of starter culture and producing excessive gas, off-flavours, slime formation or discolouration (Fig 3). The "killer factor" demonstrated in yeasts isolated from cheese brines, with its possible broad antimicrobial spectrum may affect bacteria and moulds including the starter culture. The metabolic interactions are governed by the yeasts inherent technological characteristics and biochemical activities which provide essential growth metabolites such as amino acids and vitamins, remove toxic products of metabolism, inhibit the growth of undesired microorganisms by lowering the pH, secrete alcohol, produce CO_2 and encourage the growth of the starter culture by increasing the pH due to the utilization of organic acids (Viljoen, 2001; Jakobsen and Narvhus, 1996).

Lactic acid bacteria species have nutritional requirements for many compounds, and can also be stimulated by the synthesis of vitamins or by the production of amino acids by yeasts. In addition some LAB species release galactose, which may favour the growth of lactose–negative yeasts. The capability of some *Y. lipolytica* and *D. hansenii* strains to specifically inhibit the growth of spoilage and pathogenic microorganisms is considered beneficial. The combination of low pH produced by the bacterial starter plus the alcohol and CO_2 produced by the yeasts is inhibitory to many undesirable microorganisms. The negative interactions recorded mainly concern the mutual inhibition of growth. Lactic acid bacteria produced compounds such as phenyl-lactic acid, 4-hydroxy-phenyl-lactic and cyclic peptides inhibiting yeasts; conversely, the growth of LAB is inhibited by fatty acids produced by the metabolism of lipolytic yeasts. Positive and negative interactions influencing the growth and metabolism of either LAB or yeasts may modify ripening time and/or the production of essential odours (Álvarez- Martín *et al*., 2008).

In an investigation of the interactions on growth and in the production of organic acids and volatile compounds between yeast strains and strains of typical LAB species, positive and negative interactions were observed. Both of the species were used as single and co-cultures in UHT– treated milk. The species were twelve yeast strains and four most typical LAB species found in cheese (*Lactococcus lactis subsp. cremoris* (Wg2), *Lactococcus lactis* subsp *lactis* (2BA1) *Lactobacillus paracasei* (2BC7*)*, and *Leuconostoc citreum* (VI-19)). Lactic acid bacteria species grew in milk in single cultures from around 7.0 $\log_{10}$ mL^{-1} in the inocula to higher than 9.0 $\log_{10}$ mL^{-1} after 48 h at 25 °C. The LAB strains in co-culture increased their numbers over those seen in single cultures. In particular, the growth of 2BA1 was enhanced in most co-cultures. By contrast, VI-19 was inhibited in co-culture with four yeast species: *C. famata*, *D. hansenii*, *K. lactis* and *Pichia membranifaciens*. *Lactococcus lactis* subsp. *cremoris* (Wg2) was also inhibited in a majority of co-cultures, though this inhibition was only significant in co-cultures with *C. pararugosa*, *D. hansenii* and *G. candidum*. *Debaryomyces hansenii* strain inhibited all four LABs, although the inhibition of 2BA1 was not significant. In single culture, yeast species reached between 5.93 $\log_{10}$ mL^{-1} and 7.46 $\log_{10}$$mL^{1}$.

The growth of most yeasts was significantly affected in co-culture. *Candida famata* 1AD5 was severely inhibited by all LAB strains, as were *D. hansenii* 3AD24 and *Y. lipolytica* 4AD16. In contrast, higher numbers in co-culture were scored for two (out of three) *G. candidum* and two *K. lactis* strains. Though not significant, a slight increase was also shown in all four *C. pararugosa* 3AD19 combinations. The remaining yeast strains (*C. famata* 2BD10, *G. candidum* 3AM9M, *Pichia fermentans* 3AD16 and *P. membranifaciens* 1AD89) were slightly promoted or inhibited by the two *L. lactis* strains, but were significantly inhibited by both *L. citreum* and *Lb. paracasei* strains. In agreement with their growth, all LAB strains acidified the milk in single and co-cultures, reaching a final pH of between 4.26 and 4.05. In contrast, most of the yeasts strains had no appreciable effect on pH in single cultures. However, *C. famata* 2BD10, *D. hansenii* and *P. fermentans* produced slight pH decrements and the *G. candidum* strains acidified the milk to the same extent as the LAB (final pH from 4.32 to 4.36). In general, acidification was favoured by co-culture and the pH reached lower values than in single LAB cultures. High amounts of lactic acid were produced

by all LAB strains (up to 930 mg/100mL^{-1} by the *Lb. paracasei* strain). All LAB produced variable levels of propionic and butyric acids, and a small quantity of pyruvic acid. *Leuconostoc citreum* and *Lb. paracasei* also produced noticeable amounts of acetic acid. At the same time, all LAB species consumed succinic acid and formic acids, and although not statistically significant, a part of orotic acid. Moderate amounts of pyruvic and propionic acids were produced by the majority of strains, and *P. fermentans* and *C. famata*, produced acetic acid to a level comparable to that of *L. citreum* and *Lb. paracasei*. Among the yeasts species, lactic acid was only produced by three strains of *G. candidum* (618 mg/100mL^{-1} on average). Succinic acid, which was completely metabolised by *P. membranifaciens* and *G. candidum*, was variably consumed by all other strains. *Pichia fermentans* metabolized citric acid to completion, while a part was used by *C. famata*, *G. candidum*, and *P. membranifaciens* strains.

Differences in organic acid production between single and co-cultures were observed for particular organic acids in several yeast-LAB combinations. *Lactococcus lactis* produced noticeable levels of acetic acid (around 40mg/100mL) in co-culture with *P. membranifaciens* and *Y. lipolytica*, while neither of the single cultures did. The utilization of citric acid by *L. citreum* and *Lb. paracasei* was favoured in most co-culture combinations. However, it was severely inhibited in the co-culture of *L. citreum* with *Y. lipolytica*. *Yarrowia lipolytica* also inhibited the production of acetic and butyric acids by *L. citreum*. These two effects may be explained by the significant growth reduction of *L. citreum* in this co-culture. Degradation of citric acid was also partially inhibited in the co-cultures of *Lb. paracasei* with *G. candidum* and *P. fermentans*. Either the production of acetic acid and butyric acids by *L. citreum* is repressed in co-culture with *C. famata* and *Y. lipolytica*, or they are consumed by the yeast (Álvarez-Martín *et al*., 2008).

1.3. CHEESE RIPENING

Cheese ripening involves three main processes namely, the decomposition of protein (proteolysis), the decomposition of lactose (glycolysis) and the decomposition of fat (lipolysis). Changes during cheese ripening may be divided into two general stages.

The first stage (primary fermentation) which includes changes that occur in carbohydrate, fat and protein content of the cheese curd, resulting in the accumulation of lactic acid, fatty acids and free amino acids, which are responsible for the basic textural changes and flavour that occur during ripening/ maturation. The second stage (secondary fermentation) comprises changes involving the formation of flavour, aroma and/ or volatile compounds brought about by the action of enzymes primarily from microorganisms on the primary fermentation products which include – deamination, decarboxylation and desulfurylation of amino acids, β - oxidation of fatty acids, further fermentation of the organic acids and even some synthetic changes i.e. esterification. These secondary changes are responsible for the finer aspects of cheese flavour and modify cheese texture.

The gradual breakdown of carbohydrates, lipids and protein during ripening is mediated by several agents, including:

i. Residual coagulant.
ii. Starter bacteria and their enzymes.
iii. Non- starter bacteria and their enzymes.
iv. Indigenous milk enzymes, especially proteinases and
v. Secondary inocula with their enzymes (Singh *et al*., 2003).

1.3.1. Proteolysis

The degradation of milk proteins – caseins (proteolysis) leads to peptides and free amino acids, which can subsequently be taken up by cells. The conversion of caseins is undoubtedly the most important biochemical pathway for flavour formation in hard type and semi-hard type cheese. Proteolysis occurs in all cheese varieties and is considered to be a prerequisite for good flavour development. It is affected by a number of agents including residual coagulant, indigenous milk proteinases, and the proteinases and peptidases of starter and non-starter bacteria to yield small peptides and free amino acids (FAAs). Proteolysis is the most complex of the three primary events during cheese ripening and is possibly the most important for the development of flavour and texture, especially in internal bacterium ripened cheeses. Proteolysis contributes to cheese ripening in at least four ways: (i) a direct contribution to flavour

via amino acids and peptides, some of which may cause off-flavours, especially bitterness, or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters, etc (ii) greater release of sapid compounds during mastication (iii) changes in pH via the formation of NH_3 (iv) changes in texture arising from breakdown of the protein network, increase in pH and greater water-binding by the newly formed amino and carboxyl groups .

Although the ripening of some cheese varieties (e.g. Blue and Romano) is dominated by the consequence of lipolysis, proteolysis is more or less important in all cheese varieties. In the case of Cheddar and Dutch type cheeses, and probably other varieties, many authors believe proteolysis is the major biochemical event during ripening. A high relation exists between the intensity of Cheddar cheese flavour and the concentration of FAAs. Undoubtedly, further proteolysis by coagulant, plasmin and bacterial proteinases modifies the texture further. Certain proteolytic and lipolytic enzymes derived from yeasts contribute to the ripening process and many types of yeasts are carriers of proteolytic enzymes. Species with high proteolytic activity are *K. lactis*, *Kluyveromyces fragilis, Candida pseudotropicalis* and *D. hansenii*, *Y. lipolytica* and *Candida catenulata* are species with a strong extracellular lipolytic activity. Peptidases, i.e. aminopeptidases, present in nearly all the yeast species, and also carboxypeptidases, seem to play a major role in the proteolysis of milk proteins. The peptidolytic activity of yeasts may also play an important role in the breakdown of bitter peptides by releasing smaller peptides and amino acids. In particular, *Galactomyces candida* is known to show such activity (Smit *et al.,* 2002; Fox, 1989).

1.3.1.1. Catabolism of amino acids

Protein degradation varies with the variety of cheese. Primary protein hydrolysis to amino acids occurs to some extent in all cheese varieties. It is recognized that amino acids contribute to the background flavour of cheese, but whether amino acids add to the characteristic flavour is problematic (Harper and Kristoffersen, 1956).

During ripening, proteolysis in cheese is catalyzed by enzymes from: (i) the coagulant (e.g., chymosin, pepsin, or plant/fungal acid proteinases); (ii) the milk (plasmin, cathepsin D and perhaps other somatic cell proteinases) (iii) the starter (iv) the

nonstarter or (v) the secondary starter (e.g., *P. camemberti*, *P. roqueforti, Propionibacterium* spp*., Br. linens* and other coryneforms) and (vi) exogenous proteinases and/or peptidases used to accelerate ripening. In most cheese varieties, the initial hydrolysis of caseins is caused by the coagulant and to a lesser extent by plasmin and perhaps somatic cell proteinases (e.g. cathepsin D), which results in the formation of large (water-insoluble) and intermediate sized (water-soluble) peptides which are subsequently degraded by the coagulant and enzymes from the starter and non-starter flora of the cheese. The final products of proteolysis are FAAs, the concentrations of which depend on the cheese variety, and which have been used as indices of ripening. The concentration of FAAs in cheese at any stage of ripening is the net result of the liberation of amino acids from casein and their transformation to catabolic products. The production of small peptides and FAAs is caused by the action of microbial proteinases and peptidases (McSweeney and Sousa, 2000).

Amino acids are the precursors of various volatile cheese flavour compounds, which have been identified in cheese. Medium and small peptides and FAAs contribute to the background flavour of most cheese varieties and some individual peptides have 'brothy', 'bitter', 'nutty' and 'sweet' tastes. The principal amino acids in Cheddar cheese are Glu, Leu, Arg, Lys, Phe and Ser and concentrations of amino acids generally increase during ripening, with the exception of Arg, the concentration of which is reported to decrease later in ripening. The level of peptides and FAAs soluble in cheese in 5 % phosphotungstic acid (PTA) has been considered to be a reliable indicator of the rate of flavour development and the composition of the amino acid fraction and the relative proportions of individual amino acids are thought to be important for the development of the characteristic flavour.

Although LAB (*Lactococcus*, *Lactobacillus*, *Streptococcus*) are weakly proteolytic, they possess a very comprehensive proteinase/peptidase system and are able to hydrolyze milk peptides down to FAAs (Smit *et al*., 2002; McSweeney and Sousa, 2000). Amino acid catabolism produces, in turn, a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols, which contribute as a whole to cheese flavour (Urbach, 1995); however, the roles played by each species (or even genus) of LAB in terms of those biochemical routes are not yet fully understood.

There are usually three recognizable steps in this complex process: the first one pertains to such reactions as decarboxylation, deamination, transamination, desulfuration, and hydrolysis of side-chains; the second one involves conversion of the resulting compounds (mainly amines and *α*-ketoacids), as well as some FAAs themselves, to aldehydes affected by deaminases; and the third stage corresponds to reduction of aldehydes to alcohols, or their oxidation to carboxylic acids. Sulfur containing FAAs may undergo specific chemical reactions, which are responsible mainly for the generation of methanethiol and a few other sulfur derivatives (Tavaria *et al*., 2002).

Amino acids can be converted in many ways by enzymes such as deaminases, decarboxylases, transaminases (aminotransferases), and lyases (Fig. 4). Generally, amino acid conversion to aroma compounds proceeds by 2 different pathways (Fig. 4.1.). The first one is initiated by elimination reactions catalysed by amino acid lyases which cleave the side chain of amino acids. This pathway has been observed for aromatic amino acids (ArAAs) and methionine and leads by a single step to phenol, indole and methanethiol, respectively. The second pathway goes through α-keto acid intermediates, it is mainly initiated by a transamination reaction catalysed by amino acid aminotransferases and has been observed for ArAAs, branched-chain amino acids (BcAAs) and methionine. Amino acid transamination is a key step in the amino acid conversion to aroma compounds by cheese microorganisms.

Transamination of amino acids results in α- keto acids that can be converted into aldehydes by decarboxylation and, subsequently, into alcohols or carboxylic acids by dehydrogenation. Many of these components are odour-active and contribute to the overall flavour, e.g. by hydrogenates activity towards α-keto acids resulting in the formation of hydroxyl-acids, which do hardly contribute to flavour. Aromatic amino acids, branched-chain amino acids, and methionine are the most relevant substrates for cheese flavour development. Conversion of aromatic amino acids can result in formation of undesirable flavours, so-called off-flavours, such as p-cresol, phenylethanol, phenylacetaldehyde, indole and skatole, which contribute putrid, faecal or unclean flavours in cheese. Conversion of trypophan or phenylalanine can also lead to benzaldehyde formation. This compound is found in various hard-type and

soft-type cheeses and contributes positively to the overall flavour. Branched-chain amino acids are precursors of various aroma compounds such as isobutyrate, isovalerate, 3-methylbutanal, 2-methylbutanal, and 2-methylpropanol. These compounds are found in various cheese types. Volatile compounds derived from methionine, such as methanethiol, dimethyl sulphide (DMS) and dimethyl trisulphide (DMTS), are regarded as essential components in many cheese varieties (Smit *et al*., 2002; Yvon and Rijnen, 2001).

1.3.1.2. Catabolism of peptides

The degradation of milk proteins - caseins leads to peptides and free amino acids, which can subsequently be taken up by cells. Peptide uptake occurs via oligopeptide transport systems, and di- / tri-peptide transporters. Following uptake, the peptides are degraded intracellulary by a variety of peptidases. These peptidases of LAB can be divided into endopeptidases, aminopeptidases, di- /tri-peptidases and proline-specific peptidases. The specialized peptidases in LAB for hydrolysis of Pro containing peptides have been postulated to be important for the degradation of casein-derived peptides, since these are known to have high proline content (Smit *et al*., 2002). In many bacterium-ripened cheeses, the *L. lactis* cell envelope-associated proteinase (lactocepin, EC 3.4.21.96) is the most important microbial enzyme for the conversion of large molecular-weight (water-insoluble) peptides produced by coagulant or plasmin into the small water-soluble peptides needed for flavour development (Broadbent *et al*., 2002).

Although it is known that peptides can taste bitter or delicious and that amino acids can taste sweet, bitter or broth-like, the direct contribution of peptides and amino acids to flavour is probably limited to a basic taste. The balance between formation of peptides and their subsequent degradation into amino acids is very important since accumulation of peptides might lead to a bitter off-flavour in cheese. Various bitter-tasting peptides have been identified and these should be degraded rapidly in order to prevent bitterness. Specific cultures have been selected with high bitter-tasting–peptide degrading abilities and such cultures are nowadays frequently used in preparation of various types of cheeses (Smit *et al*., 2002).

Bitterness develops when small to medium-sized hydrophobic peptides produced by the coagulant and some starter bacteria accumulate to levels that exceed desirable taste thresholds, whereas starter autolysis releases intracellular peptidases that can hydrolyze many of these peptides. However, the degree of starter autolysis and the individual activity of peptidases vary widely among lactococci (Broadbent *et al*., 2002). The rapid release of intracellular enzymes due to autolysis of lactic acid bacteria in the cheese matrix post-manufacture is thought to play a role in the acceleration of cheese ripening.

Cell lysis is therefore a necessary step to release the cytoplasmic peptidases into the cheese curd and allow access to their substrates. The earlier the peptidases are released, through lysis, the sooner they can participate in proteolysis and, hence, accelerate ripening. The rate and extent of starter culture lysis in young cheese is linked positively to the quality and rate of development of flavour in Cheddar cheese. Previous studies have shown that starter lysis in cheese results in an increase in the concentration of FAAs and to a decrease in bitterness (Hannon *et al*., 2003). Hence, the selection of highly autolytic strains for cheese manufacture not only appears to be a means to accelerate the development of cheese flavour but also to potentially improve its sensory characteristics.

1.3.1.3. Proteolytic activity of LAB and yeasts

A number of different LAB and other cheese microorganisms have been evaluated for their ability to degrade amino acids to aroma compounds. The ability has been determined by incubating resting cells or cellular extracts in cheese models or in synthetic media containing casein or free amino acids and analyzing products formed either by GC/MS or by HPLC. Many cheese micro-organisms, including LAB, coryneform bacteria, yeasts and *G. candidum*, are capable of producing aroma compounds from amino acids, but the ability is highly strain dependent (Yvon and Rijnen, 2001).

The proteolytic system of lactococci and lactobacilli consists of an extracellular proteinase (Prt P), and a range of intracellular peptidases including: oligo endopeptidases (Pep O, Pep F), general amino peptidases (Pep N, Pep C, Pep G),

glutamyl amino peptidase (PepA), pyrolidone carboxylyl peptidase (PCP), prolyldipeptidyl aminopeptidase (Pep X), proline iminopeptidase (Pep I), aminopeptidase P (Pep P), prolinase (PepR), prolidase (Pep Q), general dipeptidase (Pep V) and general tripeptidase (Pep T). The action of these endo and exopeptidases leads to the production of oligopeptides (Hannon *et al.,* 2003).

The major LAB associated with the production of aldehydes and alcohols from BcAAs is *Lactococcus lactis* var. *maltigenes*. This *maltigenes* variant of *L. lactis* is also capable of converting phenylalanine and methionine to phenylacetaldehyde and methional. Non-starter bacteria and especially some strains of *Lb. paracasei* were shown to generate low amounts of aldehydes, alcohols and acids from BcAAs. Several LAB such as *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis cremoris*, *Lb. lactis*, *Lb.* helveticus, *Lb. bulgaricus* and *Lb. casei*, are also capable of degrading methionine to methanethiol, DMDS and DMTS. However, *Micrococcaceae* and coryneform bacteria and especially *Br. linens* that are used as surface flora in various cheeses, are much better producers of methanethiol and DMDS than LAB. Moreover, these bacteria are capable of producing S-methylthioesters from methanethiol and different carboxylic acids such as acetic, propionic, isobutyric or isovaleric acids.

All these volatile sulfur compounds (VSCs): methanethiol, DMDS, DMTS and methylthioesters, are also produced in significant quantities by *G. candidum* that is commonly present in ripening cultures used in the dairy industry especially for Camembert cheese. However, the amount of methanethiol and the type of thioester produced are dependent on and specific to the strain of *G. candidum*. Moreover, most of the *G. candidum* strains produced alcohols and carboxylic acids from Leu, Ile, Val and Phe as well as yeasts isolated from Camembert which explains the presence of these compounds in Camembert (Yvon and Rijnen, 2001).

Although other AACE (amino acid converting enzymes) have been detected in LAB their occurrence in cheese and the ability of this group of bacteria to catabolise amino acids remains equivocal. In thirty-one LAB isolates that were able to utilize amino acids in one or more protein hydrolysates only isolates of *Lb. paracasei* (71 %), *Lb. curvatus* (16 %), *L. lactis* (3 %) were selected to determine the range of individual

amino acids catabolised. The number of amino acids catabolised by individual LAB isolates ranged from 1 to 22 Among the 31 LAB isolates 53 % utilised 1 - 10 amino acids, 38 % used 11 – 20 and 9 % of the isolates degraded more than 20 amino acids. None of the isolates utilised all of the 24 amino acids tested, but all of the amino acids were degraded by at least 2 isolates. Although *Lb. curvatus* B6 and I4 catabolised 15 and 7 amino acids, respectively, the other 3 *Lb. curvatus* isolates utilised only 1 or 2 of Ala, Arg or Ile. There was, however, a greater diversity of utilisation profiles among the 22 *Lb. paracasei* isolates screened. The number of amino acids catabolised ranged from 3 – 22 (mean 12) with marked intra-species differences in the utilisation profiles.

Among the *Lb. paracasei* isolates 37 % catabolised 1 – 10 amino acids, 50 % used 11 – 20 amino acids and 14 % degraded more than 20 amino acids. *Lactobacillus paracasei* strains are predominant in the NSLAB population of Cheddar cheese and as individual cheeses contain several different strains it is likely that the NSLAB population will be able to transform all of the amino acids that are likely to be released into the curd during cheese maturation. A majority of the principal amino acids were catabolised by approximately half of the isolates screened. The exceptions were tryptophan and methionine; however, these two amino acids were degraded by one-quarter and a third of the *Lb. paracasei* isolates, respectively. Twenty nine of the amino acid metabolite forming LAB isolates were screened for amino acid converting enzymes. Specific activities were determined in unfractionated cell free lysates. Branched-chain aminotransferase activity was detected in all the LAB isolates with α -ketoglutarate as acceptor and leucine as the amino acid substrate. Inter- and intra-species differences in the level of activity were detected with *Lb. paracasei* strains tending to have the highest activity of the LAB screened.

Aromatic aminotransferase (phenylalanine/α-ketoglutarate transaminase) and methionine/ α-ketoglutarate aminotransferase activities were also detected in the isolates although these activities were lower than the leucine aminotransferase. The evidence for the presence of deaminase activity in cell lysates of the LAB is less conclusive. Activity was detected in the 2 *Lc. lactis* strains, in isolates of all 5 species of *Lactobacillus* represented and in both isolate groupings from the microplate screen.

It was not established whether the deamination occurred by an oxidative or reductive mechanism also the decarboxylase activity was inconclusive. The degradation of sulfur containing amino acids was detected by the formation of thiol, which was detected in 27 of the 29 isolates and 2 isolates of the *Lb. paracasei* failed to form thiols. Thiol formation was detected in incubations without added α-ketaglutarate also with the methionine deamination product – KMTBA (α – hdroxy – γ – methylthiobutyrate). When the lysate was incubated with cystathionine, thiol formation was only detected in one strain of *L. lactis* (C 27). Thiol formation appeared to occur when many of the cell suspensions were incubated endogenously without an added substrate (Williams *et al*., 2001).

Amino acid metabolism by LAB was also studied in various cheese trials were in the cheese Serra da Estreta was found that Lys and Glu were completely degraded by all 12 LAB strains. Valine was degraded up to 59.4 %, whereas Leu was degraded up to 66.8 %. In what pertains to the other FAAs, Asp was substantially degraded by all strains (from 31 to up to 100 %), as well as Ser from 27 to up to 100 %, Thr from 33.5 to up to 100 %, and Ala from 93.6 to up to 100 % (Tavaria *et al*., 2002).

In most studies on the consequence of starter autolysis in cheese, proteolytic ripening changes have been investigated. This reflects the fact that proteolysis is the major factor in ripening of many cheese types, influencing both texture and flavour. In Cheddar cheese manufactured under the same conditions and with similar composition, the viability of *L. lactis* subsp. *cremoris* strains decreases at a faster rate than *L. lactis* subsp. *lactis* strains. Associated with this decrease is the observation that two cytoplasmic marker enzymes are released more readily into cheese made with *L. lactis* subsp. *cremoris* strains than with *L. lactis* subsp. *lactis* strains. The formation of alanine, glutamate and the branched amino acids over time at three different storage temperatures was also greatest in the latter cheese. In Saint-Paulin type cheese autolysis of two lactococcal strains and proteolysis appeared to be related, these conclusions being based on cell viability, electron microscopic studies, the release of two intracellular peptidases and amino nitrogen.

Further, the *L. lactis* subsp. *cremoris* strain, AM2, used in the study lysed to a greater extent than *L. lactis* subsp. *lactis* NCD0763 and the cheese made with the latter strain contained the higher concentration of amino nitrogen. In an investigation, in which starter viability and release of three intracellular starter enzymes into cheese were monitored, it was concluded that three *L. lactis* subsp. *cremoris* strains had different autolytic patterns. Higher levels of free amino acids were produced by the most autolytic strain (AM2) as compared to the least autolytic strain (HP). In the experiments in which different rates of loss of starter viability in cheese was achieved using phage, decreased starter viability was shown to correspond to increased autolysis. The release of intracellular enzymes and the concentrations of glutamate and the branched-chain amino acids were highest in those cheeses made with the highest phage and this was shown to correspond to increased autolysis levels (Crow *et al*., 1995).

Yeasts can be a substantial part of the microflora of different cheeses such as mould-, smear-, soft-, semi-hard and brine-ripened cheeses, due to their high proteolytic and lipolytic activities, some yeast species play an important role in the production of aroma precursors such as amino acids, fatty acids and esters. Isolation of eight strains in goat cheese and water-buffalo mozzarella cheeses, indicated weak proteolytic activity for all the *Y. lipolytica* strains considered after 8 days of incubation at 10 and 25 °C but from the 8^{th} to the 14^{th} day the proteolytic activity increased. The proteolytic activity at the end of incubation was markedly affected by temperature. At 25 °C all the strains produced more than 300 mg leucine/ 100 mL skim milk while at 10 °C, all the considered strains showed a limited activity. However, proteolytic activity at temperatures lower than 10 °C is a rare feature among yeasts. The minimum temperature for producing efficient proteinases, for a great number of *Y. lipolytica* strains isolated from different habitats, was reported to be 0 – 3 °C (Suzzi *et al*., 2001).

In a cheese model using the yeasts *K. marxianus*, *Y. lipolytica* and *D. hansenii*, L-methionine consumption was much more important in *Y. lipolytica* cultures than in others. *Yarrowia lipolytica* consumed over 77 % of L-methionine within 72 h, only 16 % and almost 4 % of the initial amount of this amino acid was utilized at this time by *K.*

marxianus and *D. hansenii*, respectively. In yeast co-culture, 22 % of the L-methionine was consumed after 72 h and 32 % after 120 h. In parallel to L-methionine degradation, a transient accumulation of the transamination product KMTBA was observed in the yeast co-culture and in *Y. lipolytica* culture. Moreover, HMTBA (α – keto – γ – methylthiobutyrate), which is the reduction product of KMTBA, was detected in *K. marxianus* and *D. hansenii* cultures, as well as in the yeast co-culture. The absence of HMTBA from *Y. lipolytica* cultures indicated that this strain is strongly oxidative.

Volatile sulfur compounds were measured in the pure cultures of the three yeasts, as well as in yeast co-culture. *Yarrowia lipolytica* was by far the most efficient of the yeasts at producing VSC, with DMDS (dimethyldisulphide) being the major sulfur compound produced. This is in agreement with the fact that *Y. lipolytica* can degrade L-methionine most efficiently among the three yeasts. The thioester methylthioacetate was produced only by *D. hansenii* and *K. marxianus*. In the yeast co-culture, VSC production was lower than in the *Y. lipolytica* culture and surpassed the VSC biosynthesis of the two other yeasts, *D. hansenii* and *K. marxianus*. This suggests that the presence of *Y. lipolytica* promotes VSC production within the yeast co-culture (Cholet *et al*., 2007).

Although the proteolytic characteristics of some oxidative yeasts of cheese surfaces have been observed, little work has been done to determine whether the proteolytic enzymes of the yeast flora have significant roles in the ripening of cheese. The protein degradation has generally been attributed to the species of *Brevibacterium* that appear on the cheese surface at the time the number of yeasts is beginning to decline (Szumksi and Come, 1962).

1.3.2. Lipolysis

The second dominant component of milk is the lipid fraction, also known as milk fat (or butter fat), which has as a very complicated composition and structure. Milk fat is relatively rich in low molecular weight fatty acids including: butyric, caproic and capric. These fatty acids are released on hydrolysis and contribute to the cheese flavour due to their volatile nature. The hydrolysis of triglycerides, which constitute more than 98

% of cheese fat, is the principal biochemical transformation of fat during ripening, which leads to the production of free fatty acids (FFAs), di- and mono-glycerides and possibly glycerol, with small amounts of other milk lipids constituting the remaining 2 % (Singh *et al*., 2003; Upreti *et al*., 2006).

Lipids play a major role in the quality of cheese:

- They affect cheese rheology and texture
- They influence flavour by:
  - Acting as a source of fatty acids which in turn may be catabolised to other flavour compounds e.g. methyl ketones, esters, thioesters and lactones.
  - Acting as a solvent for sapid compounds produced from lipids or other precursors (Collins *et al*., 2004).

Like all types of food with a high fat content, lipolytic (enzymatic hydrolysis by lipases and esterases) and oxidative (chemical) changes are likely to occur in cheese. Free fatty acids contribute to the aroma of cheese. Individual FFAs, particularly acids between C4:0 and C12:0 have specific flavours (rancid, sharp, goaty, soapy, and coconut-like). The flavour intensity of FFAs depends not only on the concentration, but on the distribution between aqueous and fat phases, the pH of the medium, the presence of certain cations (that is, Na^{+}, Ca^{2+}) and protein degradation products (Singh *et al*., 2003; Upreti *et al*., 2006).

1.3.2.1. Lipolytic agents in cheese

Lipolytic enzymes may be classified as esterases or lipases, which are distinguished according to three main characteristics: length of the hydrolysed acyl ester chain, physico-chemical nature of the substrate and enzymatic kinetics. Lipolytic enzymes are specific for fatty acids esterified at the sn-1 or sn-3 positions of the triglycerides. Initially triglycerides are hydrolysed to 1,2- and 2,3-diglycerides and later to 2-monoglyerides as well as the other short and medium chain acids, and these are mainly the sn-1 and sn-3 positions in milk lipids and thus are preferentially released by lipolytic enzymes.

Lipases in cheese originate from six possible sources:

- Milk
- Rennet paste
- Starter bacteria
- Secondary starter bacteria
- Non-starter lactic acid bacteria (NSLAB)
- Exogenous lipase preparations

Milk contains a very potent indigenous lipoprotein lipase (LPL). The lipase is of blood origin and is involved in the metabolism of plasma triglycerides; its presence in milk is due to leakage through the mammary cell membrane. Lipoprotein lipase is relatively non-specific for the acids at the sn-1 and sn-3 positions of mono-, di- and tri-glycerides. Short and medium chain fatty acids are released preferentially by LPL. In raw milk cheeses, LPL activity is significant. It is generally accepted that high-temperature short time (HTST) pasteurization (72 °C for 15 s) very extensively inactivates the enzyme. However, it may contribute to lipolysis in pasteurized milk cheese, as heating at 78 °C for 10 s is required for its complete inactivation (Collins *et al*., 2004).

1.3.2.2. Catabolism of fatty acids

Free fatty acids are precursors of many important flavour and aroma compounds, such as methyl ketones, lactones, esters, alkanes and secondary alcohols. Initially, fatty acids are released by lipases, followed by the oxidation of FFAs to β- ketoacids and decarboxylation to alka-2-ones, of one less carbon atom than the parent FFA; alkan-2-ones may be reduced to the corresponding secondary alcohol (alkan-2-ol).

Lipoprotein lipase has a preference for medium-chain triglycerides (MCT) with a 2-fold faster rate of hydrolysis of emulsions containing C6:0, C8:0, C10:0 or C12:0 compared to long-chain triglycerides (LCT) emulsions containing C16:0, C18:0, C18:1, C18:2, C18:3 or C20:0. Lipoprotein lipase is relatively non-specific for fatty acid type but is specific for the acids at the sn-1 and sn-3 positions of mono-, di- and tri-glycerides. Therefore, short - and medium – chain fatty acids are released preferentially by LPL

(Collins *et al*., 2004). Free fatty acids also act as precursor molecules for a series of catabolic reactions leading to the production of flavour and aroma compounds, such as methyl ketones, lactones, esters, alkanes and secondary alcohols (Collins *et al*., 2003).

The pathway (Fig. 5) (β-oxidation) by which methyl ketones (alkan-2-ones) are produced involves the release of fatty acids by lipolysis, their oxidation to β-ketoacids and decarboxylation to alkan-2-ones with one less C-atom. Alkan-2-ones may be reduced to the corresponding secondary alcohols (alkan-2-ols), a step which is reversible under aerobic conditions. Lactones are cyclic compounds formed by the intra-molecular esterification of hydroxy fatty acids. The principal lactones in cheese are γ- and δ - lactones which have 5- and 6- sided rings, respectively, and are stable, strongly flavoured and could be formed from the corresponding γ - or δ - hydroxy fatty acids. Formation of hydroxyl acids in the mammary gland by oxidation provides the precursors of lactones in freshly-drawn milk. The formation of γ- and δ - lactones from the corresponding hydroxyl acid is spontaneous once the fatty acid is released by lipolysis. Hydroxylation of fatty acids can result from the normal catabolism of fatty acids, and/or they can be generated from unsaturated fatty acids by the action of lipoxygenases or hydratases [FFA can react with alcohols to yield esters (which are highly flavoured) or with free sulphydryl groups to give thioesters] (McSweeney and Sousa, 2000).

A great diversity of esters, formed by the reaction of a FFA with an alcohol, is present in cheese. While methyl, ethyl, propyl and butyl esters of FFAs have been reported in various cheese varieties, ethyl esters predominate. Esterification reactions resulting in the production of esters occur between short-to medium-chain fatty acids and ethanol, derived from lactose fermentation or from amino acid catabolism and that they are formed in cheese during ripening by the trans-esterification of a FFA from partial glycerides to ethanol. Thioesters are formed when FFAs react with sulphydryl compounds and may be formed by the action of a wide range of microorganisms associated with cheese (Collins *et al*., 2004).

1.3.2.3. Contribution of microbial lipases to lipolysis

Lipases and esterases of LAB appear to be the primary lipolytic agents in Cheddar and Dutch-type cheeses made from pasteurized milk. Lactic acid bacteria possess esterolytic/ lipolytic enzymes capable of hydrolyzing a range of derivatives of FFAs, tri-, di- and mono-glyceride substrates. Despite the presence of these enzymes, LAB, especially *Lactococcus* and *Lactobacilus* are weakly lipolytic in comparison to species such as *Pseudomonas*, *Acinectobacte*r and *Flavobacterium*. However, because they are present at high numbers over an extended period, LAB are responsible for the liberation of significant levels of FFAs in many cheese varieties which do not have strongly lipolytic secondary flora. Lipases and esterases of LAB appear to be intracellular and a number have been isolated and characterized (Collins *et al*., 2004).

Strains of LAB consisting of *Streptococcus lactis*, *S. diacetilactis*, *S. cremoris*, *Lb. casei*, *Lb. plantarum*, *Lb. brevis*, *Pediococcus cerevisiae*, *and Leuconostoc mesenteroides* all tested positive to varying extents towards tributyrin, the extent of lipolysis depending upon the concentration of organisms in the cell suspension examined. An experimental Cheddar cheese made with a single strain of *S. cremoris* as starter using milk with an inactive native milk lipase resulted in appreciable amounts of FFAs > C4:0 from the beginning of maturation, gradually increasing with time (Fryer *et al*., 1967).

According to Suzii *et al*. (2001), several authors have reported on the high lipolytic activity of yeasts and their contribution to cheese ripening. The lipase specificity and the subsequent metabolism of FFAs are important for the aromatic characterization of cheeses. The qualitative presence and the quantitative concentrations of FFAs, which are largely dependent on the milk fat and lipolytic strain specificity and activity, are reported to contribute to the flavour characterization of many dairy products. Consequently, diversity among the strains in the fat hydrolysis could be exploited to obtain products having different aromatic features.

Lipase activity from various strains of *Y. lipolytica* showed interesting variations. Strains, such as LF25, LF35, LF46 and PZ67, showed very high lipolytic activity over the first 3 days of growth, producing the highest amounts of total FFAs. However,

continued incubation of these strains resulted in a significant decrease of total FFAs concentrations. In the samples inoculated with the other strains, characterised by a lower lipolytic activity at 3 days of growth, the total FFA content increased at the end.

The degree of specificity for saturated or unsaturated fatty acids as well as for even- or odd-numbered carbon FFAs also varied among the strains. The *Y. lipolytica* strains showed different routes, and consequently, different enzymatic systems for FFA metabolism. The short-chain FFAs C4:0 – C10:0 were produced by all the strains at low levels, corresponding to only about 1 – 2 % of the total FFAs. Short-chain FFAs were generally released during the first days of incubation and later, with the exception of strain PZ20, were none could be detected. A decrease in previously released longer chain FFAs, such as palmitic 16:0, palmitoleic C16:1, stearic C18:0, oleic C18:1 and linoleic C18:2 acids, was also observed after 6 days of incubation in the samples inoculated with the strains LF25, LF35, LF46 and PZ67. The linolenic acid C18:3 present after 3 days of incubation, tended to disappear in all the samples. In regard to the specificity of lipases, all the strains hydrolysed both saturated and unsaturated fatty acids from milk fats. In all the strains, after 3 days of incubation, the major FFA released was C18:1 followed by C16:0, with the exception of strains PZ67 and LF25 in which C16:0 represented the most relevant fatty acid released, followed by C18:1. After 6 days of incubation, C16:0 became the major FFA accumulated for the strains PZ63, LF46, LF35 and LF18. The other relevant FFAs were myristic acid C14:0 and stearic acid C18:0. All the strains hydrolysed the fats with the liberation of high concentrations of even-numbered carbon FFAs, while the odd-numbered FFAs represented a limited proportion of FFAs. The LF25 strain presented, with respect to the other strains, metabolic peculiarity which appeared evident after 6 days. The fatty acid profile observed was totally modified with respect to that observed after 3 days. A relevant increase of lauric acid C12:0, pentadecanoic acid C15:0 and margaric acid C17:0, presumably at the expense principally of C14:0, C16:0 and C18:0 was observed. The results suggest that the strain LF25 metabolised the FFAs released from triglycerides or in cellular components or in shorter chain compounds, such as C12:0, C15:0 and C17:0. Free unsaturated fatty acids can be transformed by the microbial enzymes lipoxygenase, epoxidase and hydratase in the relative hydroxy-acids (Suzzi *et al*., 2001).

1.3.2.4. Starter autolysis contribution to lipolysis

Contribution of the degree of autolysis to flavour development in cheese is quite limited and this has led to studies on the autolytic activities of starter cultures. Starters with enhanced autolytic abilities can accelerate proteolysis and hence ripening of cheese through the early release of intracellular enzymes. There has been an increasing interest in the isolation of lactococcal starter strains with an autolytic phenotype as early cell lysis would result in the early release of cytoplasmic components, including intracellular enzymes. Autolytic strains would release their intracellular enzymes into the curd matrix at an early stage in the cheese making process, and thus such a strain could act as a "delivery system" for ripening enzymes such as proteinases, peptidases, lipases, esterases, or other enzymes that could enhance or accelerate the development of flavour or the quality of cheese. However, starter autolysis on lipolysis still receives little attention (Collins *et al*., 2003; Crow *et al*., 1995).

In Cheddar cheese made with *Lactococcus lactis* subsp*. cremoris* AM2 (highly autolytic) and *Lactococcus lactis* subsp*. cremoris* HP (poorly autolytic), cell viability was lowest for AM2 and highest for HP. Upon autolysis of starter cells, autolysis proceeded in the order AM2>>HP. The levels of FFAs increased significantly in all the cheese, with levels of C8:0, C14:0, C16:0 and C18:0 significantly higher in cheese manufactured with AM2 than in cheese manufactured with HP. This suggested that a relation existed between the extent of starter autolysis and levels of FFA released during ripening of Cheddar cheese (Collins *et al*., 2002).

Early studies in cheese suggested that strains of *L. lactis* subsp. *lactis* survive in cheese better, and hence are less autolytic, than strains of *L. lactis* subsp.*cremoris.* Recent studies monitoring different autolytic patterns have also been observed in cheese made with different strains of *L. lactis* subsp. *cremoris.* The variations in the decrease of cell viability in cheese made with different *L. lactis* subsp. *cremoris* strains suggests that within *L. lactis* subsp. *cremoris* there is considerable variation in the rate of autolysis of strains in cheeses made under similar conditions. However, a major problem in using strains differing in autolytic rates to study the effects of autolysis on

cheese making is the probability that the strains also differ in other properties, such as levels of important cheese ripening enzymes, considerable diversity in the levels and cellular distribution of proteinase and esterase activities (Crow *et al*., 1995).

1.3.3. Glycolysis

Glycolysis is an essential biochemical event for the production of fermented milk products, including natural cheeses. It involves the conversion of lactose to constituent sugars or water-soluble organic acids mainly lactic acid by LAB. Lactococci (*Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*) are the commonly used starter LAB for Cheddar cheese manufacture because of their ability to convert about 95 % of the fermented sugar to L-lactate. Organisms with greater metabolic diversity can produce additional end-products and may result in undesirable organoleptic characteristics. Although glycolysis is important for a decrease in pH during Cheddar cheese making, it can also have significant consequences during cheese ripening. Glycolysis, in addition to the production of aroma compounds, can influence taste, principally by the production of lactic acid and its subsequent degradation to flavour compounds (McSweeney, 1997).

The complete and rapid metabolism of the lactose and its constituent monosaccharides in cheese curd is essential for the production of good quality cheese since the presence of a fermentable carbohydrate may lead to the development of an undesirable secondary flora (McSweeney and Fox, 2004). Lactose fermentation results in lactate which contributes to the flavour of acid curd cheeses and probably also contributes to the flavour of ripened cheese varieties, particularly in early maturation. Oxidation of lactate can also occur in cheese. During this process, lactate is converted to acetate and CO_2. This oxidative activity is dependent on NSLAB population and on the availability of O_2, which is determined by the size of the blocks and the oxygen permeability of the packaging material. Acetate is present at fairly high concentrations in Cheddar and is considered to contribute to cheese flavour, although a high concentration may cause off-flavour. Diacetyl and acetate produced from citrate contribute to the flavour of Dutch-type and Cheddar cheese. The principal flavour compounds produced from metabolism of citrate are acetate,

diacetyl, acetoin, and 2, 3-butandiol. Diacetyl is usually produced in small amounts, but acetoin is generally produced in much higher concentration (10 to 50 fold higher than diacetyl concentration). Acetate is produced from citrate in equimolar concentrations (Singh *et al*., 2003; Upreti *et al*., 2006).

1.3.4. Formation of cheese flavour

The flavour of Cheddar cheese is particularly hard to define, since as a product it is considered marketable any time between 2 and 12 months ripening (Green and Manning, 1982). The formation of flavours in fermented dairy products is a complex and, in the case of cheese ripening, rather a slow process involving various chemical and biochemical conversions of milk components. The characteristic flavour, aroma, texture and appearance of individual cheese varieties developed during ripening are predetermined by the manufacturing process: (i) composition, especially moisture, pH, salt (ii) microflora - starter, non-starter microflora and adjunct cultures (Singh *et al*., 2003).

Accelerating or diversifying flavour development in cheese is of major economical interest since final flavour of cheese partly determines consumer choice and because flavour development is a time consuming and expensive process that is still not well mastered (Smit *et al*., 2002; Yvon and Rijnen, 2001). Most flavour notes that specifically characterize each type of cheese develop during ripening, due to the presence of a number of actively metabolizing microorganisms (with starter and non-starter roles), as well as enzymes secreted by these microorganisms (or released there from after lysis) coupled with enzymes indigenous in milk (or added to as part of the rennet). The main sapid compounds in cheese are produced through the primary reactions of glycolysis, lipolysis, and proteolysis (Tavaria *et al*., 2002).

In most cheese varieties breakdown of protein is the most important flavour development pathway. The primary cheese protein, casein, is degraded enzymatically to short peptides and free amino acids while lactose and milk fat, are degraded to lactic acid and fatty acids respectively, including other compounds (Table 2). The agents primarily responsible for these conversions are the residual rennet that is retained in the cheese curd at the end of the manufacturing phase and the proteinases

and peptidases that are associated with the starter bacteria. While the rate and degree of proteolysis are of vital significance for desired flavour development, the direct products of proteolysis do not fully define cheese flavour (Beresford, 1999).

1.3.4.1. Volatile compounds in cheese

The flavour of cheese originates from microbial, enzymic and chemical transformations. The breakdown of milk proteins, fat, lactose and citrate during ripening gives rise to a series of volatile and non-volatile compounds which may contribute to cheese flavour. The formation of volatiles occurs concurrently with proteolysis, hydrolysis of fat and carbohydrate breakdown. Both enzymic and non-enzymic modification pathways (Fig. 6) have been suggested for the formation of volatile flavour compounds from amino acids, free fatty acids and lactic acid in cheese. Volatiles belong to six major groups: fatty acids, esters, aldehydes, alcohols, ketones and sulfur compounds. The volatile aroma components of various cheeses have received a great deal of attention and a large number of volatiles have been detected in individual types of cheese, since their flavour attributes range from pleasant-fruity for esters to putrid-unclean for sulfur compounds (Engels *et al*., 1997).

Fatty acids are important components in the flavour of many cheese types. Short-chain fatty acids impart a desirable peppery taste to Blue cheese flavour, while acetic and butyric acids play a significant role in the flavour of Parmesan and Swiss Gruyère cheese, however large amounts of butyric acid which might originate from butyric acid fermentation are undesirable (Engels *et al*., 1997). Short-chain free fatty acids play a significant role in the flavour of cheese. Mixtures of alkanoic acids with carbon chains from C 2:0 to C 8:0 or C 4:0 to C 10:0 appear to impart cheese-like flavours either to naturally maturing cheese or to flavour mixtures for processed cheese. Short-chain fatty acids including butyric, caproic, and capric, which are formed from milk fat degradation, are considered among the necessary constituents of Cheddar cheese flavour. Concentrations of individual VFA (volatile fatty acids) are affected by cheese age and cheese composition. Volatile fatty acids have qualitative and quantitative profiles that vary during ripening and can indicate some metabolic reactions taking place during ripening. In addition, the determination of VFA is a useful parameter for

evaluation of the quality of a specific type of cheese characterized by lipolysis (Tungjaroenchai *et al*., 2004).

The role of fatty acids as precursors for other flavour compounds is also of importance with esters, methyl ketones and secondary alcohols formed from fatty acids. Esterification takes place by an enzymic or chemical reaction of fatty acids with primary alcohols. Esters have a sweet-fruity aroma and ethyl esters are known for their important role in the formation of a fruity character in cheeses. Primary alcohols are reported to be present in various cheese types, e.g. Parmesan, Roquefort and Domiati. They are considered to originate from the corresponding aldehydes following a reaction pathway involving alcohol dehydrogenases. The strong reducing conditions in hard cheeses may favour the formation of alcohols from aldehydes. Secondary alcohols are formed from cheese by enzymic reduction of methyl ketones, which are themselves produced from fatty acids. These alcohols are typical components of the flavour of blue cheeses. In Cheddar cheese, the production of 2-propanol from acetone has been reported, as well as the production of 2-butanol from butanone. Straight–chain aldehydes, such as butanal, pentanal, hexanal, heptanal and nonanal, were detected in the WSF (water soluble fraction) of cheeses and these are formed during β-oxidation of unsaturated fatty acids. Branched aldehydes probably originated from amino acid degradation. In Cheddar cheese, 2-methyl propanal, 2-methyl butanal and 3-methyl butanal, produced from valine, isoleucine and leucine respectively were responsible for unclean and harsh flavours.

Ketones are common constituents in most dairy products. Methyl ketones are primarily recognized for their contribution to the flavour of mould-ripened cheeses, such as blue cheese. The significance of methyl ketones for the flavour of other cheeses is not yet established completely. However, 2-pentanone may impart an orange-peel aroma to Cheddar cheese, while in Parmesan and Mozzarella cheeses, methyl ketones are also thought to play an important role as constituents. Methyl ketones are formed in cheese by enzymic oxidative decarboxylation of fatty acids. Due to the reducing cheese environment, enzymic reduction of methyl ketones to secondary alcohols will also occur. One of the important ketone flavour compounds is

diacetyl (2, 3-butanediol), which has a buttery, nut-like flavour. The reduction of diacetyl leads to acetoin, a compound with a woody and mildew aroma.

The decomposition of sulfur-containing amino acids is of interest for cheese flavour formation. Decomposition of sulfur amino acids during cheese ripening produces volatile sulfur compounds such as hydrogen sulphide and methanethiol. Oxidative reactions can convert the latter to DMDS and DMTS. Both DMSD and DMTS are considered to be very important for cheese flavour and especially the odour of DMTS has been described as “overripened-cheese-like” (Engels *et al*., 1997).

Yeasts play a role in the development of aroma through the production of a wide variety of volatile compounds. The yeasts contribute to the flavour indirectly by the production of proteolytic and lipolytic enzymes and directly by the production of aroma components. During ripening, the roles of yeasts in the appearance and in the flavour continue in relation to the growth of the other flora (Leclercq-Perlat *et al*., 2004).

Volatile sulfur compounds (VSCs) are present in many foods, and it is estimated that VSCs represent about 10 % of the volatile components detected in food and beverages. These compounds are commonly found in dairy products, including yoghurt and ripened cheeses, and they comprise a structurally diverse class of molecules which provides a whole range of characteristic aromatic notes (e.g. “cheesy” and “garlic”) in a particular cheese. Their low odour thresholds and the pronounciation of their sensory properties at low concentrations make an important contribution to the odour and aroma of cheeses and may interact with the organoleptic properties of cheeses.

The origin of many sulfur compounds in cheese is believed to form at the late stages of ripening by surface bacteria, the most common *Br. linens*. The occurrence of the VSCs in such an ecosystem is mainly the result of the degradation of sulfur-containing amino acids by microflora or the processing conditions. Yeasts may also contribute in a direct way to the formation of VSCs, since they can grow in acidic environments. For example the yeast *K. lactis* is able to produce and/ or accumulate acetyl-CoA - a common precursor of MTA (methyl thioacetate) and EA (ethylacetate) albeit it produces limited amounts of methanethiol (MTL). The importance of VSCs derives

mainly from their reactivity, their high volatility and their potency at very low concentrations (Bondar *et al*., 2005; Spinnler *et al*., 2001; Arfi *et al*., 2002).

Methanethiol is generally believed to result from the degradation of L-methionine by a one – step degradation pathway catalyzed by L-methionine-γ- demethiolase. Another possible metabolic sequence leading to the formation of MTL from L-methionine is a two-step degradation pathway initiated by an aminotransferase also named transaminase. It gives KMTBA as the first biotransformation product, the latter being converted to MTL most probably by a γ-demethiolase. Another possible two-step mechanism for L-methionine to MTL conversion is the oxidative deamination of L-methionine to KMTBA and ammonia, KMTBA being in turn converted to MTL. Further catabolism of MTL leads to the generation of a range of sulfur compounds which contribute significantly to the aroma of cheese, including DMDS, DMTS by auto-oxidation of MTL, and also thioesters such as MTA and S-methyl thiobutyrate (MTB) (Spinnler *et al*., 2001). The production of VSCs was measured in the pure cultures of three yeasts, as well as in yeast co-cultures of *D. hansenii*, *K. marxianus* and *Y. lipolytica*. *Yarrowia lipolytica* was by far the most efficient of the yeasts at producing VSCs, with DMDS being the major sulfur compound produced. The thioester MTA was produced only by *D. hansenii* and *K. marxianus*. In yeast co-cultures, VSC production was lower than in *Y. lipolytica* cultures and surpassed the VSC biosynthesis of the two other yeasts, *D. hansenii* and *K. marxianus* (Cholet *et al*., 2007).

The catabolic products of sulfur amino acids have been implicated as major contributors to the flavour of Cheddar and many other cheese varieties, but their importance in smear and surface-ripened cheeses appears to be accentuated by their high concentration in the surface. Sulfur compounds are thought to interact with each other and with other compounds in cheese, generating other volatile flavour compounds. Methanethiol is present in Camembert together with other sulfur compounds, such as 2, 4-dithiapentane; 3, 4-dithiahexane; 2, 4, 5-trithiahexane and 3-methylthio-2, 4- dithiapentane, and these compounds are responsible for the garlic note which can be found in well-ripened Camembert cheese. *Brevibacterium linens* is one of the principal microorganisms found on the surface of smear-ripened cheeses,

and is also present on the surface of mould-ripened varieties (e.g. Camembert), and can produce MTL enzymatically.

In cheeses such as Cheddar, which lacks a surface microflora, flavour is produced by starter and non-starter bacteria and their enzymes, and the production of MTL is thought to be a chemical process although suggested that the secondary flora, particularly in Cheddar and Emmental, are likely to be more important than chemical reactions for the formation of sulfur compounds. DMS, DMDS and DMTS are thought to be important contributors to cheese flavour (Table 3). DMS is a product of the metabolism of propionic acid formed from methionine, and is a component of Swiss cheese flavour. Dimethylsulfide concentrations in Cheddar cheese remain constant for up to 6 months, but decrease thereafter. Dimethyldisulfide can be formed as an end-product of Strecker degradation, and has been identified in Parmesan, Cheddar and surface- ripened cheeses. Dimethyldisulfide concentrations in Cheddar correlate reasonably well with flavour scores. Dimethyltrisulfide has been associated with the aroma of cooked cabbage, broccoli, or cauliflower, and has been identified in Parmesan and Cheddar cheeses. Dimethylsulfide and DMDS could be produced directly from MTL, but it is unclear how DMTS is produced in cheese (McSweeney and Sousa, 2000).

More than 50 volatile compounds were identified in single and co-cultured fermented milk. Key components of cheese flavour such as ethanol, diacetyl, acetoin, acetaldehyde, 2-methyl-butanal, 3-methyl-butanal, 2-methyl propanol, acetone and 2-propanol were identified in the fermented milk. Ethanol was the major volatile compound produced by both yeasts and LAB, although the production varied from 2.2 to 253.23 mgL^{-1}. *Leuconostoc citreum* was the LAB that produced the highest levels (72 mg L^{-1} of milk) of ethanol, and *P. fermentans* was the strongest ethanol-producing yeast (up to 237 mgL^{-1}). High acetaldehyde levels were found in milk samples with *L. lactis* (1.25 mgL^{-1}), *K. lactis* (1.63 mgL^{-1}) and *P. fermentans* (1.40 mgL^{-1}). Diacetyl was mainly produced by LAB, among which *L. lactis* subsp. *cremoris* Wg2 produced the most (1.01 mgL^{-1}). This aromatic compound was also produced by some yeasts, especially *P. fermentans* (0.43 mgL^{-1}), but these microorganisms usually reduce it into acetoin and 2-3- butandiol. Methyl alcohol and methyl aldehyde were mainly

produced by the yeasts, although different species produced different amounts. Most of the single and co-culture fermented milk samples had a good appearance and a pleasant flavour. Pleasant organoleptic characteristics were obtained in the co-cultures of *C. famata* 1AD5, *D. hansenii* 3AD24 and in *G. candidum* 3AM4 with all four strains of LAB (Álvarez-Martín *et al*., 2008).

1.4. CONCLUSION

The cheese microbiota, whose community structure evolves through a succession of different microbial groups, plays a central role in cheese-making. The subtleties of cheese character, as well as cheese shelf-life and safety, are largely determined by the composition and evolution of this microbiota. Lactic acid bacteria starter cultures are mainly used during cheese making while yeasts will be encountered for as microbial contaminants, albeit the use of yeast as adjunct starters in recent literature. Yeasts are frequently isolated from cheese surfaces and their contribution to ripening has been studied by various authors all over the world, however there is still mystery regarding their specific attributes to cheese flavour, aroma and texture development. Although it is established that yeasts in excess numbers can cause off-flavours, spoilage, development of odour and slime formation in cheese, however they are still regarded as essential components in cheese as they support the function of the starter culture during cheese ripening, inhibit undesirable microorganisms present, metabolize/ assimilate lactic acid leading to an increase in pH and have proteolytic and lipolytic enzymes, excrete growth factors, like B-vitamins, pantothenic acid, niacin, riboflavin and biotin, which promote the growth of lactic acid bacteria and produce gas leading to curd openness.

The presence of yeasts in a cheese ecosystem results in the formation of a microbial interaction between the starter culture and the yeasts. These interactions may be beneficial or detrimental. A study of a co-inoculum of *Y. lipolytica* (strain RO21) and starter culture did not show any inhibition of both inoculated and naturally occurring lactic acid bacteria; on the contrary, the strain RO21 seemed to stimulate the proliferation of the lactic acid bacteria (Guerzoni *et al*., 1996). Incorporation of *D. hansenii* as an adjunct starter in matured Cheddar as the sole co-inoculum, resulted in

the yeast numbers decreasing gradually during the ripening period to a minimum value of 4.25 X 10^2 cfu/g after six months of maturation. The cheese developed a fruity flavour after two months of ripening in addition to an advanced development of the desired Cheddar flavour compared to the control cheese and after six months, the cheese developed a bitter taste. Sole-inoculation of *Y. lipolytica* resulted in the number of yeasts gradually decreasing and ceasing to survive after four months of the ripening period. The cheese developed an enhanced Cheddar flavour after two months of ripening and the strong Cheddar flavour and fruity taste could still be detected after six months. Co-inoculation of *D. hansenii* and *Y. lipolytica* together resulted in cultures remaining significantly higher >2 - 3 log units compared to the cheeses with individual inoculated yeasts during the initial two months of maturation. With the addition of both yeast species to the starter culture, the cheese developed a good, slightly sweet Cheddar taste after two months of ripening, and after four months, there was a stronger Cheddar taste development compared to the control cheese (Ferreira and Viljoen, 2003). Thus the application of yeasts has some advantageous properties to the microbial composition of the cheese as well as flavour development based on the three biochemical pathways involved during cheese ripening.

The main constituents of cheese ripening are carbohydrates, proteins and milk fat. The degradation of these constituents leads to the development of the final flavour, aroma and texture of the cheese in conjunction with the activity of the microbiota in cheese. The final aroma and flavour of the cheese is partly attributed to the formation of organic acids, aroma and flavour compounds formed from the degradation of the milk carbohydrates. The texture of cheese is mostly contributed to the proteins-caseins of the milk, while the milk fat will contribute to the formation of flavour as some of the fatty acids produced from lipolysis are volatile and produce volatile compounds which form specific flavours through enzymic reactions and chemical reactions. Cheese making involves a complex series of biochemical and chemical reactions, thus a balance between these reactions is necessary as excessive proteolysis can lead to the formation of bitterness and excessive lipolysis can lead to rancidity.

Table 1: Main yeast species encountered in/on the surface of cheese (Chamba and Irlinger, 2004)

Perfect form	Imperfect form
Galactomyces geotrichum	*Geotrichum candidum*
Debaryomyces hansenii	*Candida famata*
Kluveromyces marxianus var. lactis	*Candida sphaerica*
Kluyveromyces marxianus var. marxianus	*Candida kefyr*
Pichia membranifaciens	*Candida valida*
Pichia fermentans	*Candida lambica*
Saccharomyces cerevisiae	*Candida robusta*
Saccharomyces dairensis	*Candida dairensis*
Torulaspora delbrueckii	*Candida colliculosa*
Yarrowia lipolytica	*Candida lipolytica*
Zygosaccharomyces rouxii	*Candida mogii*

Other minor species: *Candida catenulata, Candida intermedia, Candida rugosa, Candida sake, Candida vini, Candida zeylanoides*.

Table 2: Flavour compounds generated from the three principal milk constituents during ripening of cheese (Singh *et al*., 2003)

Casein	Milk fat	Lactose & Citrate
Peptides	Fatty acids	Lactate
Amino acids	Keto acids	Pyruvate
Acetic acid	Methyl ketones	CO_2
Ammonia	Lactones	Diacetyl
Pyruvate		Acetoin
Aldehydes		2, 3 – butandiol
Alcohols		Acetaldehyde
Carboxylic acid		Acetic acid
Sulfur compounds		Ethanol

Table 3: Catabolic products formed from sulfur containing amino acids (Singh *et al*., 2003)

Catabolic Products	Precursor	Aroma note
3- (Methylthio) propanal	Methionine	Cooked/ boiled potato
3- (Methylthio) propanol	Methionine	Cooked/ boiled potato
Methanethiol	Methionine/ Cysteine	Cabbage, boiled cabbage, sulfurous
Methylsulfide	S- containing	Cabbage, sulfurous
Dimethylsulfide	S-containing	Onion
Dimethyldisulfide	S-containing	Garlic
Dimethyltrisulfide	S-containing	Cabbage

Figure 1: Cheddar cheese manufacture (Lawrence *et al*., 2004)

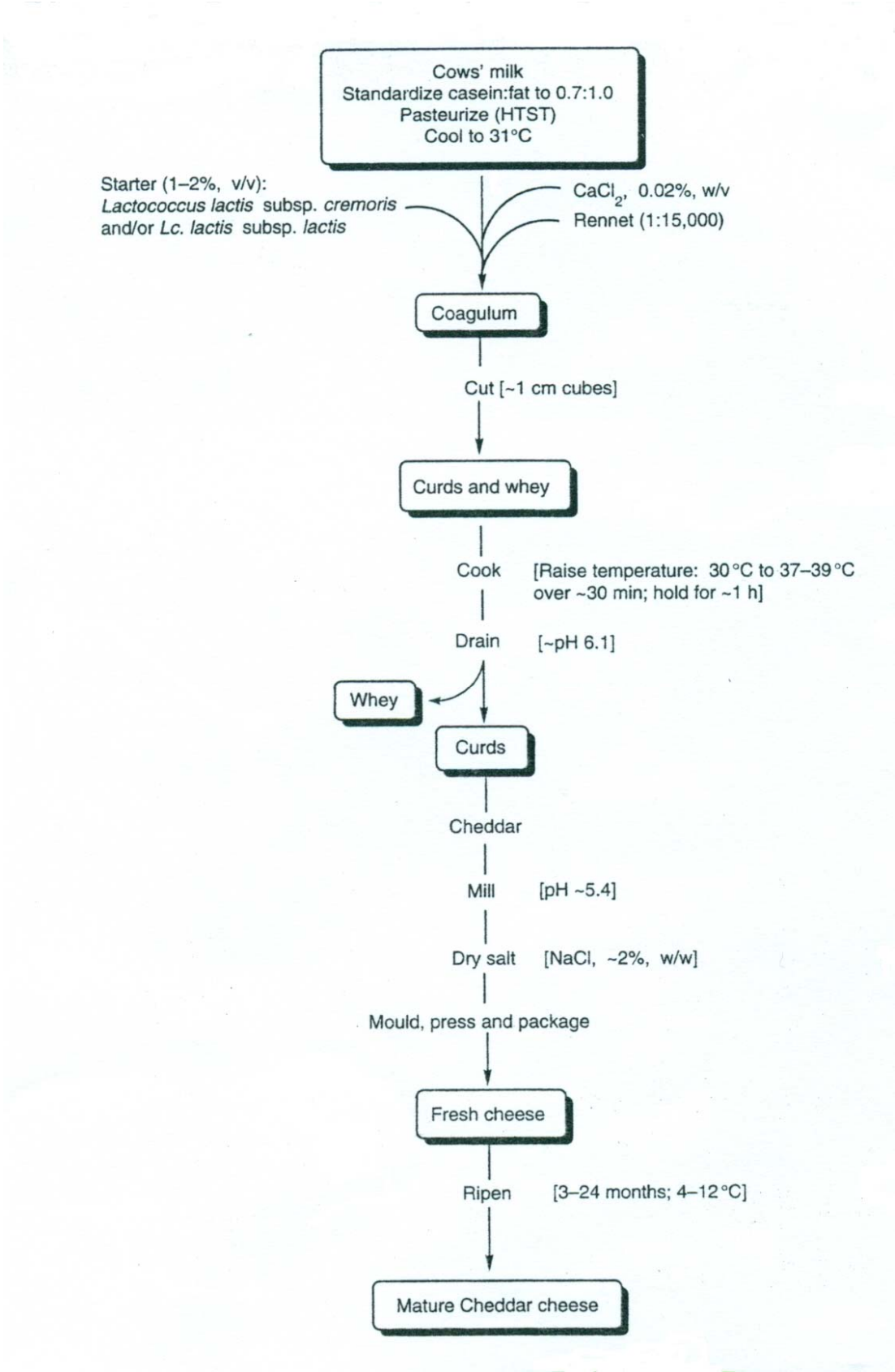


Figure 2: Cheese ripening biochemistry (Law, 2001)

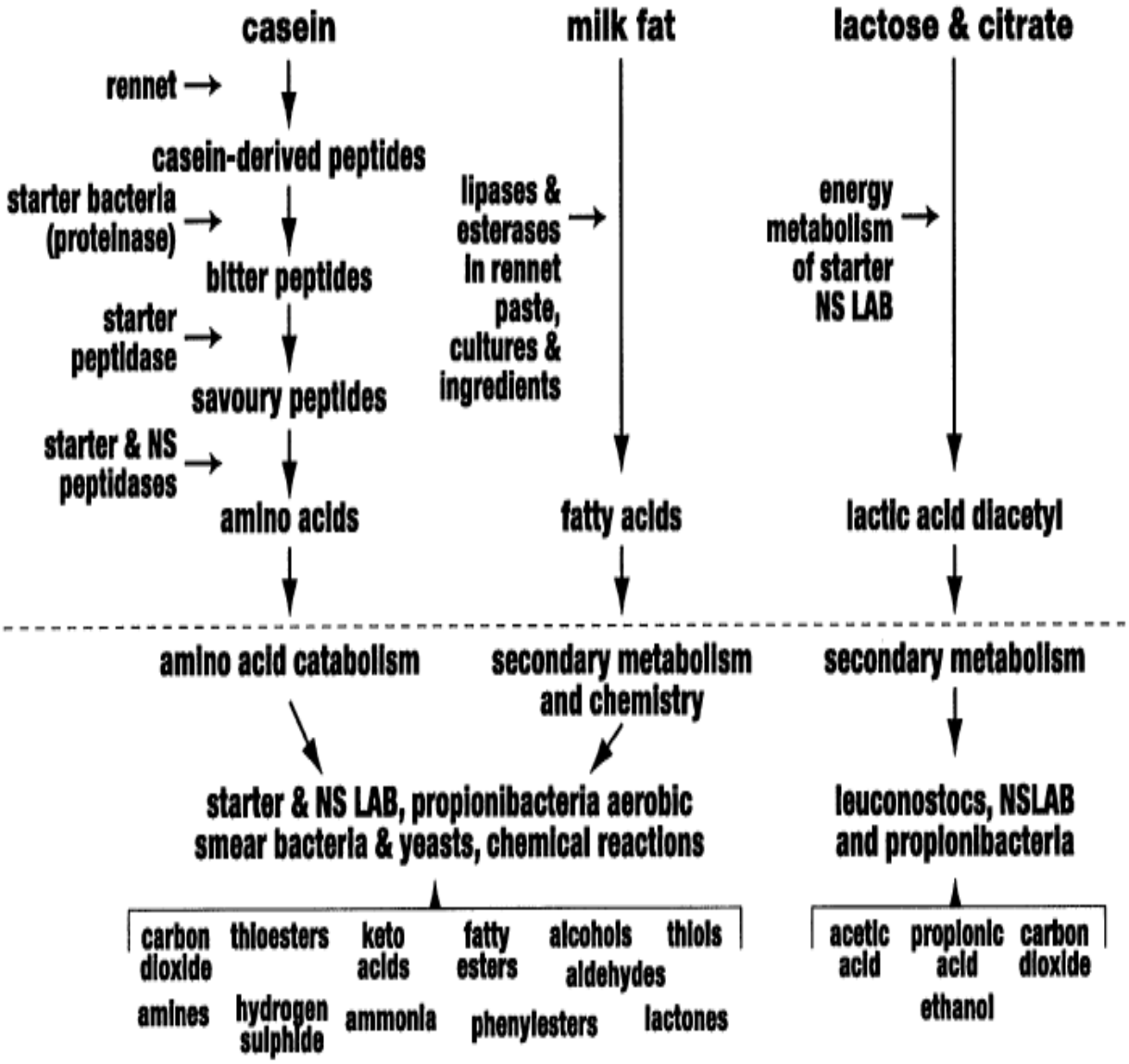


Figure 3: Microbial succession and functions of the different microbial groups involved during cheese making (Irlinger and Mounier, 2009).

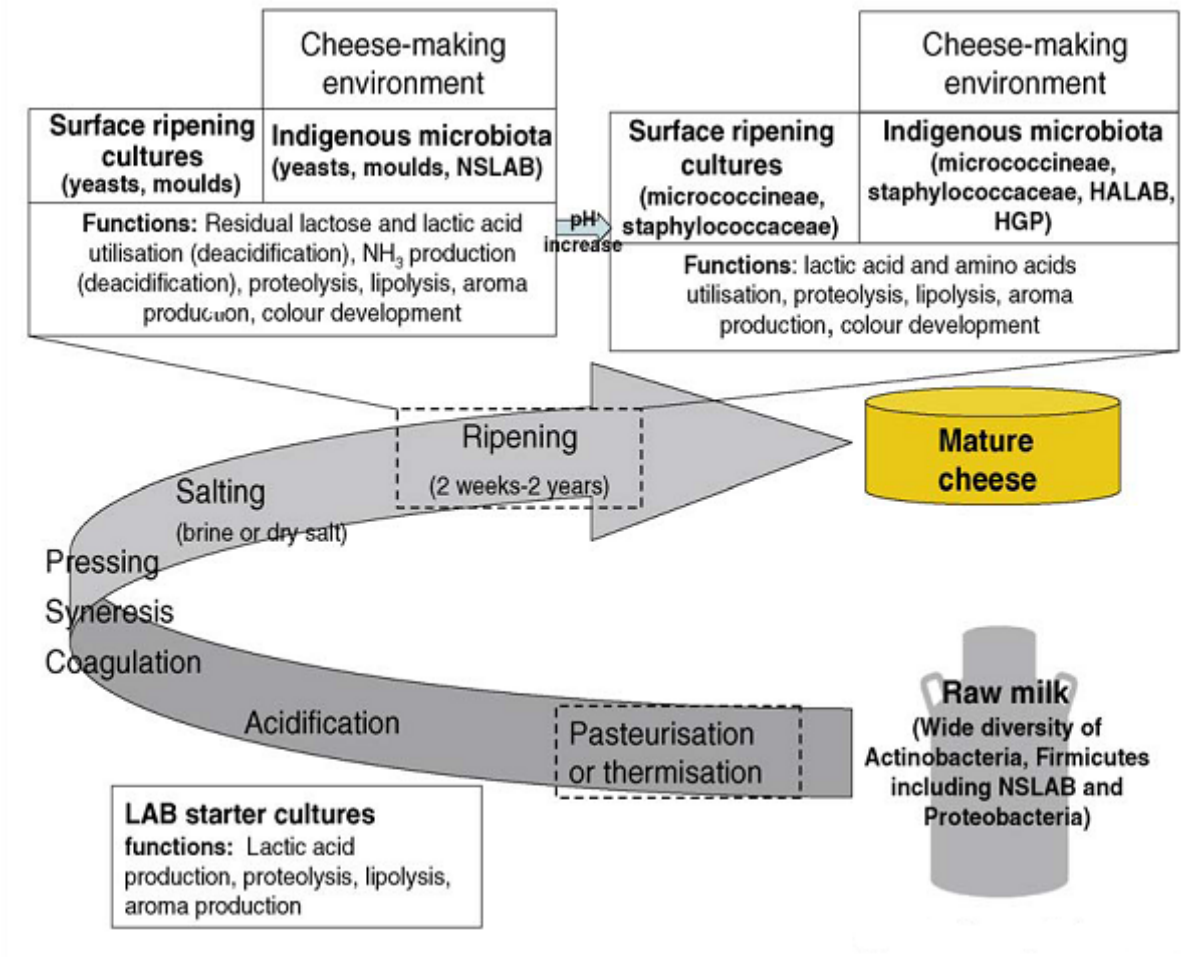


Figure 4: General pathways for the catabolism of free amino acids (McSweeney and Sousa, 2000)

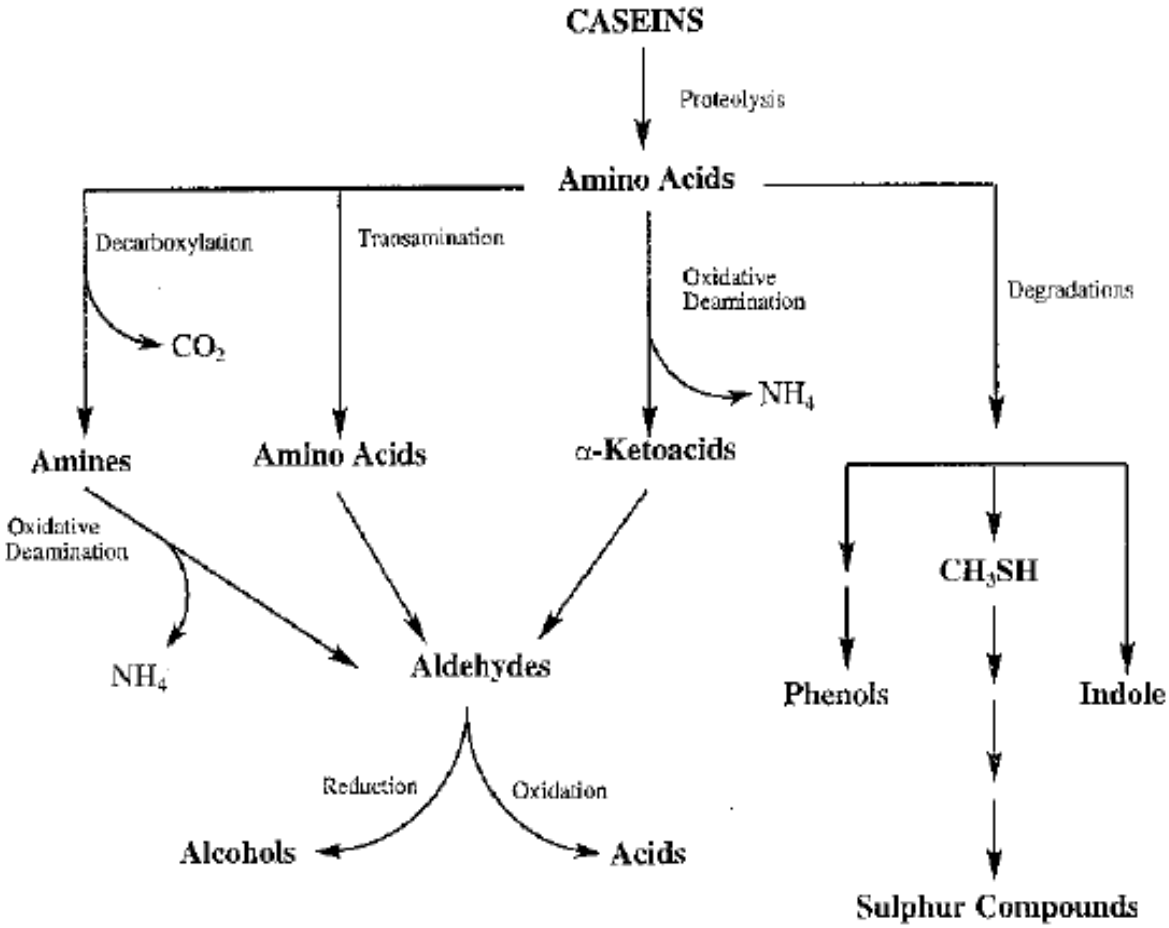


Figure 4.1: Amino acid conversion to aroma compounds (Yvon and Rijnen, 2001)

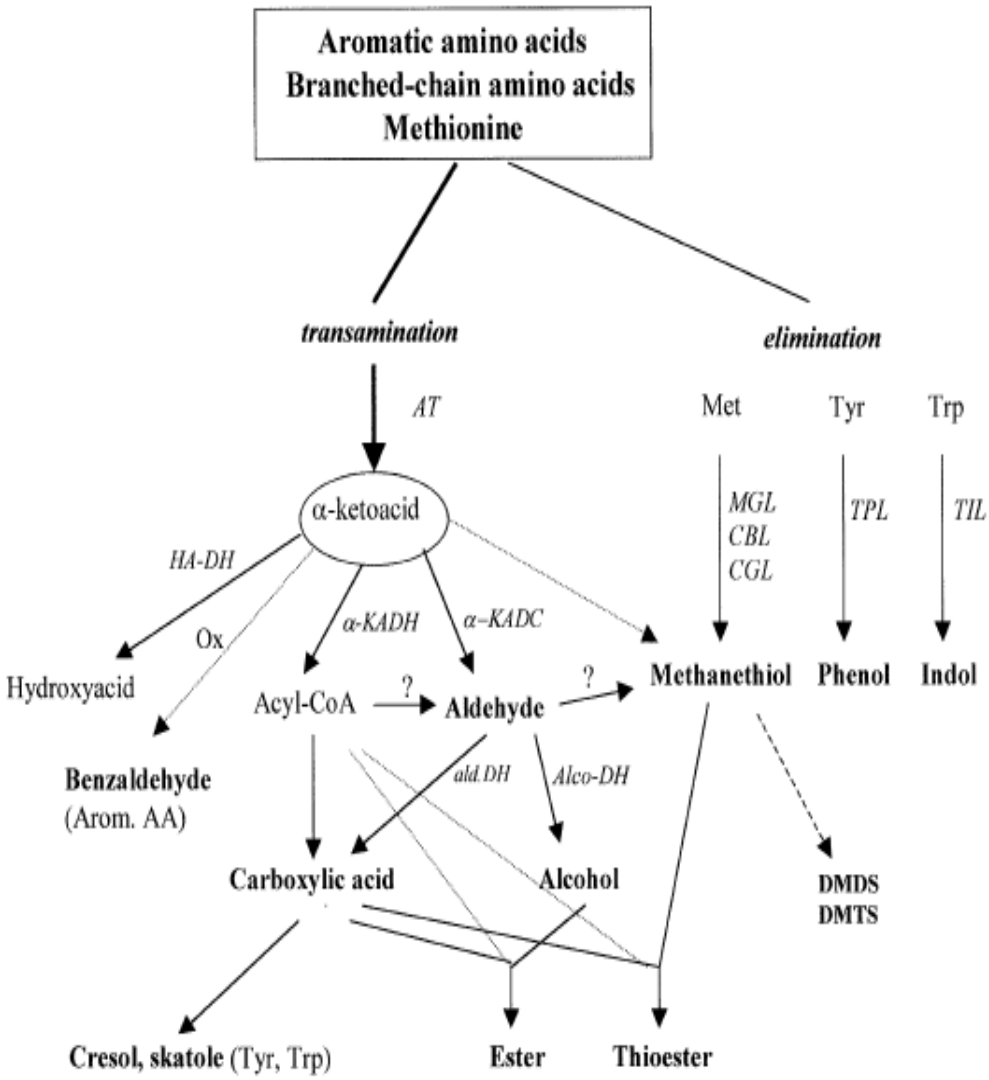


Figure 5: Catabolism of free fatty acids (Collins *et al*., 2003)

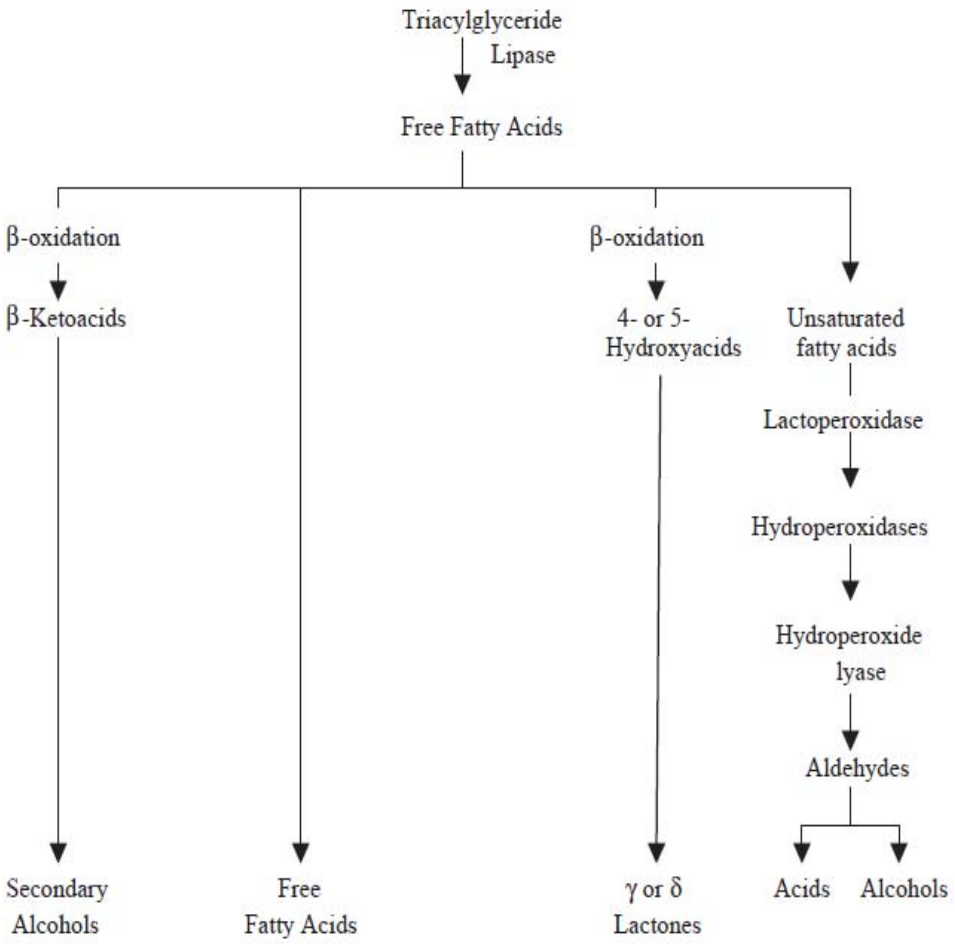
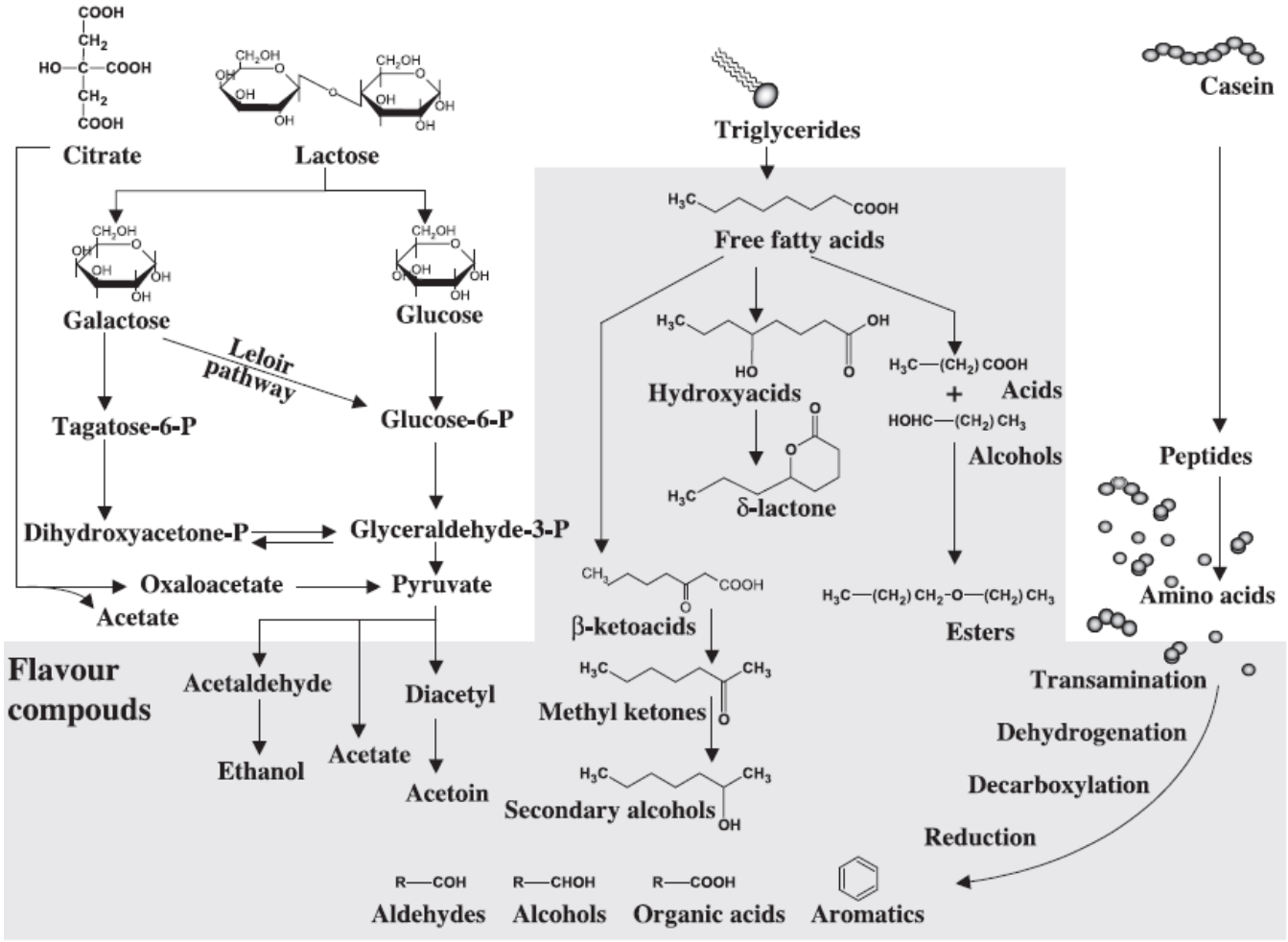


Figure 6: Biochemical pathways leading to the formation of flavour compounds (Marilley and Casey, 2004).



1.5. REFERENCES

Álvarez-Martín, Pablo, Ana Belén Flórez, Ana Hernández-Barranco & Baltasar Mayo (2008). Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation. Food Control, **19**, 62 - 70.

Arfi, K., Spinnler, H. E., Tache, R. & Bonnarme, P. (2002). Production of volatile compounds by cheese ripening-yeasts: requirement for a methanethiol donor for S-methyl thioacetate synthesis by *Kluyveromyces lactis*. Applied Microbial Biotechnology, **58**, 503 – 510.

Bondar, Daniela Cernat; Beckerich, Jean-Marie & Bonnarme, Pascal (2005). Involvement of a branced-chain aminotransferase in production of volatile sulfur compounds in *Yarrowia lipolytica*. Applied and Environmental Microbiology, **71**, 4585 – 4591.

Beresford, T. P., (1999). Identification of the key compounds responsible for Cheddar cheese flavour (Key flavour compounds of Cheddar cheese). End of Project Report, ARMIS No. 4337, DPRC No. 27. The Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork.

Beresford, T. P., Cogan, T. M., Fitzsimons, N. A. & Brennan, N. L. (2000). Recent advances in cheese microbiology. International Dairy Federation: cheese ripening and technology, 30.

Broadbent, J. R., Barnes, M., Brennand, C., Strickland, M., Houck, K., Johnson, M. E. & Steele, J. L. (2002). Contribution of *Lactococcus lactis* cell envelope proteinase specificity to peptide accumulation and bitterness in reduced-fat Cheddar cheese. Journal of Applied and Environmental Microbiology, **68**, 1778 – 1785.

Cholet Orienne, Hénaut Alain, Casaregola Serge & Bonnarme Pascal (2007). Gene expression and biochemical analysis of cheese-ripening yeasts: focus on L-methionine, lactate and lactose catabolisms. Applied Environmental Microbiology, **73**, 2561 - 2670.

Chamba, J. F. & Irlinger, F. (2004). Secondary and adjunct cultures. In *Cheese: chemistry, physics and microbiology, general aspects* Vol. 1, 3rd. Fox, Patrick F., McSweeney, Paul L. H., Cogan, Timothy M. & Guinee, Timothy P (Eds.) , pp 192. London: Chapman and Hall.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2002). Autolysis of starter bacteria and lipolysis in Cheddar cheese. The Australian Journal of Dairy Technology, **57**, 117.

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.

Collins, Y. F., McSweeney, P. L. H. & Wilkinson, M. G. (2004). Lipolysis and catabolism of fatty acids in cheese. In *Cheese: Chemistry, Physics and Microbiology, General Aspects* Vol. 1, 3rd. Fox, Patrick F., McSweeney, Paul L. H., Cogan, Timothy M. & Guinee, Timothy P (Eds.), pp374 - 389. London: Chapman and Hall.

Corsetti, A., Rossi, J. & Gobbetti, M. (2001). Interactions between yeasts and bacteria in the smear surface-ripened cheese. International Journal of Food Microbiology, **169**, 1 - 10.

Crow, V. L., Coolbear, T., Gopal, P. K., Martely, F. G., McKay, L. L. & Riepe, H. (1995). The role of autolysis of lactic acid bacteria in the ripening of cheese. International Dairy Journal, **5**, 855 – 875.

Engels, W. J. M., Dekker, R., de Jong, C., Neeter, R. & Visser, S. (1997). A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. International Dairy Journal, **7**, 255 – 263.

Fatichenti, F., Bengere, J. L ., Deiana, P. & Farris, G. A. (1983). Antagonistic activity of *Debaryomyces hansenii* towards *Clostridium tyrobutyricum* and *Clostridium butyricum*. Journal of Dairy Research, **50**, 449 – 457.

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.

Fleet , G. H. & Mian, M. A. (1987). The occurrence and growth of yeasts in dairy products. International Journal of Food Microbiology, **4**, 145 – 155.

Fox, P. T. (1989). Proteolysis during cheese manufacturing and ripening. Journal of Dairy Science, **72**, 1379 – 1400.

Fox, P. F., Wallace, J. M., Lynch, C. M., Niland, E. J. & Tabin, J., S. Morgan (1996). Acceleration of cheese ripening. Antonie van Leeuwenhoek **70**, 271 - 297.

Fryer, T. F., Reiter, B. & Lawrence, R. C. (1967). Lipolytic activity of lactic acid bacteria. Journal of Dairy Science, **50**, 399 - 389.

Guerzoni, M. E., Gobbetti, M., Lanciotti, R., Vannini, L. & Lòpez, Chaves C. (1996). *Yarrowia lipolytica* as a potential ripening agent in milk products. In *Yeasts in the dairy industry: positive and negative aspects*, Jakobsen, M., Narvhus, J., Viljoen, B.C. (Eds.), Copenhagen, Denmark. International Dairy Federation, Brussels, Belgium, 70 - 77.

Green, M. L. & Manning, D. J. (1982). Development of texture and flavour in cheese and other fermented products. Journal of Dairy Research, **49**, 737 - 748.

Hannon, J. A., Wilkinson, M. G., Delahunty, C. M., Wallace, J. M., Morrissey, P. A. & Beresford, T. P. (2003). Use of autolytic starter systems to accelerate the ripening of Cheddar cheese. International Dairy Journal, **13**, 313 - 323.

Harper, W. J. & Kristoffersen, T. (1956). Biochemical aspects of cheese ripening. Journal of Dairy Science, **39**, 1172 - 1775.

Irlinger, I. & Mournier, J. (2009). Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology, **20**, 142 – 148.

Jakobsen, M. & Narvhus, J. (1996). Yeasts and their possible beneficial and negative effects on the quality of dairy products. International Dairy Journal, **6**, 755 - 768.

Law, B. A. (2001). Controlled and accelerated cheese ripening: the research base for new technologies. International Dairy Journal, **11**, 383 - 398.

Lawrence, R. C., Gillies, J., Creamer, L. K., Crow, V. L., Heap, H. A., Honoré, Johnston, K. A. & Samal, P. K. (2004). In: *Cheese: Chemistry, Physics and Microbiology, General Aspects* Vol. 2 (Major Cheese Groups), 3rd Edition. Fox, Patrick F., McSweeney, Paul L. H., Cogan, Timothy M. & Guinee, Timothy P (Eds.). Academic Press, London, pp. 71 - 102.

Leclercq- Perlat, M. N., Corrieu, G. & Spinnler, H. E. (2004). Comparison of volatile compounds produced in model cheese medium deacidified by *Debaryomyces hansenii* and *Kluyveromyces marxianus*. Journal of Dairy Science, **87**, 1545 – 1550.

Marcellino, N., Beuvier, E., Grappin, R., Gueguen, M. & Benson, D. R. (2001). Diversity of *Geotrichum candidum* strains isolated from traditional cheese making fabrications in France. Applied and Environmental Microbiology, **67**, 4752 - 4759.

Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. International Journal of Food Microbiology, **90**, 139 – 159.

Martin, N., Savonitto, S., Molimard, P., Berger, C., Braisse, M. and Spinnler, H. E. (1999). Flavour generation in cheese curd by co-culturing with selected yeast, mould and bacteria. Journal of Dairy Science, **82**, 1072 – 1080.

McSweeney, P. L. H., (1997). The flavour of milk and dairy products: III. Cheese: taste. International Journal of Dairy Technology, **50**, 123 - 128.

McSweeney, P. L. H. & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In: *Cheese: Chemistry, Physics and Microbiology, General*

Aspects Vol. 1, 3rd. Fox, Patrick F., McSweeney, Paul L. H., Cogan, Timothy M. & Guinee, Timothy P (Eds.). Academic Press, London, pp. 361 – 371.

McSweeney, P. L. H. & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A Review. *Lait,* **80**, 293 – 324.

Roostista, 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.

Smit, Gerrit, van Hylckama, Vlieg Johan E. T., Smit, Bart A., Ayad, Eman H. E. & Engels, Wim J. M. (2002). Fermentative formation of flavour compounds by lactic acid bacteria. The Australian Journal of Dairy Technology, **57**, 61 - 67.

Singh, T. K., Drake, M. A. & Cadwallader, K. R. (2003). Flavour of Cheddar cheese: A Chemical and Sensory Perspective. Comprehensive Reviews in Food Science and Food Safety, **2**, 139 - 162.

Shakeel-Ur-Rehman, McSweeney, P. L. H., & Fox, P. F. (1999). Study on the role of indigenous microflora in ripening of Cheddar cheese. *Milchwissenschaft*, ***54***, 388 - 392.

Spencer, J. F. T., Ragout de Spencer, A. L. & Laluce, C. (2002). Non-conventional yeasts. Applied Microbiology and Biotechnology, **58**, 174 - 156.

Spinnler, H. E., Berger, C., Lapadatescu, C. & Bonnarme, P. (2001). Production of sulfur compounds by several yeasts of technological interest for cheese ripening. International Dairy Journal, **11**, 245 - 252.

Suzzi, G., Lanorte, M. T., Galgano, F., Andrighetta, C., Lombardi, A., Lanciotti, R. & Guerzoni, M. E. (2001). Proteolytic, lipolytic and molecular characterization of *Yarrowia lipolytica* isolated from cheese. International Journal of Food Microbiology, **69**, 69 - 77.

Szumski, S. A. & Cone, J. F. 1962. Possible role of yeast endoproteinases in ripening surface ripened cheeses. Journal of Dairy Science, **45**, 349 - 353.

Tamime, A.Y. (2002). The Microbiology of Milk Products. In Dairy Microbiology, Vol. 2, Robinson R. K., 113.

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

Tungjaroenchai, W., White, C. H., Holmes, W. E., & Drake, M. A. (2004). Influence of adjunct cultures on volatile fatty acids in reduced–fat Edam cheese. Journal of Dairy Science, **87**, 3224 – 3234.

Upreti, P., McKay, L. L. & Metzger, L. E. (2006). Influence of calcium and phosphorus, lactose, and salt- to- moisture ratio on Cheddar cheese quality: Changes in residual sugars and water- soluble organic acids during ripening. Journal of Dairy Science, **89**, 429 - 443.

Urbach, G. (1995). Contribution of lactic acid bacteria to flavour compound formation in dairy products. International Dairy Journal, **5**, 877 – 903.

Viljoen, B. C. (2001). The interaction between yeasts and bacteria in dairy environments. International Journal of Food Microbiology **69**, 37 - 44.

Vlijoen, B. C. & Greyling, T. (1995). Yeasts associated with Cheddar and Gouda making. International Journal of Food Microbiology, **28**, 79 – 88.

Welthagen, J. J. & Viljoen, B. C. (1996). The presence of yeasts in different cheese types. In *Yeasts in the dairy industry: positive and negative aspects*, Jakobsen, M., Narvhus, J., Viljoen, B.C. (Eds.), Copenhagen, Denmark. International Dairy Federation, Brussels, Belgium, 78 - 87.

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

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 metabolism. International Dairy Journal, **11**, 185 - 201.

CHAPTER 2

Yeasts as adjunct starters in matured Cheddar cheese

Abstract

High numbers of yeasts are frequently observed in cheeses and are believed to make a significant contribution to the maturation process due to their ability to grow at low temperatures, assimilation/fermentation of lactose, the assimilation of organic acids, resistance against high salt concentrations, tolerance of low pH values and low water activities. The use of yeasts as adjunct starters in matured Cheddar cheese was investigated over a maturation period of eight months. The yeasts *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Dekkera bruxellensis* and *Torulaspora delbreuckii* were inoculated as adjunct starters in the making of matured Cheddar cheese in four experimental cheeses and a control cheese was manufactured with the LAB starter culture (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*) only.

The growth of both the yeasts and LAB during maturation, as well as the fermentation of lactose, accumulation of the organic acids and sensory analysis was investigated. Both the yeast and LAB cultures grew in a mutualistic interaction without inhibition of growth of either culture. Yeast populations between 4.1 log cfu/g and 7.5 log cfu/g cheese and a LAB population between 6.9 log cfu/g and 10.3 log cfu/g cheese were recorded for all the cheeses during maturation. Fermentation of lactose was observed during the manufacturing stages as indicated by the complete depletion of lactose at 4 months in all the cheeses from concentrations between 2 and 6 %. Organic acids - lactic, acetic and pyruvic acid in all the cheeses increased with maturation. Concentrations > 1000 µg/g for lactic acid and < 14 000 µg/g for citric acid were detected at maturation stages. Citric acid concentration in all the samples decreased with maturation, as well as orotic and uric acid which were detected at low concentrations compared to the other organic acids. Sensory analysis of the cheeses resulted in desired Cheddar cheese character in yeast matured cheeses compared to the control. Cheese samples inoculated with *Torulaspora delbrueckii* and *Dekkera bruxellensis* had favourable scores for aroma, texture/appearance and mouthful.

2.1. INTRODUCTION

Cheese making began about 8000 years ago and now there are in excess of 1000 cheese varieties worldwide, each unique with respect to its flavour and form. Manufacture of most cheese varieties involves a combination of ingredients: milk, rennet, microorganisms and salt, which are processed through a number of common steps such as gel formation, whey expulsion, acid production and salt addition, followed by a period of ripening/ maturation (Beresford *et al*., 2001).

Cheese manufacture is essentially a dehydration process in which the fat and casein in milk are concentrated between 6- and 12-fold, depending on the variety. The manufacture of rennet-coagulated cheeses, such as Cheddar, can be divided into the conversion of milk to curd and ripening of the curd. Modern cheese manufacturing commences with the use of pasteurized milk, through which acidification as one of the primary events in the manufacture of most, if not all, cheese varieties is achieved. Selected lactic acid bacteria are applied to begin the fermentation process of lactose to lactic acid. The rate and point of the process at which lactic acid is principally produced is characteristic of the cheese variety (Singh *et al*., 2003).

Microorganisms are essential components of all natural cheese varieties and are divided into both starters and secondary microflora. Starter cultures are homofermentative lactic acid bacteria which are added to bring about a fermentation process, consisting of one or more species of bacteria. Starter culture isolates produce sufficient acid to reduce the pH of milk to < 5.3 in 6 h at 30 – 37 °C. Starter cultures may either be in blends of defined strains or as in the case of many cheeses manufactured by traditional methods, composed of undefined mixtures of strains which either are added at the beginning of manufacture or are naturally present in the cheese milk (Beresford *et al.*, 2000; 2001).

Primary starter cultures are homofermentative mesophilic and heterofermentative lactic acid starter cultures. Homofermentative mesophilic starter cultures produce lactic acid only and are widely used in the cheese industry and have an optimum temperature between 20 - 30 °C. Heterofermentative thermophilic lactic acid starter cultures (optimum temperature 37 - 45 °C) produce lactic acid, carbon dioxide, aroma

compounds (e.g. ethanol and acetic acid) from glucose rapidly at high temperatures. Either mesophilic or thermophilic starter cultures are used, depending on the cheese being manufactured. Mesophilic starter cultures are used in the production of Cheddar, Gouda, Edam, Blue and Camembert, while thermophilic cultures are used for high temperature (50 - 55 °C) cooked hard cheeses such as Emmental, Gruyere, Parmesan and Grana. Both mesophilic and thermophilic cultures can be subdivided into mixed (undefined) cultures in which the number of strains is unknown and defined cultures, which are composed of a known number of strains. Thermophilic starters are composed of either single or multiple strains of *Streptococcus thermophilus* and thermophilic lactobacilli such as *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus* while mesophilic starters include *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Beresford *et al.*, 2001; Tamime, 2002).

The growth of secondary flora among cheeses during manufacture initiates the unintentional development of adjunct starter cultures which do not necessarily have starter activity. The source of secondary flora in cheese is the result of contamination during cheese production from the environment, equipment as well as the handling of the cheese ingredients. Adjunct cultures include yeasts, bacteria and NSLAB which are frequently isolated during the maturation of cheese. Yeast adjunct cultures include *Geotrichum candidum*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Yarrowia lipolytica, Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Torulaspora delbrueckii* and various species of *Candida;* moulds i.e. *Penicillium camemberti, Penicillium roqueforti;* bacteria i.e. *Corynebacterium, Staphylococcus, Micrococcus, Propionibacterium* sp. and NSLAB such as *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnococcus* and *Lactobacillus curvatus*. Cheese adjunct cultures are added for their impact on cheese quality, due to their production of lipolytic and proteolytic enzymes, fermentation of residual lactose, and utilization of lactic acid (Chamba and Irlinger, 2004; Welthagen and Viljoen, 1999; Wyder and Puhan, 1999; Beresford *et al.*, 2001).

The primary function of the starter bacteria is to produce acid during the fermentation process, although they also contribute to cheese ripening where their enzymes are involved in proteolysis and conversion of amino acids into flavour compounds. Production of a homogenous, high quality Cheddar cheese requires uniform lactose fermentation, lipolysis and proteolysis. Lactic acid bacteria contribute relatively little to lipolysis, and most dairy LAB strains possess extracellular proteinase, several do not and are mainly dependent on the other strains in the starter culture for the production of peptides and amino acids (Beresford *et al.*, 2001; Smit *et al.*, 2002). Inherent yeast characteristics such as the possession of lipolytic and proteolytic enzymes, assimilation/fermentation of lactose, assimilation of organic acids like succinic, lactic and citric acid favour the desired activity of yeasts in the further maturation of cheese to obtain a homogenous, high quality cheese.

Dairy products offer a special ecological niche that selects for the occurrence of yeasts. The low pH, low moisture content, low temperature of the cheese environment and the ability of yeasts to grow at low temperatures, resistance against high salt concentrations and resistance to cleaning compounds favours their growth in dairy products and environments (Viljoen, 2001; Beresford *et al.*, 2001; Ferreira and Viljoen, 2003). The growth of yeasts in dairy products is not widely accepted due to the fact that continued lactose fermentation by yeasts at maturation stages leads to an increase in acidity, gas content and fruity flavours, while continued hydrolysis of protein and fat softens the texture of the product and produces bitter and rancid flavours which can cause spoilage (Welthagen and Viljoen, 1999; Jakobsen and Narvhus, 1996; Ferreira and Viljoen, 2003).

The yeasts *Y. lipolytica* and *D. hansenii* are regarded as good candidates for ripening agents in cheese, fulfilling specific criteria to be regarded as co-starters in cheese making. *Yarrowia lipolytica* occurs frequently in milk products and the species has the ability to predominate over naturally occurring yeasts and is compatible with starter cultures. The yeast *D. hansenii* occurs in high numbers in cheese mainly due the species ability to grow at low temperatures, high salt concentrations, low a_w values and has a synergistic effect with lactic acid bacteria in cheese (Ferreira and Viljoen, 2003). According to Welthagen and Viljoen (1996), on the presence of yeasts in

different types of cheese, *Torulaspora delbrueckii* was the second predominant yeast strain present in nine different cheese varieties. Upon isolation of yeasts in Cheddar and Gouda cheese, Viljoen and Greyling (1995) found that *T. delbrueckii* cannot assimilate lactose, lactic acid, citric acid and that *Torulaspora* strains are typically fermentative yeasts, tolerant to high salt, high sugar concentrations and low pH, a_w values and can ferment lactose (Welthagen and Viljoen, 1996). It is thus the aim of the study to investigate yeasts as adjunct starter cultures in the making of matured Cheddar cheese.

2.2. MATERIALS AND METHODS

2.2.1. Starter culture preparation

Eight yeast cultures (Table 1) from the UFS cultivar collection were collected and used as adjunct starters. The yeast starters were cultured in 200 mL YM - broth under agitation at 30 °C for 24 h. The cells were counted (cells/mL broth) by means of a Haemocytometer (3 times each) and a yeast suspension containing 1 X 10^8 cells was prepared from the broth. The cells were collected by centrifugation of the broth for 5 min at 6000 rpm in sterile centrifuge tubes. The cells were re-suspended and kept in UHT milk, stored at 4 - 5 °C and used within 24 h (Ferreira and Viljoen, 2003).

2.2.2. Cheddar cheese manufacture

The cheese was manufactured by a fellow student, Mai Nguyen, for a study on the enzymes of yeast adjuncts on Cheddar cheese. Cheeses were made according to de Wit *et al*., (2005) scaled down to 4 L milk, with R 704 FD Chr. Hansen's/ Bulk Starter containing the strains *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis.* The yeast adjunct starter cultures were added 5 min after the addition of the starter culture (Table 1). Samples were obtained after ripening/maturing at specific stages and treated as described below.

2.2.3. Sampling description

A total of five experimental cheeses were prepared (Table 2), each inoculated with the starter culture and a yeast culture except for the control cheese, which was inoculated

with starter culture only. The cheese was kept under controlled conditions (8 - 12 °C) and samples were taken at selected points during the manufacturing of matured Cheddar cheese - from the inoculation of the milk, addition of starter culture, during and after the cheddaring process and also during the maturation stages at day 0, week 1, 2, 3 and after 1, 2, 3, 4, 6 and 8 months.

2.2.4. Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by aseptically opening the cheeses and taking representatives of each batch, and cutting with a sterile knife. For each sample, 1 g cheese was aseptically weighed and added to 9 mL sterile peptone water into Whirl Pak bags (Nasco, USA) and homogenised for 2 minutes using a Colworth 400 Stomacher (London, UK). Further decimal dilutions were carried out as required for microbial assays and plated by the spread plate technique onto selective media. MRS (Merck) agar plates were prepared which are elective for lactic acid bacteria and were incubated aerobically for 48 h at 30 °C, Violet Red Bile Agar (Merck) plates elective for coliforms were incubated for 24 h at 37 °C and Rose Bengal Chloramphenicol – Agar (Merck) plates, selective for yeasts, were incubated for 72 h at 30 °C.

2.2.5. Sample analysis

All plates containing between 30 and 300 colony forming units (cfu) on the highest dilution (or the highest number if below 30) were counted and the mean values were determined from the duplicate plates (Ferreira and Viljoen, 2003).

2.2.6. Chemical analysis

25 mL of 0.01N H_2SO_4 (mobile phase) was added to 5 g of Cheddar cheese and homogenized for 2 min using a Colworth 400 Stomacher for chemical analysis. The extract was centrifuged at 5000 x g for 10 min. The supernatant was filtered through a 0.20 µm membrane filter (Gema Medical S.L). Duplicate samples were performed for all samples. Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector, whereas organic acids

were quantified by means of a HPLC system equipped with a variable wavelength detector set at 220 nm. A Biorad-aminex 87H column with a 0.01N H_2SO_4 at 0.6 ml/min eluent was used (Bouzas *et al*., 1991). The pH was measured at 25 ° C with a Cyberscan pH meter. Organic acid analysis was investigated during the maturation stages of day 0, 2 months, 4 months and 8 months; the sugar analysis was investigated during all the manufacturing and maturation stages.

2.2.7. Sensory Analysis

The sensory analysis of the cheeses was evaluated by an expert panel and executed by the fellow student, Mai Nguyen. For purposes of discussion, referral to her data is essential and therefore these data are added as an appendix (Appendix 1). The sensory quality of the cheeses was evaluated based on an aroma, texture/appearance, mouth feel, taste, after taste and after feel. The sensory score ranged from 1 - 10, with 1 being undesired, 5 desired, 10 highly desired. The cheeses were evaluated after 4 months of maturation.

2.3. RESULTS AND DISCUSSION

2.3.1. Microbial population

Dairy products offer a special ecological niche that selects for the occurrence and activity of specific yeasts, and quite a number of cheese varieties are characterised by the development of a specific surface microflora. Yeasts are therefore frequently found within the microflora of many cheese types and it is still generally not accepted that these yeasts contribute significantly to the quality of the final product (Viljoen, 2001).

The microbial population of the cheese samples indicated a supportive growth medium as indicated by the survival of the yeasts and LAB species in the cheeses. The manufacturing stages of the cheeses initialled with the inoculation of the milk with starter culture (0.34 g/ 10 L) as per supplier instructions and a yeast culture (1 X 10^8 cells) for the experimental cheeses. LAB counts of the inoculated milk were > 10 log cfu/g in all cheeses except for SC 4 (9.8 log cfu/g) and the yeast population was between 3.8 log cfu/g and 7.6 log cfu/g during manufacturing (Fig. 1. (a – e)). The

maturation of Cheddar cheese is by far the most critical stage based on the microbial population growing at this stage. During the maturation stage a high starter culture biomass and yeast population represent considerable biocatalytic potential for cheese ripening reactions as it determines the potential biochemical and enzymic reactions that can occur for the development of the matured Cheddar taste and flavour profile of the cheese (Beresford *et al*., 2001).

The maturation stages of the cheeses indicated an overall LAB population > 6.9 log cfu/g for all the cheese samples. LAB are known to attain densities of 10^8 cfu/g within hours at the beginning of manufacture and 10^9 - 10^7 cfu/g at 2 – 12 months of maturation, respectively in Cheddar cheese (Beresford *et al*., 2001; Lues *et al*., 1999) and that viable starter cell counts are sometimes undetectable (< 10 cfu/g) after about 3 to 4 months although this is dependent on the starter strain used (Swearingen *et al*., 2001). From the investigation, high starter culture populations were evident for the samples C and SC1 which initialled with a LAB population of 10.3 log cfu/g at the beginning of maturation but were reduced at the end of maturation to 7.7 log cfu/g for both samples. The other samples - SC 2, SC 3, and SC 4 had LAB populations that ranged between 8.7 – 9.7 log cfu/g at the beginning of maturation and 6.9 - 8.4 log cfu/g at the end of maturation. Prentice and Brown (1983), found that lactobacilli were present in cheese at concentrations of 2 X 10^4 - 2 X 10^6 colonies/g after 24 h and that their numbers increased to 10^7 colonies/g in 10 - 60 days.

Harper and Kristoffersen (1956), reported a decrease in LAB added for acidity development shortly after the cheese has been made, when the microorganisms responsible for secondary fermentation became more dominant. Ferreira and Viljoen (2003) also found that the LAB in the control cheese decreased by more than one log unit when compared to that of the experimental cheeses during maturation. During the present study the sample SC 4 showed an overall decreased LAB population during manufacturing compared to the other samples including the control. The LAB population of SC 4 remained between 9.8 log cfu/g to 7.7 log cfu/g from the beginning to the end of maturation, respectively. The overall LAB population of the samples showed a decline during maturation and this was attributed to the competition for nutrients based on the presence of yeasts at the same time.

High numbers of yeasts are frequently observed in cheeses and are believed to make a significant contribution to the maturation process. Their occurrence may be attributed to the yeast's ability to grow at low temperatures, the assimilation/ fermentation of lactose, the assimilation of organic acids, their proteolytic and lipolytic activities, resistance against high salt concentrations and resistance to cleaning compounds and sanitizers. Furthermore yeasts have the ability to tolerate low pH and water activity values (Ferreira and Viljoen, 2003).

There are numerous references to the occurrences of yeasts in retail cheese where counts are often 10^5 - 10^6 cfu/g and in some cheese varieties as high as 10^7 - 10^8 cfu/g. Yeasts in cheese are considered insignificant at the earlier stages of cheese production, but play a significant role in later stages being present as natural contaminants in the curd during maturation (Welthagen and Viljoen, 1999). Enumeration of the yeasts during the present study resulted in a decrease in the yeast counts during maturation. The highest yeasts counts were observed for SC 2 at the start of maturation with counts of 7.5 log cfu/g which declined to reach counts of 5.1 log cfu/g at the end of maturation. Throughout the maturation of the cheese samples a decrease in the yeast counts was observed compared to the initial counts at manufacturing and at the beginning of maturation. The samples SC 4 and C were however in contrast to the observation, as the yeast counts increased during the late maturation stages.

Yeasts are generally assumed to be post-pasteurisation contaminants and that in cheese can rise to levels as high as 10^5 cfu/g without any deterious effect on the quality of the product (Prentice and Brown, 1983), this was evidenced by the survival of the yeast counts in the experimental cheeses and the control cheese with no hydrolytic rancidity, fruity odours, slime formation or discolouration observed on the cheeses. Ferreira and Viljoen (2003) reported on a decrease in *D. hansenii* counts and the cessation of survival of *Y. lipolytica* after four months of maturation. In the present study it was observed that none of the yeasts or LAB species ceased to survive during the maturation stages.

2.3.2. Chemical analysis

The role of carbohydrate hydrolysis products as indicators of cheese quality and maturation is a research field that is not well established. The reason is that the compounds associated with glycolysis are perceived not to contribute significantly to the sensorial properties of cheese, like texture and aroma compared to that of proteolysis or lipolysis. Organic acids and related compounds have, however, been utilized as classification parameters for different cheeses in models to predict ripening time or the glycolytic age and reflect the status of microbial metabolism. Whether they contribute to or impair cheese quality, it can be deduced that organic acids and related compounds play an integral role in Cheddar cheese flavour (Lues, 2000).

From a maturation point of view the carbohydrate catabolism may be divided into two phases – the primary fermentation and the secondary fermentation, with the LAB responsible for the primary fermentation and the other microorganisms i.e. yeasts, adventitious NSLAB responsible for the secondary fermentation. The primary fermentation covers the fermentation of the cheese milk taking place in the cheese vat and the fermentation during the first 24 hours or so during which the residual lactose in the cheese is primarily fermented into lactic acid by the starter cultures to create proper acidic conditions.

The conversion of lactose to lactic acid during and after manufacturing is essential in all cheese varieties. The lactic acid plays an important part in the character of the cheese with the primary fermentation indirectly determining the maturation of cheese. During primary fermentation all lactose is consumed with only trace amounts of glucose and galactose detectable for the next 7 to 14 days. In addition some galactose may accumulate if starters containing *Streptococcus thermophilus* and/or *Lactobacillus bulgaricus* have been used or may be fermented by the adventitious NSLAB flora (Fagen *et al.*, 1952). Yeasts are traditionally associated with fermentation, and nearly half of the presently known species lack the ability to ferment sugars (Ferreira and Viljoen, 2003) and the yeast cultures used in this study do not ferment lactose with only *D. hansenii* weakly assimilating lactose. According to literature the monosaccharide glucose is readily utilised directly by the starter cultures

(Ferreira and Viljoen, 2003) while the accumulation of galactose is important for the growth of the lactose-negative yeasts as reported by Álvarez- Martín *et al*., (2008).

In the experimental cheeses (Fig 2 (a – e)) the utilization of lactose was similar to that of the control. Lactose concentration declined from inoculation of the milk through to the complete degradation of the sugar at four months of maturation. Lactose concentration was initially present between 2 – 6.0 % and galactose concentration remained below 2 % during the manufacturing stages and complete depletion of the sugars was observed at the maturation stages of all the cheeses.

According to Bouzas *et al*. (1991) lactose in Cheddar cheese decreased with maturation time from an initial high level of 7.25 to 3.85 mg/g cheese after 8 days of storage and after 48 days no lactose was found. Fagen *et al., (*1952) also found that there was a strong positive test for lactose up to the time the cheese was pressed and comparison of cheese made from raw milk with that made from pasteurised milk resulted in similar lactose degradation were the lactose was completely depleted at day 53. The complete and rapid metabolism of lactose and its constituent monosaccharides in cheese curd is essential for the production of good quality cheese since the presence of fermentable carbohydrate may lead to the development of an undesirable secondary flora (McSweeney and Fox, 2004).

The utilization of organic compounds for flavour and aroma compounds is of crucial importance to the overall flavour development of cheese. Cheese varieties vary as to when the major part of the lactic acid is produced in the manufacturing process. Varieties such as Cheddar and Provolone require almost complete acid development before the manufacturing process is completed. Each cheese variety has its own chemical pattern consisting of certain metabolic products. The difference may be quantitative or qualitative depending on how closely related the varieties are to one another. All the products from lactose degradation are well known metabolic products, but most of them are characterised as intermediates – elaborating that they are between the beginning and the end of fermentation. These include acetic acid, propionic acid, diacetyl, CO_2 and ethanol which are the secondary carbohydrate fermentation products. Cheese deviating from the desired variety characteristics

usually shows variation in the intermediate metabolic pattern. The best secondary reaction in cheese involves the conversion of lactic acid to propionic acid in Swiss cheese (McSweeney and Fox, 2004; Swearingen *et al*., 2001; Harper and Kristoffersen, 1956).

Depiction (Fig 3) of the production of organic acids from lactose fermentation results in the accumulation of flavour and aroma compounds from the organic acids. Organic acids mainly encountered in the fermentation process vary according to the product of interest as well as the fermentation metabolism of the species involved in the fermentation. During Cheddar cheese maturation organic acids such as lactic, citric, pyruvic, acetic acid take preference over other accumulating organic acids. Also most of the organic acids accumulated are not necessary essential in the development of flavour for that particular cheese, i.e. propionic acid is significant in Swiss cheese more than in Cheddar cheese.

From the results (Table 3 (a – e)) obtained during the analysis of the accumulated organic acids in the cheese samples, it can be deduced that the cheese developed the desired curd acidity. The organic acid concentrations of the cheese samples showed varied concentrations. Pyruvic acid, the main organic acid after the fermentation of lactose showed an increasing concentration in all the samples. The organic acids lactic and acetic acid showed a continued increase during the maturation stages. Citric acid decreased in all the cheese samples throughout maturation. Singh *et al.* (2003) reported that the concentration of citrate in Cheddar cheese decreases slowly to almost zero at 6 months, presumably as a result of metabolism by lactobacilli. Cheddar cheese contains ca. 0.2 - 0.5 % of citrate and is responsible for the characteristic eyes in Dutch-type cheeses and for the undesirable openness and floating curd defects in Cheddar and Cottage cheese. Diacetyl and acetate (present at fairly high concentrations in Cheddar) produced from citrate contribute to the flavour of Dutch-type and Cheddar cheeses (Beresford *et al*., 2001; McSweeney and Fox, 2004; Singh *et al.,* 2003).

Álvarez- Martín *et al*. (2008) reported on high amounts of lactic acid being produced by LAB, with *L. citreum* and *L. paracasei* producing noticeable amounts of acetic acid

and consuming most of the citric acid. Among the yeast species investigated, lactic acid was produced by three strains of *G. candidum* and citric acid was metabolized to completion by *P. fermentans* and a part was used by *C. famata*, *G. candidum* and *P. membranifaciens* strains. Citric and lactic acid concentrations of 235, 292, 263 mg/100mL^{-1} and 733, 87, 83 mg/100mL^{-1} for milk fermented by *Lactococcus lactis* 2BA1, Wg2; *D. hansenii* 3AD24; *Y.lipolytica* 4AD16 respectively were reported. The propionic acid concentration of the yeast fermented milk was 77 mg/100mL^{-1} for both *D. hansenii* 3AD24 and *Y. lipolytica* 4AD16 while formic acid was 59 mg/100mL^{-1} and 57 mg/100mL^{-1} for the former and the latter. Bouzas *et al*., (1991) reported an increase in lactic acid from 19.5mg/g at time 0 to 28.3mg/g after 48 days in Cheddar cheese.

Lactic acid concentration in the experimental cheeses (Table 3 (a – e)) increased during maturation to concentrations > 1000 µg/g and the same was observed for the control. A decrease in the citric acid concentration was also observed, however it never decreased to less than 700 µg/g. Propionic acid was detected only at day 0 in the C sample at 170.21 µg/g and for the sample SC 2 at 251.03 µg/g, with the sample SC 4 having concentrations of 32.26 µg/g and 532.89 µg/g at day 0 and 8 months of maturation respectively. Concentrations of orotic, uric and formic acid decreased throughout maturation for all samples and were < 619.44 µg/g. Bouzas *et al. (*1991) reported on low concentrations (< 2 mg/g cheese) of these acids during the period from day 0 to 60 days. Acetic acid continually increased with maturation time in all the samples. Comparison of the organic acid concentrations of the control and the experimental cheeses indicated similar utilization of the organic acids, with concentrations falling within the same range.

Due to the accumulation of organic acids, cheese curd post manufacture has a pH ranging between 4.5 and 5.3, which does not allow for the survival of acid sensitive species (Beresford *et al.*, 2001). Most yeasts tolerate a wide range of pH and grow rapidly at values between 3 and 8. In general, yeasts prefer a slightly acidic medium, with an optimum between 4.5 and 6.5. While yeasts will grow well under neutral and alkaline conditions, they do not compete well with bacteria at these pH values and are therefore found as spoilage agents in dairy products at pH values of 5.5 and below.

The actual pH range of growth for a particular species depends upon which organic acids are present in the substrate, since propionic, acetic and lactic acids are generally more inhibitory than the other organic acids (Viljoen, 1996)

From the experimental data (Table 4) it is observed that the pH of the cheeses decreased with the production of organic acids. The pH of all the cheese samples during maturation was between the range of 4.66 – 5.14. This decrease in pH is in agreement with reports (Wyder and Puhan, 1999; Singh *et al.*, 2003; Amos, 2007) where the cheese curd slurry or cheese had a pH of 4.90 - 6.00. Singh *et al*. (2003) reported pH for Cheddar cheese to be between 5.0 - 5. 2. The decrease in pH is attributed to the production of the various organic acids during the secondary fermentation phase of cheese maturation.

2.3.3. Sensory analysis

The sensory analysis of the cheeses (Appendix 1) were obtained from the fellow student, Mai Nguyen, and was based on comparison of the experimental cheeses with the control, which was manufactured using cheese starter cultures. The control cheese resulted in scores below 5 except for texture/appearance, mouth feel and after feel which sample SC 4 showed desired scores for all the sensory characters concerned. Amos, (2007) reported on *Dekkera bruxellensis* having a sturdy texture and an off-flavour, creamy taste with a relative good Cheddar taste. Cheeses matured with *Debaryomyces hansenii* and *Torulaspora delbrueckii* had an overall good taste, while cheese with *Yarrowia lipolytica* developed a better Cheddar taste. The standard cheese by the investigation of Amos, (2007) resulted in a soft, clean creamy texture and had a good development taste.

In the present investigation SC 1 scored below 5 and the scores were also below that of the control cheese. SC 2 and SC 3 samples compared well to that of the C sample. From the investigation SC 4 indicated to be the cheese with the desired Cheddar character compared to the other yeast matured cheeses as well as the control cheese.

2.4. CONCLUSION

The objective of the study was to investigate the use of yeasts as adjunct starter cultures in matured Cheddar cheese. The investigation of lactose degradation resulted in complete lactose fermentation in all the cheeses. It was observed that lactose fermentation was continual during the manufacturing stages and resulted in the complete degradation of the sugar during maturation. This fermentation of lactose yielded the accumulation of organic acids at the maturation stages which are precursors of flavour and aroma compounds in cheese. Thus the accumulation of these organic acids indicated development of the cheese aroma and flavour compound due to the activity of the starter culture and the assimilating abilities of the yeast cultures.

The growth of both the yeasts and LAB resulted in a long and enhanced survival of the LAB in the presence of the yeasts, and this was attributed to the yeasts ability to support the starter culture and the ability of the LAB to ferment lactose favouring survival of the yeasts through their utilization of the organic acids. Due to the accumulation of the organic acids and the presence of yeasts, the occurrence of spoilage and contamination of undesirable microorganisms was inhibited throughout manufacturing and maturation. The sensory analysis corresponded with enhanced taste achieved with the yeast inoculated cheeses. It had desired Cheddar cheese sensory character, attributed to the biochemical activities of the yeasts during the maturation of the cheeses. Therefore, the application of yeasts as adjunct cultures in cheese making seemed to be promising.

Table 1: Yeast species used as inoculum

Yeast species	**Culture ref. number 1**	**Culture ref. number 2**
Debaryomyces hansenii	UOFS y-0610	CSIR y-953
Yarrowia lipolytica	UOFS y-1138	CBS 599
Torulaspora delbrueckii	UOFS y-137	CBS 133
Dekkera bruxellensis	UOFS y-0937	CSIR y-506

Table 2: Sample cheeses prepared for microbial, chemical and sensory analysis

Cheese sample	**Cultures inoculated in cheese**
C	Control (*Lactococcus lactis* subsp. *cremoris* + *Lactococcus lactis* subsp. *lactis*)
SC 1	*Yarrowia lipolytica*
SC 2	*Debaryomyces hansenii*
SC 3	*Torulaspora delbrueckii*
SC 4	*Dekkera bruxellensis*

Table 3: Organic acid concentrations (µg/g) at Day 0; 2, 4 & 8 months of cheese maturation

(a) Control (C)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	1318.67	14.17	44.54	272.96	9343.77	110.49	120.05	3.68	170.21
2 Months	nd	nd	nd	nd	nd	nd	nd	nd	nd
4 Months	1237.07	12.22	112.27	619.44	13552.7	9.63	187.6	1.14	nd
8 Months	1007.97	9.77	117.95	247.95	12015.81	nd	127.68	1.55	nd

(b) *Yarrowia lipolytica* (SC1)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	783.21	32.12	29.22	1123.42	11866.85	nd	35.36	32.95	nd
2 Months	nd	nd	nd	nd	nd	nd	nd	nd	nd
4 Months	1181.13	10.76	183.33	85.73	14173.28	26.33	190.54	0.95	nd
8 Months	1314.45	9	190.94	202.96	10244.91	nd	627.94	0.88	nd

(c) *Debaryomyces hansenii* (SC 2)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	nd	nd	nd	nd	nd	nd	nd	nd	nd
2 Months	1317.34	11.1	57.1	432.75	14103	43.74	157.58	1.04	nd
4 Months	910.14	9.14	64.9	181.59	10747.46	nd	289.03	0.38	nd
8 Months	982.75	9.46	124.75	34.27	12501.22	nd	532.81	1.29	251.03

(d) *Torulaspora delbrueckii* (SC 3)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	1061.85	11.25	113.41	445.75	13097.17	72.87	121.99	4.65	nd
2 Months	1090.53	11.24	43.95	484.32	12019.82	62.15	137.96	0.96	nd
4 Months	nd	nd	nd	nd	nd	nd	nd	nd	nd
8 Months	985.69	12.73	157.41	267.76	13160.29	nd	203.98	4.4	nd

(e) *Dekkera bruxellensis* (SC 4)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	738.4	10.29	23.67	83.66	5216.84	11.49	60.24	3.92	32.26
2 Months	1027.79	8.4	34.62	136.07	6593.03	nd	54.61	0.36	nd
4 Months	1401.39	12.83	85.53	232.19	12003.11	30.14	193.05	1.26	nd
8 Months	1175.79	11.27	151.24	310.25	11443.27	nd	401.89	2.12	532.89

Table 4: pH measurements during cheese maturation at Day 0; 2, 4 & 8 months

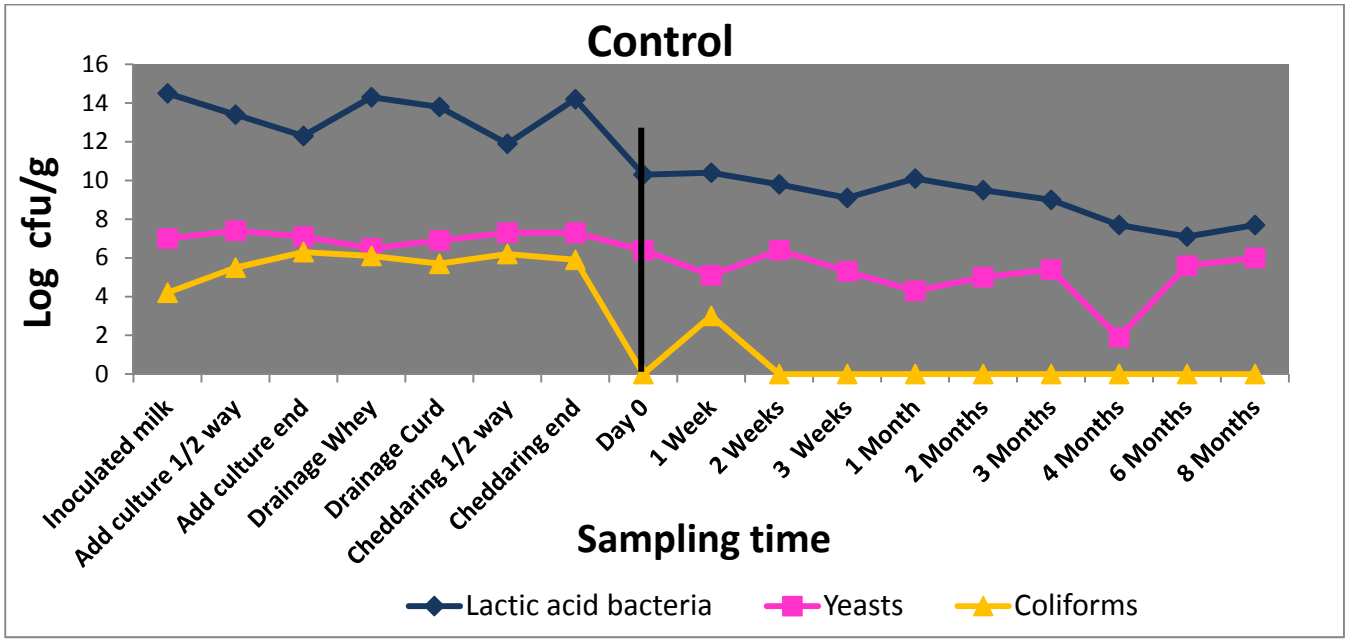
Cheese Sample	**Day 0**	**2 Months**	**4 Months**	**8 Months**
Control (C)	4.87	4.47	4.81	5.12
Yarrowia lipolytica (SC 1)	5.09	4.66	4.77	5.14
Debaryomyces hansenii (SC 2)	5.12	4.74	4.69	5.07
Torulaspora delbrueckii (SC 3)	5.11	4.72	4.8	5.1
Dekkera bruxellensis (SC 4)	4.85	4.74	4.7	5.11

Appendix 1: Sensory analysis in single inoculated cheeses at 4 months, performed by a fellow student, Mai Nguyen, for a study on the enzymes of yeast adjuncts on Cheddar cheese.

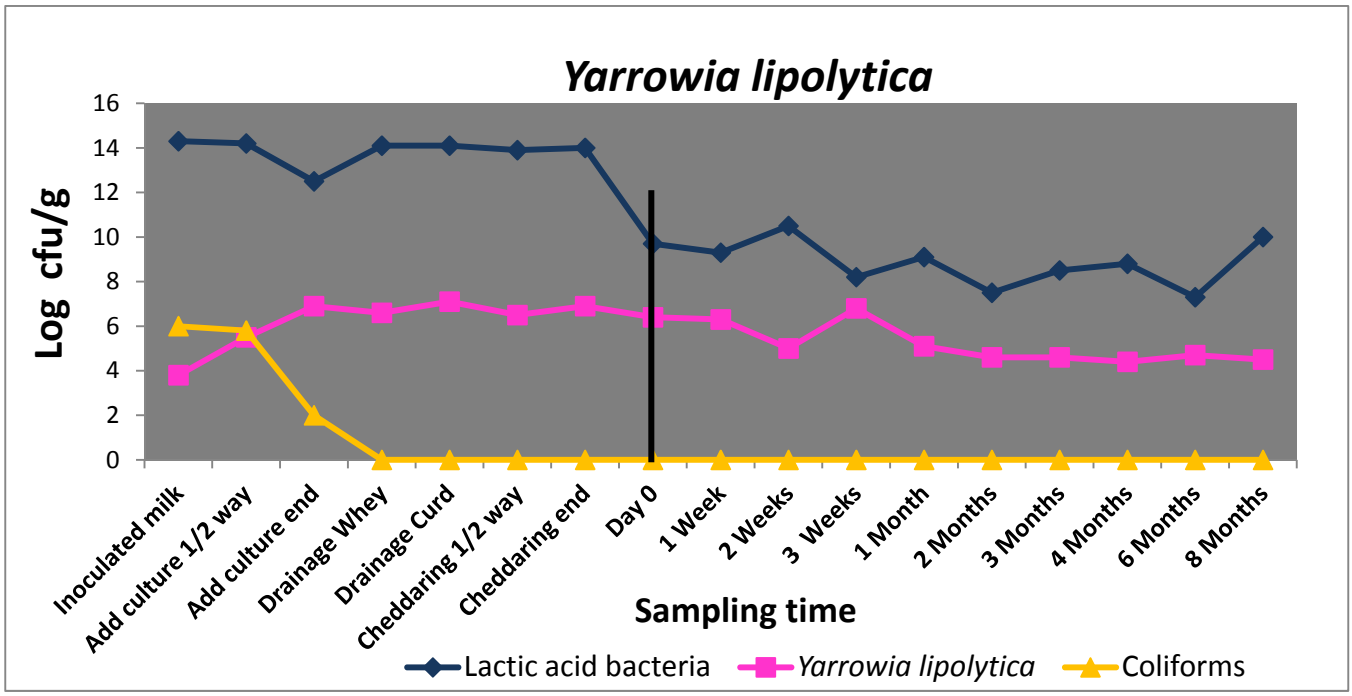
Cheese sample	**Aroma**	**Texture/ Appearance**	**Mouthfeel**	**Taste**	**After taste**	**After feel**
Control (C)	4.7	6.5	6.1	4.1	4.6	5.8
Yarrowia lipolytica (SC 1)	3.8	5.3	4.2	3.3	2.7	3.8
Debaryomyces hansenii (SC 2)	4.8	5.3	4.7	4.1	4.3	4.3
Torulaspora delbrueckii (SC 3)	5.6	6.1	5.1	4.3	3.6	4.6
Dekkera bruxellensis (SC 4)	7.2	6.6	6.8	6.6	6.1	6.3

Figure 1: Microbial population during the manufacturing and maturation of Cheddar cheese

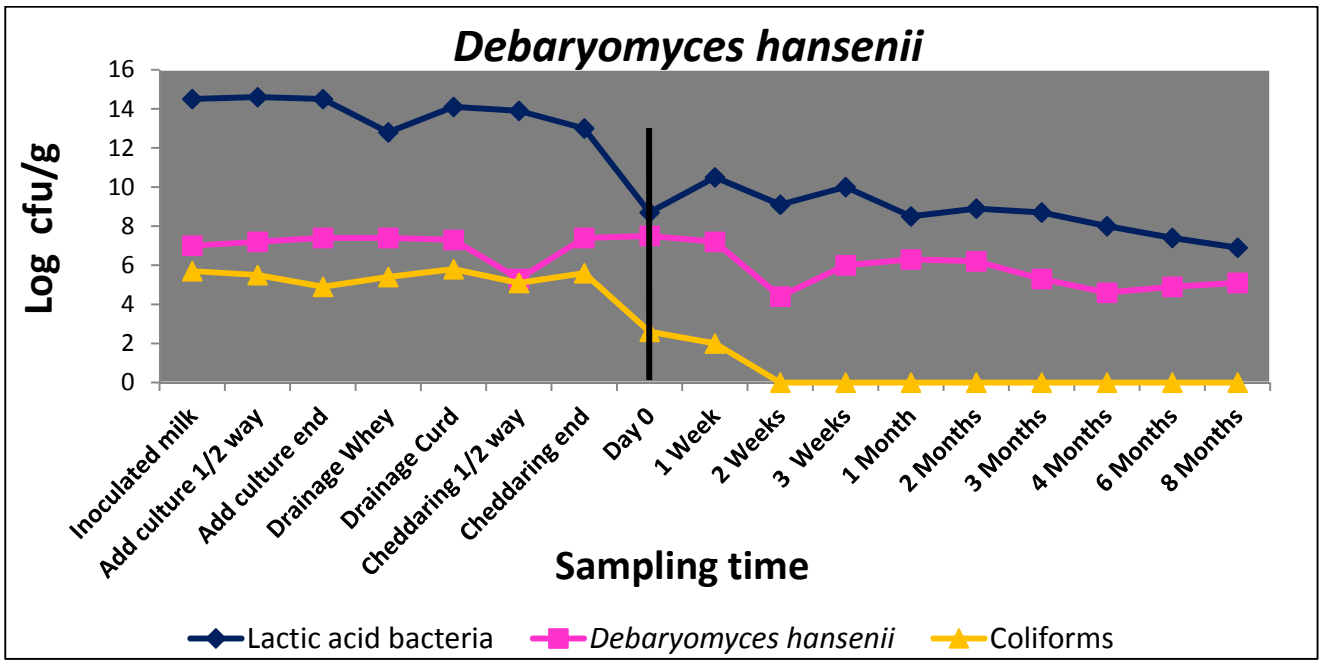
(a) Control (C)



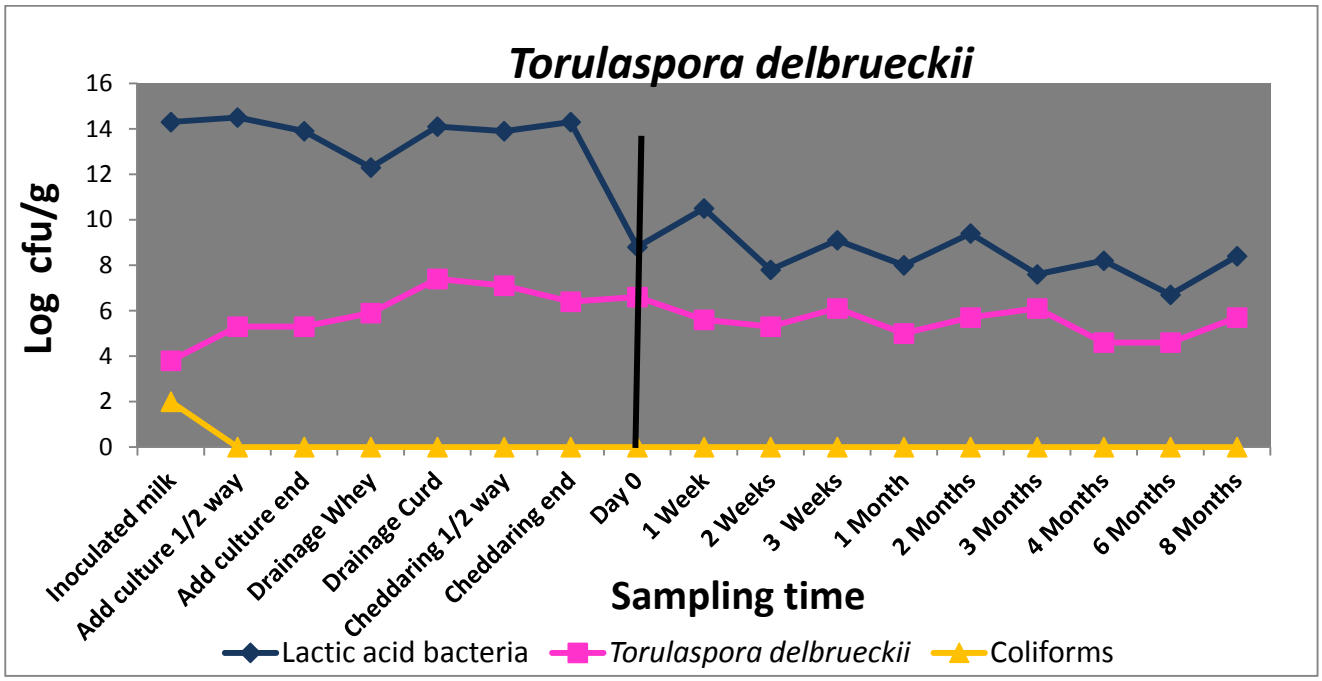
(b) *Yarrowia lipolytica* (SC 1)



(c) *Debaryomyces hansenii* (SC 2)



(d) *Torulaspora delbrueckii* (SC 3)



(e) *Dekkera bruxellensis* (SC 4)

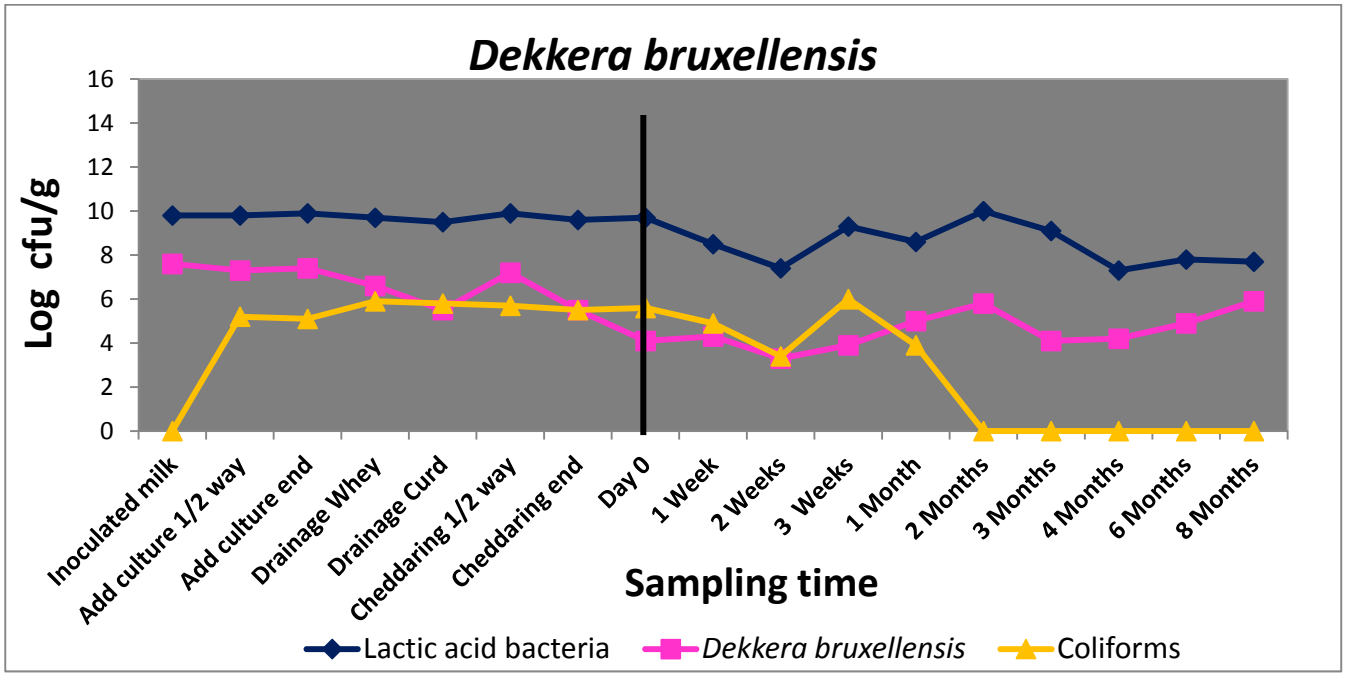
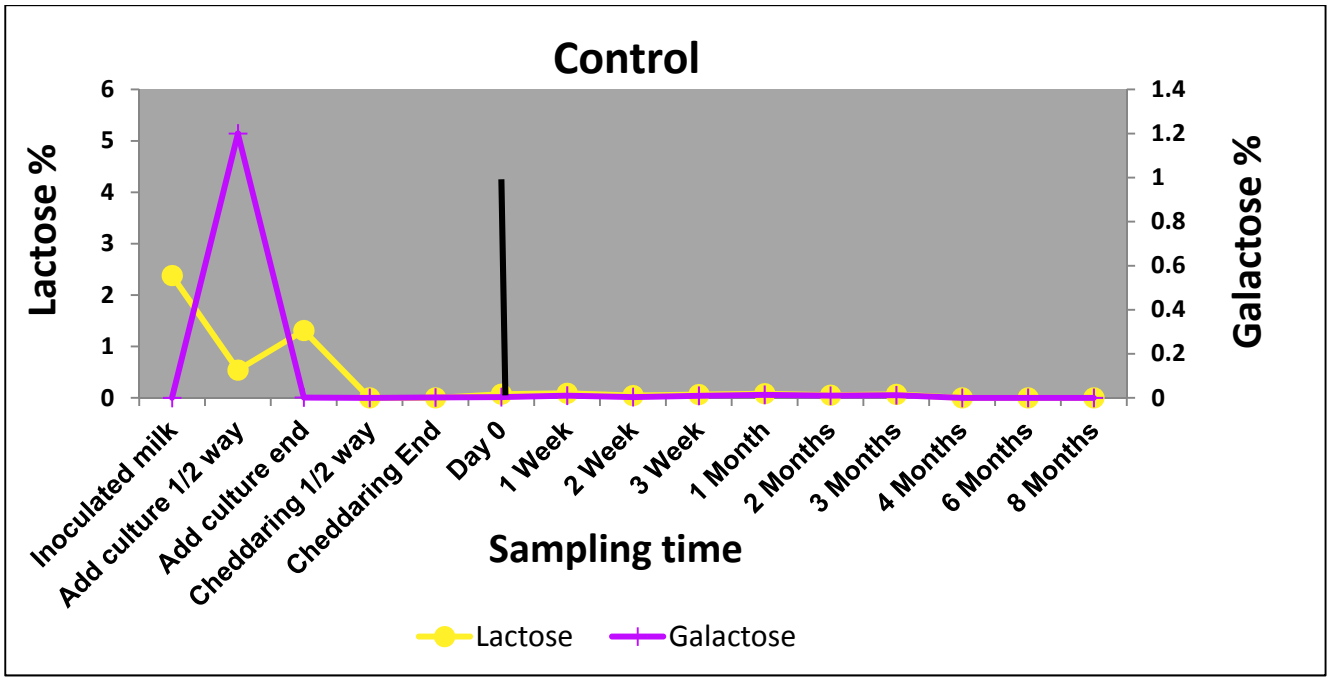
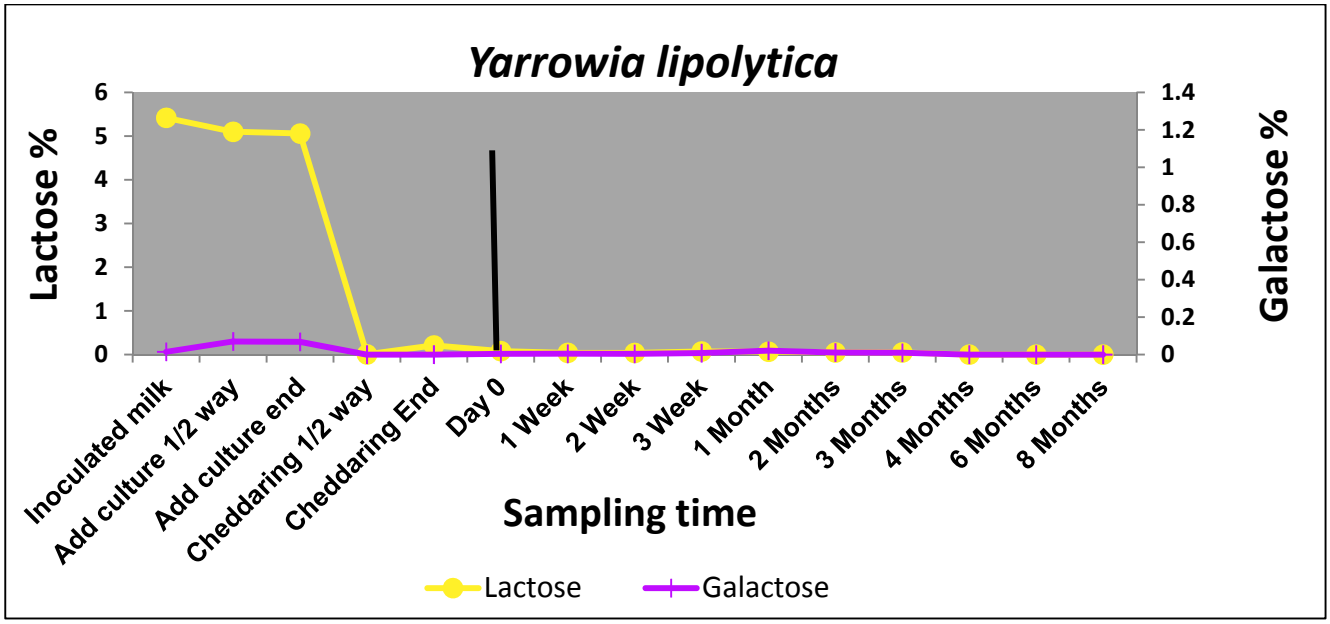


Figure 2: Sugar analysis during manufacturing and maturation of Cheddar cheese

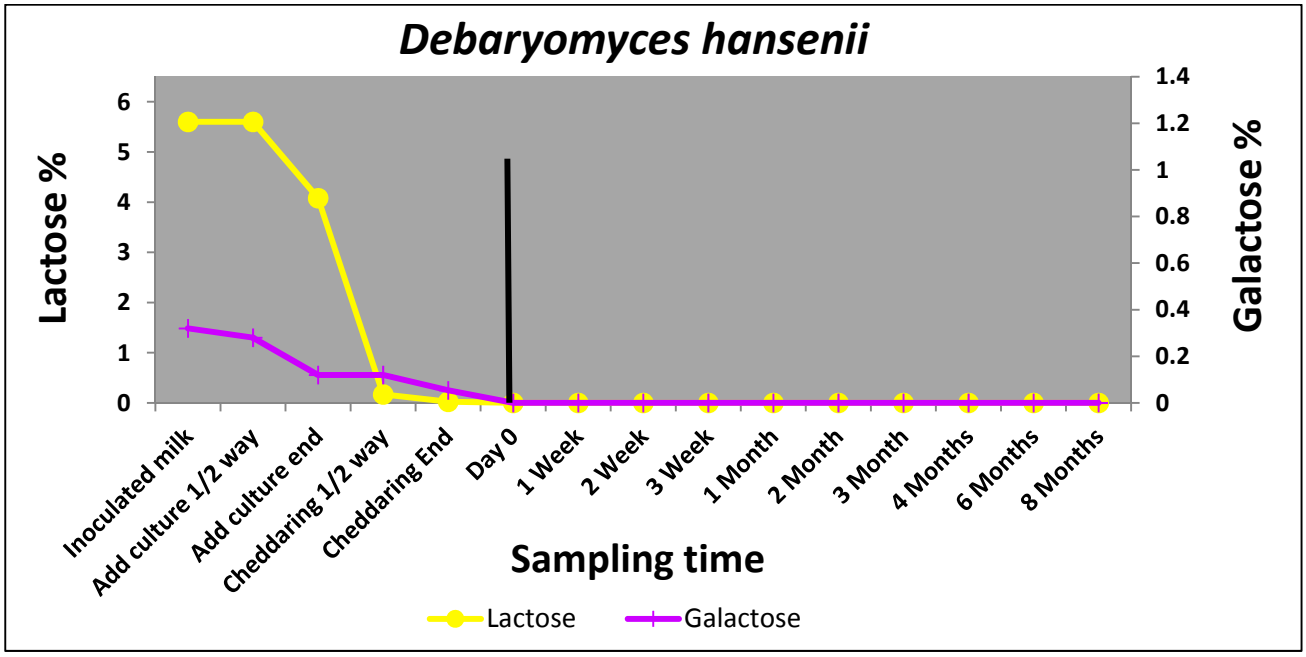
(a) Control (C)



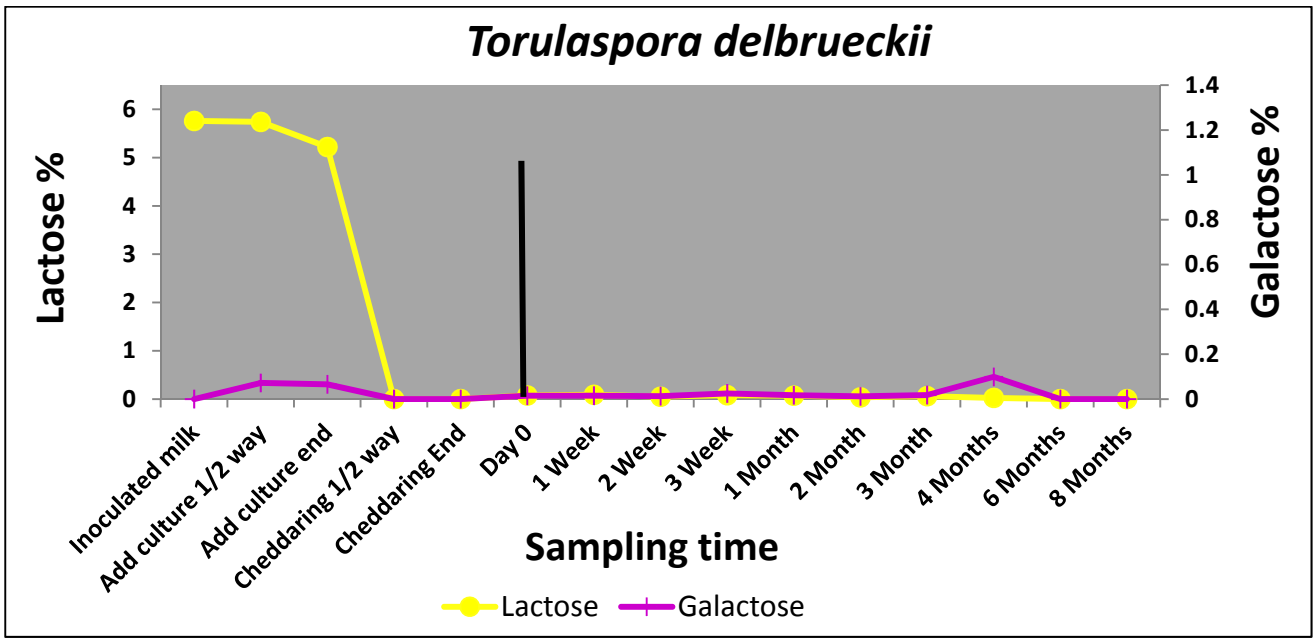
(b) *Yarrowia lipolytica* (SC 1)



(c) *Debaryomyces hansenii* (SC 2)



(d) *Torulaspora delbrueckii* (SC 3)



(e) *Dekkera bruxellensis* (SC 4)

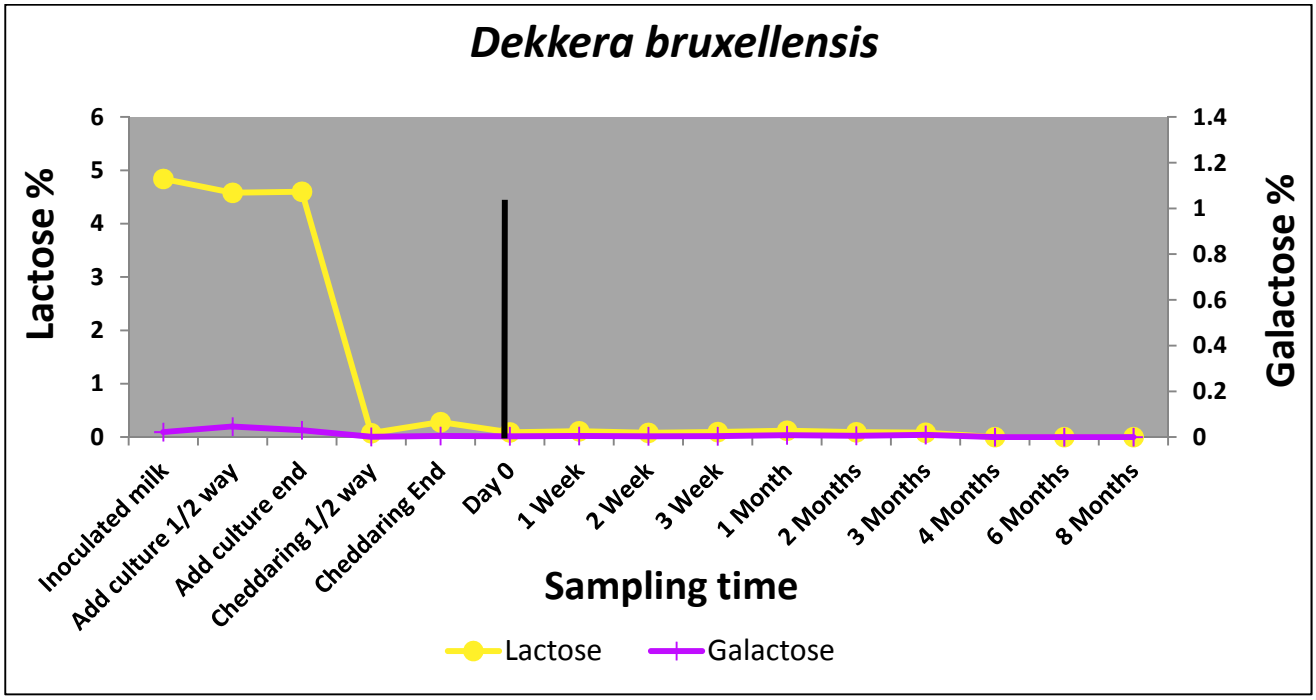
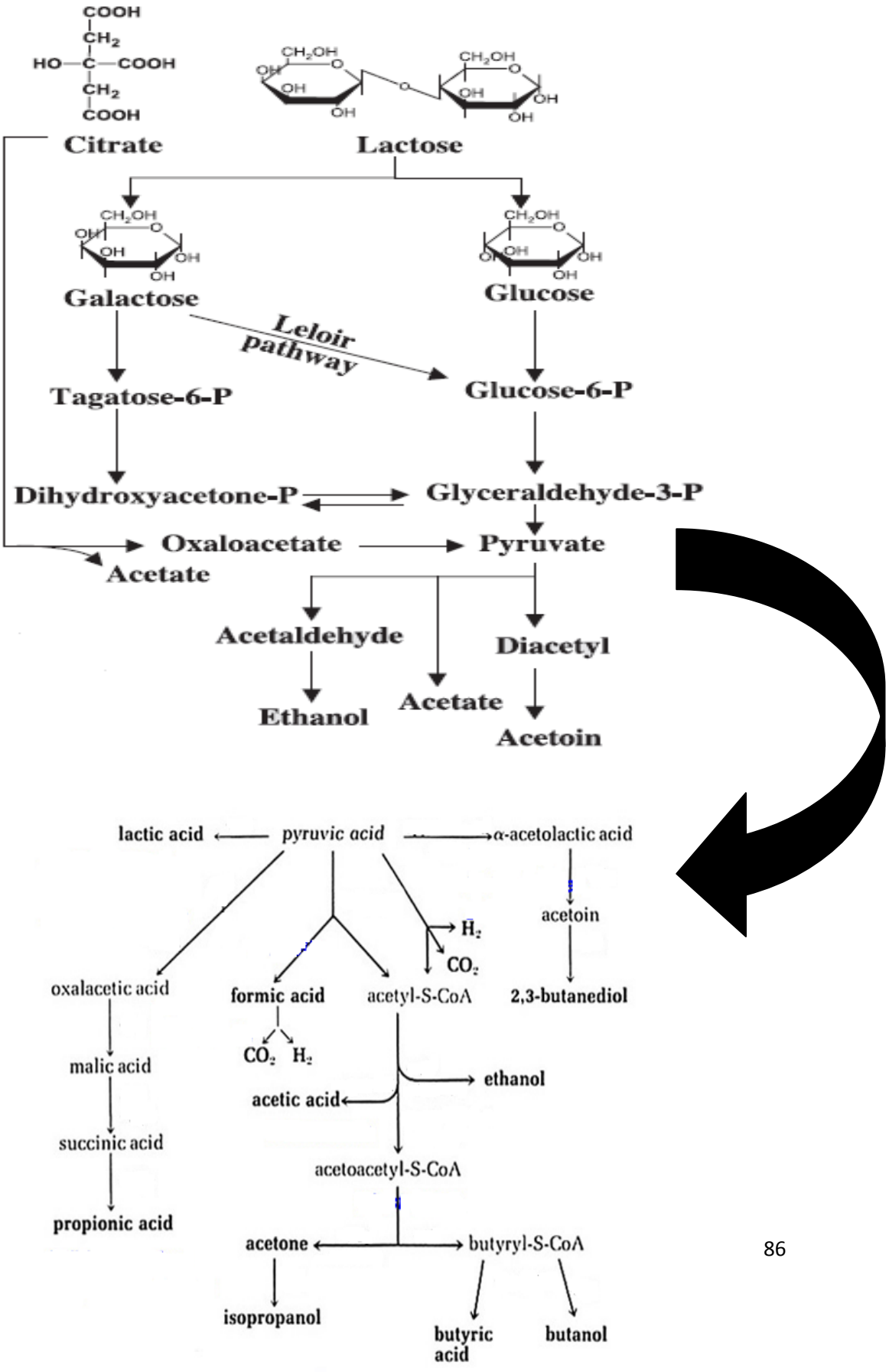


Figure 3: Flavour and aroma (organic acid) accumulation during sugar fermentation (Marilley and Casey, 2004; Todar, 2009)



2.5. REFERENCES

Álvarez-Martín, Pablo, Ana Belén Flórez, Ana Hernández-Barranco & Baltasar Mayo (2008). Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation. Food Control, **19**, 62 - 70.

Amos, Lize-Mari (2007). Manufacture and sensory evaluation of model cheeses with and without yeast adjuncts in *Enzymes from yeast adjuncts in proteolysis during Cheddar cheese ripening*, Magister Scientae, University of the Free State, Bloemfontein, South Africa, 71 – 83.

Beresford, T. P., Cogan, T. M., Fitzsimons, N. A. & Brennan, N. L. (2000). Recent advances in cheese microbiology. International Dairy Federation: Cheese Ripening and Technology, 30.

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

Bouzas, J., Kantt, A. C., Bodyfelt, F. & Torres, J. A. (1991). Simultaneous determination of sugars and organic acids in Cheddar cheese by High- Performance Liquid Chromatography. Journal of Food Science, **56**, 276 - 278.

Chamba, J. F. & Irlinger, F. (2004). Secondary and adjunct cultures. In *Cheese: chemistry, physics and microbiology, general aspects* Vol. 1, 3rd. Fox, Patrick F., McSweeney, Paul L. H., Cogan, Timothy M. & Guinee, Timothy P (Eds.) , pp 192. London: Chapman and Hall.

de Wit, M., Osthoff, G., Viljoen, B.C. & Hugo, A. (2005). A comparative study of proteolysis in Cheddar cheese and yeast-inoculated Cheddar cheese during ripening. Enzyme and Microbial Technology, **37**, 606 - 616.

Fagen, H. P., Stine, J. B. & Hussong, R. V. (1952). Identification of reducing sugars in Cheddar cheese during the early stages of ripening. Journal of Dairy Science, **35**, 779 - 782.

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

Harper, W. J. & Kristoffersen, T. (1956). Biochemical aspects of cheese ripening. Journal of Dairy Science, **39**, 1172 - 1775.

Jakobsen, M. & Narvhus, J. (1996). Yeasts and their possible beneficial and negative effects on the quality of dairy products. International Dairy Journal, **6**, 755 - 768.

Lues, J. F. R. (2000). Organic acid and residual sugar variation in a South African Cheddar cheese and possible relationships with uniformity. Journal of Food Composition and Analysis, **13**, 819 - 825.

Lues, J. F. R., Smit, E. J. & van Zyl, J. M. (1999). Patterns of non-starter microflora during ripening of selected South African Cheddar cheeses manufactured by the open vat procedure. Food Microbiology, **16**, 645 - 651.

Marilley, L. & Casey, M. G. (2004). Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. International Journal of Food Microbiology, **90**, 139 – 159.

McSweeney, P. L. H. & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In *Cheese: Chemistry, Physics and Microbiology, General Aspects Vol 1,3rd Edition.* Fox, P. F., McSweeney, P. L. H., Cogan, T. & Guinee, T. P. (Eds).pp 361 – 371. London: Chapman and Hall.

Prentice, G. A. & Brown, J. V. (1983). The microbiology of Cheddar cheese manufacture. Dairy Industries International, **48**, 23 - 26.

Singh, T. K., Drake, M. A. & Cadwallader, K. R. (2003). Flavour of Cheddar cheese: A Chemical and Sensory Perspective. Comprehensive Reviews in Food Science and Food Safety, **2**, 139 - 162.

Smit, G., van Hylckama Vlieg, J. E. T., Smit, B. A., Ayad, E. H. E. & Engels, W. J. M. (2002). Fermentative formation of flavour compounds by lactic acid bacteria. The Australian Journal of Dairy Technology, **57**, 61 - 67.

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.

Tamime, A. Y. (2002). The Microbiology of Milk Products. In *Dairy Microbiology* Vol 2, R. K. Robinson (Ed), Wiley Interscience, New York, 113.

Todar, Kenneth (2009). Diversity of metabolism in prokaryotes. In Todar's Online textbook of bacteriology, 1 – 8. http://www.textbookof bacteriology.net/metabolism.html

Viljoen, B. C. (2001). The interaction between yeasts and bacteria in dairy environments. International Journal of Food Microbiology, **69**, 37 - 44.

Viljoen, B. C. & Greyling, T. (1995). Yeasts associated with Cheddar and Gouda making. Food Microbiology, **28**, 70 – 88.

Welthagen, J. J. & Viljoen, B. C. (1996). The presence of yeasts in different cheese types. In *Yeasts in the dairy industry: positive and negative aspects*, Jakobsen, M., Narvhus, J., Viljoen, B.C. (Eds.), Copenhagen, Denmark. International Dairy Federation, Brussels, Belgium, 78 - 87.

Welthagen, J. J. & Viljoen, B. C. (1999). Isolation and identification of yeasts obtained during manufacture and ripening of Cheddar cheese. Food Microbiology, **16**, 63 – 73.

Wyder, Marie-Therese & Puhan Zdenko (1999). Role of selected yeasts in cheese ripening: an evaluation in aseptic cheese curd slurries. International Dairy Journal, **9**,117 - 124.

CHAPTER 3

Yeast co-cultures applied as adjunct starters during Cheddar cheese maturation

Abstract

The yeasts *Debaryomyces hansenii*, *Torulaspora delbrueckii* and *Dekkera bruxellensis* were co-cultured with *Yarrowia lipolytica* and used as adjunct starter cultures in matured Cheddar cheese manufacture. Microbial population investigation of the cheeses resulted in enhanced survival of both the yeast and starter culture present during the maturation stages of the cheeses, with populations > 5 log cfu/g cheese. Although no inhibition of either culture at all stages of manufacturing and maturation, the LAB growth of the cheese inoculated with *D. bruxellensis* + *Y. lipolytica* ceased to survive at 6 and 8 months of maturation. The overall yeast populations of the cheeses were > 5 log cfu/g cheese, of which in literature such population is speculated to cause spoilage. However no surface run-off, odour, slime or indications of spoilage were detected in the cheeses in the present study. Undesired microorganisms i.e. coliforms in the cheese were undetected during maturation and this was attributed to the biochemical activities in the cheese.

The complete fermentation of lactose sugar by the starter culture (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*) resulted in the accumulation of lactic acid and other organic acids through the secondary fermentative pathways. The lactic acid, acetic and pyruvic acid increased during maturation of the cheeses. Organic acids - succinic, citric, formic, propionic, uric, orotic varied in concentrations during maturation but indicated an overall decreasing trend. The overall pH activity of the cheeses increased with maturation time except for the cheese with *D. hansenii* + *Y. lipolytica* which decreased. The sensory analysis scores of the cheese with *D. bruxellensis* + *Y. lipolytica*, compared to the control cheese (inoculated with starter culture only) and the other cheeses, resulted in high scores - thus attaining the desired Cheddar cheese characteristics.

3.1. INTRODUCTION

The occurrence and growth patterns of a restricted group of yeast species during cheese making and ripening have been described for different cheese types (Rosalba *et al*., 2005). Cheese curd can be viewed as a complex biochemical habitat in which a variety of microorganisms co-exist, anabolising some substances and catabolising other substances in order to live and grow (Arfi *et al*., 2005). Specific surveys have that the most frequently occurring species in cheeses are *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Pichia membrananefaciens*, *Pichia fermentans*, *Candida famata*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Geotrichum candidum, Rhodotorula mucilaginosa, Torulaspora delbrueckii* and *Trichosporon cutaneum (beigelii)* (Gardini *et al*., 2006; Rosalba *et al*., 2005; Wouters *et al*., 2002; Bockelmann, 2002; Wyder and Puhan, 1999; Roostita and Fleet, 1996b; Welthagen and Viljoen, 1996; Seiler and Busse, 1990). Yeast species belonging to the genera *Candida*, *Yarrowia*, *Torulaspora*, *Rhodotorula, Cryptococcus*, *Trichosporon*, *Saccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Issatchenkia* and *Debaryomyces* were found in Cheddar and Gouda cheeses according to (Welthagen and Viljoen, 1999; Viljoen and Greyling, 1995).

Dairy products offer a special ecological niche that selects for the occurrence of yeasts. The immediate ecosystem of dairy products, environmental conditions prevailing and the pasteurization of raw milk contribute to the selection of a uniform and well-defined yeast domain that initially originates as environmental contaminants (Viljoen, 2001). Yeasts grow well during the manufacturing and ripening of fermentation dairy products due to their tolerance of low pH, low a_w, high salt concentrations (Álvarez-Martin *et al*., 2008), low moisture content, low temperature of the cheese environment and the ability of yeasts to assimilate/ferment lactose, assimilate organic acids like succinic, lactic and citric acid, as well as having proteolytic and lipolytic activities, resistance against high salt concentrations and resistance to cleaning compounds (Rosalba *et al*., 2005; Viljoen, 2001; Beresford *et al*., 2001; Ferreira and Viljoen, 2003).

The growth of yeasts in dairy products is not widely accepted due to the fact that continued lactose fermentation by yeasts at maturation stages leads to an increase in acidity, gas content and fruity flavours. Excessive yeast growth is reported to cause the rind to become soft and smeary, resulting in poor development of the other members of the surface flora in surface ripened cheeses. The condition is usually associated with an unpleasant yeasty or esterlike odour. Excessive hydrolysis of protein and fat softens the texture of the product and produces bitter and rancid flavours which can cause spoilage and the cheese surface to run off. The role of yeasts as spoilage organisms in dairy products is linked with their nutritional requirements, certain enzymic activities and the belief that they make a significant contribution to the maturation process by effecting desirable biochemical changes when present in high numbers in cheeses (Welthagen and Viljoen, 1999; Seiler and Busse, 1990, Jakobsen and Narvhus, 1996; Ferreira and Viljoen, 2003).

Yeasts contribute substantially to the final product and this is attributed to the various interactions between the yeasts, LAB and the secondary flora of bacteria and moulds (Viljoen, 2001). Cheese-surface de-acidification, lactic acid utilisation, alkaline metabolites (NH_3) production is attributed to yeasts, while ripening bacteria contribute to the growth of acid-sensitive bacteria through commensalistic interactions. Lactic acid bacteria acidify the curd, produce organic acids (i.e. lactic acid) and bacteriocins, and spoilage and pathogenic bacteria via amensalistic interactions inhibit acid sensitive bacteria and cause the lysis of pathogenic and spoilage bacteria. Reduced colonisation capacity of auxotrophic strains and limited colonisation by *Listeria monocytogenes* through competition of nutrients result in the harvest of iron and the Jameson effect in cheese through the action of siderophore containing bacteria, auxotrophic bacteria and *L. monocytogenes*. The failure of cheese fermentation is caused by parasitism due to the action of phage bacteria resulting in the inactivation of dominant strains in the cheese (Sieuwerts *et al*., 2008; Irlinger and Mounier, 2009).

The yeasts, as part of the interactions, either contribute to the fermentation by supporting the starter cultures, inhibiting undesired microorganisms causing quality defects or adding to the final product by means of desirable biochemical changes like the production of aromatic compounds, proteolytic and lipolytic activities (Jakobsen

and Narvhus, 1996; Viljoen, 2001). *Debaryomyces hansenii* acts synergically with the lactic microflora and is reported to inhibit the germination of the spores of *Clostridium tyrobutyricum* and *Clostridium butyricum* by nutritional competition and through the production of exo- and endo-cellular metabolites (Deiana *et al*., 1984).

In surface-ripened cheese, yeasts are the first microorganisms to develop on the external part of the cheese surface and dominate during the early stages of ripening, followed by bacterial domination of the surface flora. The metabolism of lactate and the formation of alkaline metabolites, such as ammonia from amino acid de-amination lead to the de-acidification of the cheese surface enabling the growth of less acid-tolerant, but more proteolytic, salt-tolerant microorganisms such as micrococci, *Brevibacterium linens*, *Arthobacter* and *Corynebacterium* spp. and it is reported that when the pH of the cheese surface rises to 5.85 due to the growth of the yeasts the growth of *B. linens* commences (Corsetti *et al*., 2001).

Production of mould-ripened cheeses, such as the Camembert and Blue-veined varieties, involves a maturation stage that is characterised by the growth of a complex ecology of yeasts, bacteria and filamentous fungi. During the manufacturing of Camembert species of *Klebsiella oxytoca*, *Citrobacter freundii*, *Pseudomonas putida, Bacillus* spp., *Staphylococcus xylosus* and *Micrococcus* v*arians* were isolated, with the *Staphylococcus* species showing some tendency to grow. Yeasts were not detected in the product until 2 – 4 days into maturation thereafter; they grew to 10^5 – 10^6 cfu/g at the outer curd. *Debaryomyces hansenii* was the dominant species but *Y. lipolytica* was inconsistently isolated at much lower populations. The populations of *D. hansenii* were about 100-fold greater in Camembert B than in Camembert A. For both cheeses A and B, significant growth of *Acinetobacter*, *Staphylococcus* and *Micrococcus* species did not occur until after growth of the yeast *D. hansenii*. Throughout the production of Blue-veined cheese, no *Acinetobacter* spp. were detected but growth of *S. xylosus* and *Micrococcus* spp. principally *M.* v*arians* was significant. Such growth occurred in the later stages of maturation, after growth of the yeasts, principally *D. hansenii* and to a lesser extent *Y. lipolytica*. *Penicillium roqueforti* developed to 10^8 cfu/g throughout maturation and was the only mould species detected with *D. hansenii* predominating after 20 days of maturation (Addis *et al*., 2001). Therefore the

interactions between yeasts and other microbial groups in cheese and the yeasts inherent characteristics play a substantial role in the development of cheeses. We, therefore investigated the co-culture of yeasts as adjunct starters during the maturation of Cheddar cheese.

3.2. MATERIALS AND METHODS

3.2.1. Starter culture preparation

Eight yeast cultures (Table 1) from the UFS cultivar collection were collected and used as adjunct starters. The yeast starters were cultured in 200 mL YM-broth under agitation at 30 °C for 24 h. The cells were counted (cells/mL broth) by means of a Haemocytometer (3 times each) and a yeast suspension containing 1 X 10^8 cells was prepared from the broth. The cells were collected by centrifugation of the broth for 5 min at 6000 rpm in sterile centrifuge tubes. The cells were re-suspended and kept in UHT milk, stored at 4 - 5 °C and used within 24 h (Ferreira and Viljoen, 2003).

3.2.2. Cheddar cheese manufacture

The cheese was manufactured by a fellow student, Mai Nguyen, for a study on the enzymes of yeast adjuncts on Cheddar cheese. Cheeses were made according to de Wit *et al*.,(2005) scaled down to 4 L milk, with R 704 FD Chr. Hansen's/ Bulk Starter containing the strains *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis.* The yeast adjunct starter cultures were added 5 min after the addition of the starter culture (Table 1). Samples were obtained after ripening/maturation at specific stages and treated as described below.

3.2.3. Sampling description

A total of four sample (control and experimental) cheeses were prepared (Table 2), each inoculated with the starter culture and yeast except for the control cheese. The cheese was kept under controlled conditions (8 - 12 °C) and samples were taken at selected points during the manufacturing of matured Cheddar cheese - from the inoculation of the milk, addition of starter culture, during and after the cheddaring

process and also during the maturation stages of day 0, week 1, 2, 3 and consecutively after 1, 2, 3, 4, 6 and 8 months.

3.2.4. Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by aseptically opening the cheeses and taking representatives of each batch, and cutting with a sterile knife. For each sample, 1 g cheese was aseptically weighed and added to 9 mL sterile peptone water into Whirl Pak bags (Nasco, USA) and homogenised for 2 minutes using a Colworth 400 Stomacher (London, UK). Further decimal dilutions were carried out as required for microbial assays and plated in duplicate by the spread plate technique onto selective media. MRS agar plates were prepared which are elective for lactic acid bacteria and they were incubated aerobically for 48 h at 30 °C, Violet Red Bile Agar plates elective for coliforms were incubated for 24 h at 37 °C and Rose Bengal Chloramphenicol – Agar (Merck) plates, selective for yeasts, were incubated for 72 h at 30 °C.

3.2.5. Sample analysis

All plates containing between 30 and 300 colony forming units (cfu) on the highest dilution (or the highest number if below 30) were counted and the mean values were determined from duplicate plates (Ferreira and Viljoen, 2003).

3.2.6. Chemical analysis

25 mL of 0.01 N H_2SO_4 (mobile phase) was added to 5 g of Cheddar cheese and homogenized for 2 min using a Colworth 400 Stomacher for chemical analysis. The extract was centrifuged at 5000 x g for 10 min. The supernatant was filtered through a 0.20 µm membrane filter (Gema Medical S.L). Duplicate samples were performed for all samples. Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector, whereas organic acids were quantified by means of a HPLC system equipped with a variable wavelength detector set at 220 nm. A Biorad-aminex 87H column with a 0.01N H_2SO_4 at 0.6 ml/min eluent was used (Bouzas *et al*., 1991). The pH was measured at 25 ° C with a

Cyberscan pH meter. Organic acid analysis was investigated during the maturation stages of day 0, 2 months, 4 months and 8 months; the sugar analysis was investigated in all the manufacturing till four months of maturation.

3.2.7. Sensory analysis

The sensory analysis of the cheeses was evaluated by an expert panel and executed by the fellow student, Mai Nguyen. For purposes of discussion, referral to her data is essential and therefore these data are added as an appendix (Appendix 2). The sensory quality of the cheeses was evaluated based on aroma, texture/appearance, mouth feel, taste, after taste and after feel. The sensory scores ranged from 1 - 10, with 1 being undesired, 5 desired, 10 highly desired. The cheeses were evaluated at 4 months maturation. Referral to these data was done to explain the outcome of the yeasts contributions to the final product.

3.3. RESULTS AND DISCUSSION

3.3.1. Microbial population

Inoculation of the milk with the starter culture in the control (C) and experimental cheeses (CC1, CC2 & CC3) resulted in high > 9 log cfu/ g cheese LAB in all the cheeses (Fig. 1 (a - d)). No differences in the LAB population during manufacturing between the control and the experimental cheeses was observed, except for sample CC 3 which had initial counts of 9.0 – 9.6 log cfu/g cheese from the beginning to the end of manufacturing respectively. Initial yeast counts starting from 7.0 log cfu/g cheese were observed for the control and the experimental cheeses except for the sample CC 2 which initialled with counts as low as 2.8 log cfu/g cheese. In all the samples the counts reached > 7.0 log cfu/g cheese at the end of the manufacturing stages including the CC 2 sample. It was noted that during the manufacturing stages the coliform counts in all the cheeses were > 4.2 log cfu/g.

During the maturation stages of the cheeses, it was observed that the increased LAB population was still dominant in the cheeses. LAB populations as high as 10.5 log cfu/g cheese were observed at the start of cheese maturation. This increased starter

culture population is desired as it contributes to the accumulation of intermediate compounds necessary for the further aroma and flavour development of the cheese through lactose fermentation. For the experimental cheese CC 3 the LAB ceased to survive at 6 and 8 months of maturation.

At the beginning of the maturation stages yeasts counts as high as 7.3 log cfu/g cheese were observed for the sample CC 2. For the control cheese, the yeast population was between 6.4 – 6.0 log cfu/g cheese at the beginning to the end of maturation. It was also observed that the coliform counts in all the cheese samples had ceased to survive, this was attributed to the action of the yeasts in inhibiting undesirable microorganisms as well as the production of organic acids during the period of maturation. LAB population was greater than the yeast population in all the samples, and was noted to have stabilised counts when the yeast counts increased.

The authors Prentice and Brown, (1983) reported that yeast levels as high as 10^5 cfu/g without any deleterious effect on the quality of the product were found in Cheddar cheese. *Candida catenulata, C. lipolytica, K. marxianus, D. hansenii* and *S. cerevisiae* were reported to grow in milk at 25 °C reaching maximum populations of 10^7 - 10^8 cfu/mL in 2 - 3 days (Roostita and Fleet, 1996a). Viljoen (2001) reported that the large number of yeasts present during the later stages of ripening is indicative of a possible mutualistic interaction between the yeasts and the starter culture. It was also reported that yeast continue to increase at a faster rate than the starter cultures but with no inhibition of either population.

However Álvarez-Martin *et al*., (2008) reported on *Candida famata* 1AD5 being severely inhibited by all LAB strains as were *D. hansenii* 3AD24 and *Y. lipolytica* 4AD16. Among the four LAB strains investigated, the growth of *Lc. lactis* subsp. *lactis* 2BA1 was enhanced in contrast to *Lb. paracasei* VI – 19 which was inhibited in co-culture with four yeast species: *C. famata*, *D. hansenii*, *K. lactis* and *Pichia membranifaciens*. The strain *Lc. lactis* subsp *cremories* Wg 2 was also inhibited in a majority of co-cultures; though the inhibition was only significant in co-cultures with *C. pararugosa*, *D. hansenii* and *G. candidum*. *Debaryomyces hansenii* inhibited all four LAB, although the inhibition of *Lc.lactis* 2BA1 was not significant.

Co-inoculation of *D. hansenii* + *Y. lipolytica* (Ferreira and Viljoen, 2003) in Cheddar cheese initially decreased during manufacturing followed by a substantial increase (> 3 log units) during the first 48 h of ripening. After the initial two months of ripening the counts decreased to a value of 2 X 10^2 cfu/g after 6 months of maturation. It was also noted that *D. hansenii* predominated the growth of *Y. lipolytica* by counts higher than 3 log units and during the final three months only *D. hansenii* was found. From the data received no inhibition of either species was detected. The population of LAB was dominant in the manufacturing stages and stabilized with the increasing growth of yeasts at maturation stages. The growth of yeasts and LAB in a microbial community is governed by the yeasts inherent technological characteristics and biochemical activities of providing essential growth metabolites such as amino acids, vitamins; removing toxic-end products of metabolism; inhibit the growth of undesired microorganisms by lowering the pH; secretion of alcohol; CO_2 production or by encouraging the growth of the starter culture by increasing the pH due to the utilization of organic acids (Viljoen, 2001).

3.3.2. Chemical analysis

According to Narvhus and Gadaga, (2003) few isolates of yeasts are lactose-positive, but most strains are able to utilise galactose, lactate or citrate. From a maturation point of view the carbohydrate catabolism may be divided into two phases – the primary fermentation (fermentation of lactose to lactic acid by LAB) and secondary fermentation (products from lactose degradation i.e. acetic acid, propionic acid, diacetyl, CO_2 and ethanol produced through the activities of yeasts, adventitious NSLAB) (McSweeney and Fox, 2004).

Lactose degradation (Fig. 2 (a – d)) in all the samples continued in a similar manner, were a concentration between 4 and 6 % was observed during the manufacturing stages. In all the samples, complete depletion or below limit of detection of the lactose sugar was observed to occur up to cheddaring ½ way expect for the sample CC 1 were the sugar was depleted at culture end.

The main function of LAB is the continuous and uniform production of lactic acid from the lactose degradation. From the results (Table 3) the organic acid concentrations of

the samples resulted in increasing concentrations for the organic acids pyruvic acid, lactic acid and acetic acid. The acids citric acid, orotic acid, formic acid and uric acid decreased in all the samples except for the sample CC 2 where citric acid only decreased at 8 months. The concentration of succinic acid was varied within the samples, with C and CC 2 increasing while for the samples CC 1 and CC 3 the organic acid decreased. Propionic acid was detected in the C sample at day 0 as 170.21 µg/g, CC 1 at 8 months as 192.24 µg/g, and in sample CC 2 was not detected while it was 200.83 µg/g in CC 3 at 8 months. Sample CC 1 had > 10 000 µg/g concentration of lactic acid throughout the maturation stages compared to the other samples, however the citric acid concentration indicated an variable trend, increasing and decreasing throughout maturation.

From the samples the organic acid concentrations for the control were not detected at 2 months. The control sample resulted in an increase in lactic acid concentration and a decrease in citric acid concentration throughout the maturation stages. Overall concentrations of formic (< 110.49 µg/g), propionic (170.21 µg/g at day 0 only) and orotic acid (< 15 µg/g) were detected throughout maturation as indicated in brackets, except for propionic acid. Increased concentrations in the acids pyruvic, succinic and acetic were observed and they ranged between 12.00 µg/g – 619.44 µg/g. In all the experimental cheeses (CC 1, CC 2 and CC 3) the lactic acid increased through maturation of which a maximum was reached at 4 months as at 8 months the concentration was decreased for all the samples. The maximum concentrations reached at 4 months were > 10 000 µg/g and at 8 months the concentrations were between 7000 µg/g and 9000 µg/g except for CC 1 which the concentration was 1110.4 µg/g. Citric acid concentrations of the experimental cheeses resulted in varied concentrations, with CC 2 having greater concentrations at 2 and 4 months maturation, CC 1 having decreasing concentration but elevated at 4 months, while with the sample CC 3 the concentrations were not uniform in increasing or decreasing. Pyruvic acid in all the samples increased except for CC 1 where it was varied. Acetic acid increased by more than 2 fold in all the samples by the end of the maturation stages. Succinic and formic acid had decreasing concentrations throughout maturation and these varied in level and the sample.

Co-inoculation of yeast cultures with *Lactococcus lactis* 2BAI, WG 2 resulted in lactic acid accumulation > 600 mg/100mL. The findings were the same for the co-inoculation with other LAB cultures. The *L. lactis* produced acetic acid at level of 40mg/100mL in co-culture with *Pichia membranifaciens* and *Yarrowia lipoytica* (Álvarez-Martín, 2008). In nine cheese-making trials, lactic acid was in much greater abundance than citric, formate or acetic acids; the range for lactic acid was 10.52 - 13.61 mg l00 g $^{-1}$. An overall variation of concentrations of organic acids in the cheeses and between samples of the same variety of cheese was observed. A narrow range was observed for lactic acid within the samples from some specific cheese types, e.g. Mozzarella, Colby, Brick, and commercial Cheddar. In Emmental cheese, significant amounts of succinic and propionic acids were also found. This was due to the presence of gas-forming cultures being used. Trace amounts of succinic acid were found in the Blue cheese samples (Mullin and Emmons, 1997).

The results obtained supported the investigation of the organic in the yeast co-inoculated cheeses, were lactic acid was the dominant organic acid. Variation in organic acids among cheeses is attributed to the different organic acid assimilating activities of the yeasts. Yeasts investigated in milk and cheese slurries, resulted in the utilisation of organic acids at varying capacities as reported by Roostita and Fleet, 1996a; Wyder and Puhan, 1999.

Most yeasts tolerate a wide range of pH and grow rapidly at values between 3 and 8. In general, yeasts prefer a slightly acidic medium, with an optimum between 4.5 and 6.5. While yeasts will grow well under neutral and alkaline conditions, they do not compete well with bacteria at these pH values and are therefore found as spoilage agents in dairy products at pH values of 5.5 and below. The actual pH range of growth for a particular species depends upon which organic acids are present in the substrate, since propionic, acetic and lactic acids are generally more inhibitory than the other organic acids (Viljoen, 1996). The pH of all the cheese samples increased during maturation between 5.05 - 5.21 except for CC 1 which decreased in pH to reach 5.12 at 8 months. This pH is in agreement with reports by Wyder and Puhan, 1999; Singh *et al*, 2003; Amos, 2007 were the cheese or cheese curd slurry had a pH

of 4.90 - 6.00 and Singh *et al*., 2003 reported a pH for Cheddar cheese to be between 5.0 - 5. 2.

3.3.3. Sensory analysis

The sensory analysis of the cheese were obtained from the fellow student, Mai Nguyen, and was based on comparison of the experimental cheeses with the control, which was manufactured using cheese starter cultures. The control cheese resulted in scores below 5 except for texture/appearance, mouth feel and after feel which were above 5. Sensory analysis scores from CC 2 were very low in all areas analyzed except for texture/appearance. The CC 1 sample scored comparatively with the control however only texture/ appearance scored above 5. The sample CC 3 had a greater than 5 score in all areas, and was concluded to have the desired Cheddar cheese characteristics based on the scores.

Co-inoculation of the yeasts *D. hansenii* and *Y. lipolytica*, the cheese developed a good, slightly sweet Cheddar taste after 2 months of ripening, and after 4 months there was a stronger Cheddar taste development. At the end of the ripening period, the development of a mature taste was more significant and the cheese had a clean, slightly sweet, pleasant taste and retained its good strong flavour after 9 months (Ferreira and Viljoen, 2003). Amos, (2007) studied the sensory characteristics of model cheeses with yeast adjuncts in 3, 6 and 9 months ripening period. From the results, cheese co-inoculated with *D. hansenii* + *Y. lipolytica* resulted in a good overall taste in all the sampling times. Cheese inoculated with *T. delbrueckii* + *Y. lipolytica* resulted in very bitter, rancid (3 months); very bitter, hides the cheese taste (6 months) and bitter at (9 months) of ripening. The cheese inoculated with *D. bruxellensis* + *Y. lipolytica* had a good overall taste from 3 months throughout to 9 months.

3.4. CONCLUSION

Yeasts are present during the maturation of Gruyere, Saint-Nectaire, Parmesan and Cheddar cheese and therefore may contribute to the final product. Yeasts are indicated to play a significant role during ripening of cheese by supporting the function of the starter culture (Viljoen, 2001).

From the study the growth of the yeasts and the LAB resulted in a mutualistic interaction as none of the cultures were inhibited. During the maturation stages the growth of the yeasts was enhanced with the stabilized population of LAB. The mutualistic interaction observed is contributed to the yeasts inherent characteristics as well as the environment of the cheese ecosystem. The LAB contributed to the maturation of the cheese by the complete degradation of lactose at manufacturing which resulted in the accumulation of organic acids, which are precursors of flavour and aroma compounds. The yeasts indicated assimilation activity of the organic acids as viewed by the increase in pH for samples C, CC2 and CC3 as well as the increasing concentrations of the acetic acid which is known to be an aroma and flavour precursor in cheese. An increase in pH is not only the result of lactic acid degradation but also of alkaline metabolism products originating from proteolysis. The presence of the yeasts in samples C, CC 2, CC 3 was attributed to the increase in pH due to their proteolytic activity and the assimilating capabilities of the yeasts. Sensory analysis of the cheeses varied for the cheeses and the experimental cheeses had significant higher sensory scores than the control and these are also attributed to the ability of yeasts to form aroma and flavour compounds from the secondary intermediate products of lactose fermentation. Overall the experimental cheeses had desired biochemical and microbiological characteristics to be included as co-inoculated adjunct starters in cheese.

Table 1: Yeast species used as inoculum

Yeast species	**Culture ref. number 1**	**Culture ref. number 2**
Debaryomyces hansenii	UOFS y-0610	CSIR y-953
Yarrowia lipolytica	UOFS y-1138	CBS 599
Torulaspora delbrueckii	UOFS y-137	CBS 133
Dekkera bruxellensis	UOFS y-0937	CSIR y-506

Table 2: Sample cheeses prepared for microbial, chemical and sensory analysis

Cheese sample	**Cultures inoculated in cheese**
C	Control (*Lactococcus lactis* subsp. *cremoris* + *Lactococcus lactis* subsp. *lactis*)
CC 1	*Debaryomyces hansenii* + *Yarrowia lipolytica*
CC 3	*Torulaspora delbrueckii* + *Yarrowia lipolytica*
CC 4	*Dekkera bruxellensis* + *Yarrowia lipolytica*

Table 3: Organic acid concentrations (µg/g) at Day 0; 2, 4 & 8 months of cheese maturation

(a) Control (C)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	1318.67	14.17	44.54	272.96	9343.77	110.49	120.05	3.68	170.21
2 Months	nd	nd	nd	nd	nd	nd	nd	nd	nd
4 Months	1237.07	12.22	112.27	619.44	13552.7	9.63	187.6	1.14	nd
8 Months	1007.97	9.77	117.95	247.95	12015.81	nd	127.68	1.55	nd

(b) *Debaryomyces hansenii* + *Yarrowia lipolytica* (CC 1)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	1122.03	12.26	98.46	456.84	12449.23	64.59	96	3.69	nd
2 Months	938.54	11.54	49.42	255.88	13310.31	43.77	237.88	1.23	nd
4 Months	1328.32	12.86	126.16	60.18	13987.26	nd	466.25	1.17	nd
8 Months	657.39	6.53	85.22	39.16	11101.4	nd	529.95	0.8	192.24

(c) *Torulaspora delbrueckii* + *Yarrowia lipolytica* (CC 2)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	918.63	15.33	38.55	49.98	8526.33	8.46	nd	6.71	nd
2 Months	1074.74	7.57	44.02	362.77	10085.82	152.93	111.09	2.74	nd
4 Months	1317.52	11.89	107.82	577.04	13170.17	36.29	146.25	0.35	nd
8 Months	403.76	18.07	221.25	650.8	8444.45	nd	78.16	19.55	nd

(d) *Dekkera bruxellensis* + *Yarrowia lipolytica* (CC 3)

Sampling Time	Citric Acid	Orotic Acid	Pyruvic Acid	Succinic Acid	Lactic Acid	Formic Acid	Acetic Acid	Uric Acid	Propionic Acid
Day 0	1344.02	23.53	33.17	237.82	9476.3	nd	26.29	14.92	nd
2 Months	1094.24	9.73	45.93	160.27	9567.38	nd	78.73	0.99	nd
4 Months	1203.85	11.13	82.5	306.08	12737.27	28.3	140.23	1.28	nd
8 Months	935.89	9.15	111.31	202.77	7879.69	nd	191.83	1.02	200.88

Table 4: pH measurements during cheese maturation at Day 0; 2, 4 & 8 months

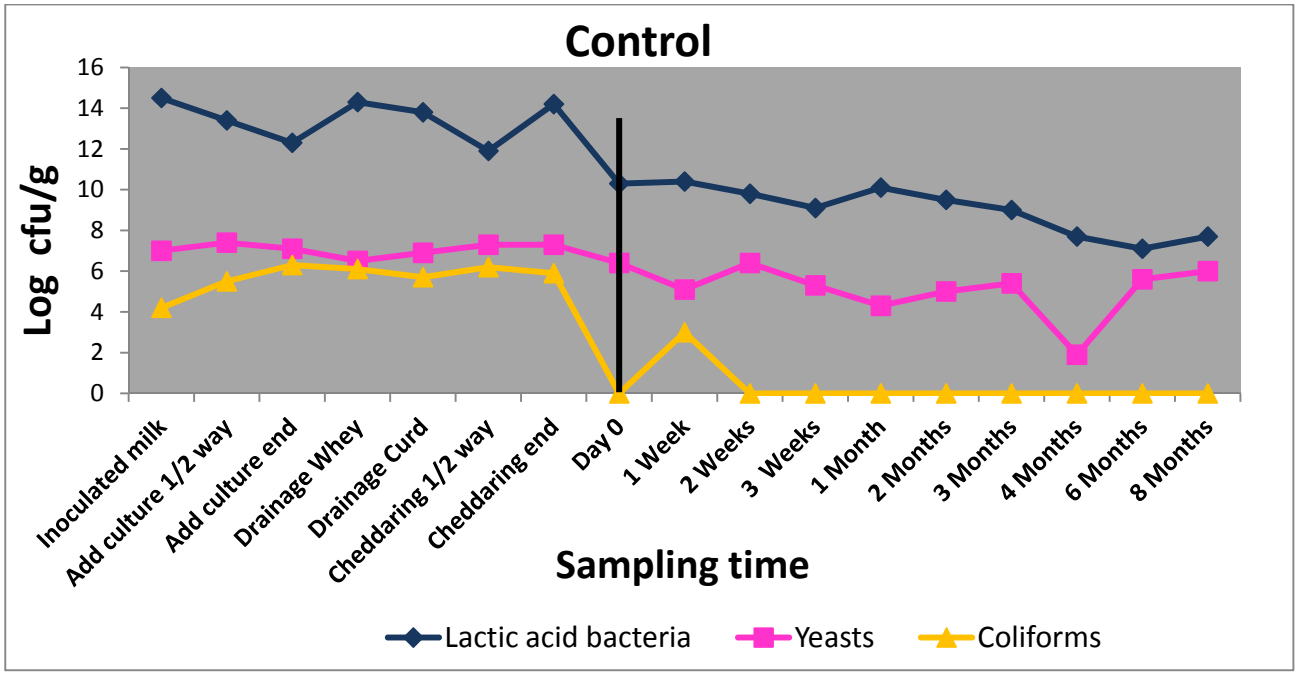
Cheese Sample	**Day 0**	**2 Months**	**4 Months**	**8 Months**
Control (C)	4.87	4.47	4.81	5.12
D. hansenii + *Y. lipolytica* (CC 1)	6.02	4.37	4.65	5.05
T. delbrueckii + *Y. lipolytica* (CC2)	5.02	4.9	4.87	5.21
D. bruxellensis + *Y. lipolytica* (CC3)	4.86	4.83	4.84	5.14

Appendix 2: Sensory analysis in co-inoulated cheeses at 4 Months, performed by a fellow student, Mai Nguyen, for a study on the enzymes of yeast adjuncts on Cheddar cheese.

Cheese sample	**Aroma**	**Texture/ Appearance**	**Mouthfeel**	**Taste**	**After taste**	**After feel**
C	4.7	6.5	6.1	4.1	4.6	5.8
CC 1	4.5	5.3	4.8	3.4	2.8	3.9
CC 2	2.2	5.6	2.8	1.1	1.1	2.4
CC 3	7.3.	7.2	6.5	6.2	5.9	5.9

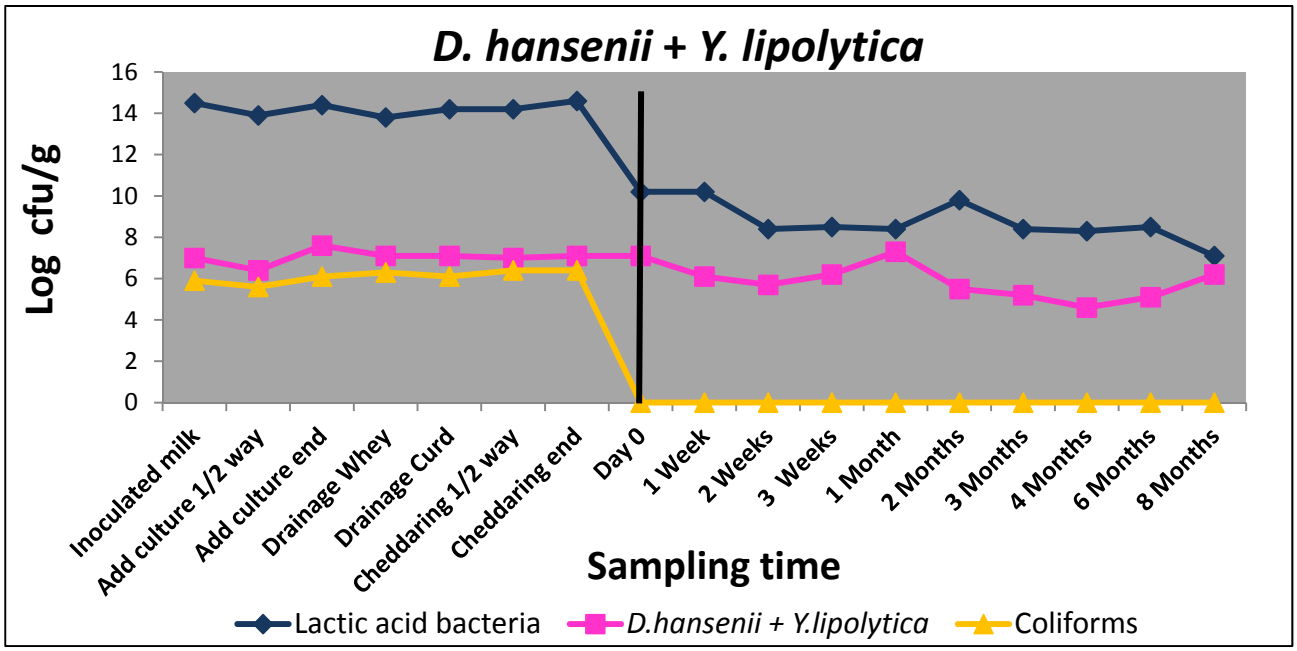
Figure 1: Microbial population during manufacturing and maturation of Cheddar cheese

(a) Control C

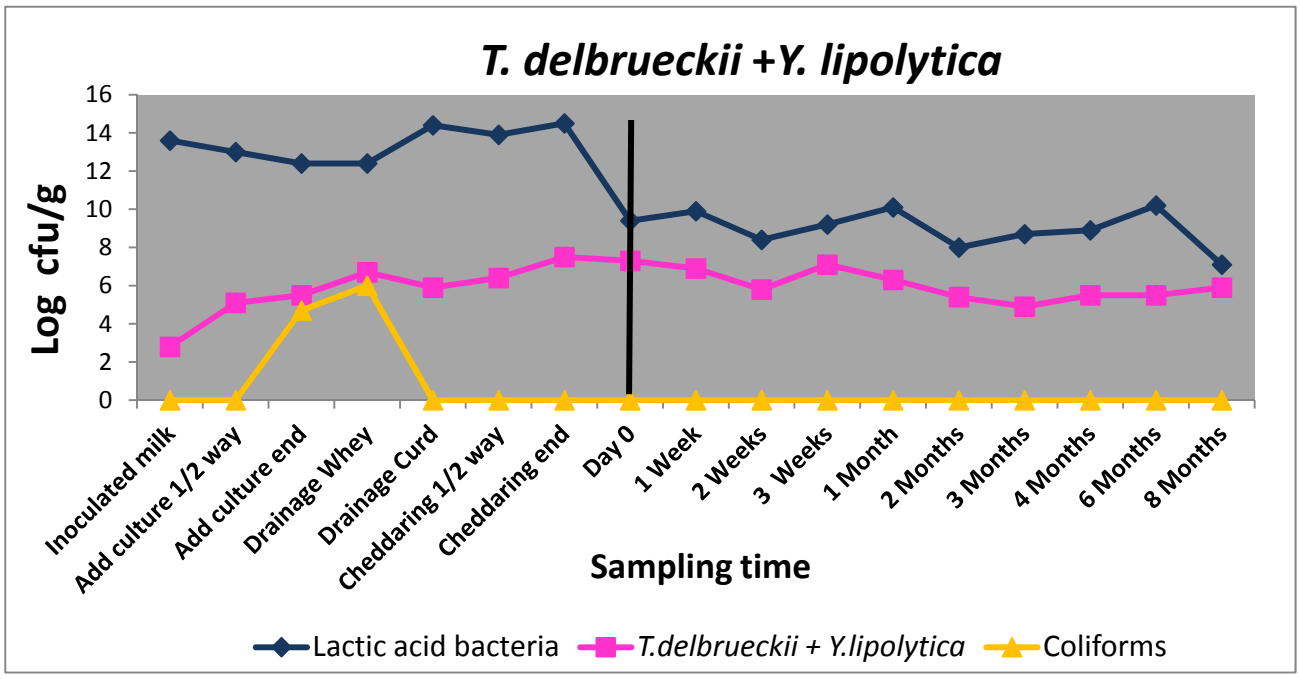


F

(b) *Debaryomyces hansenii + Yarrowia lipolytica* (CC 1)



(c) *Torulaspora delbrueckii + Yarrowia lipolytica* (CC 2)



(d) *Dekkera bruxellensis + Yarrowia lipolytica* (CC 3)

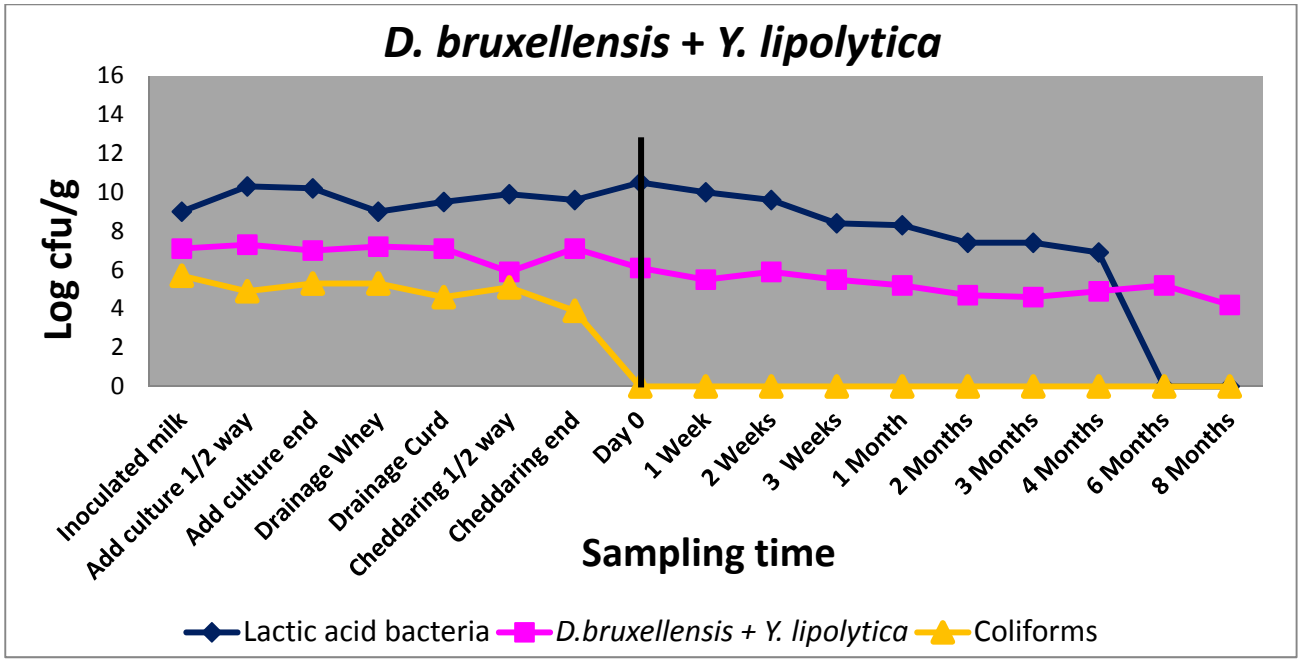
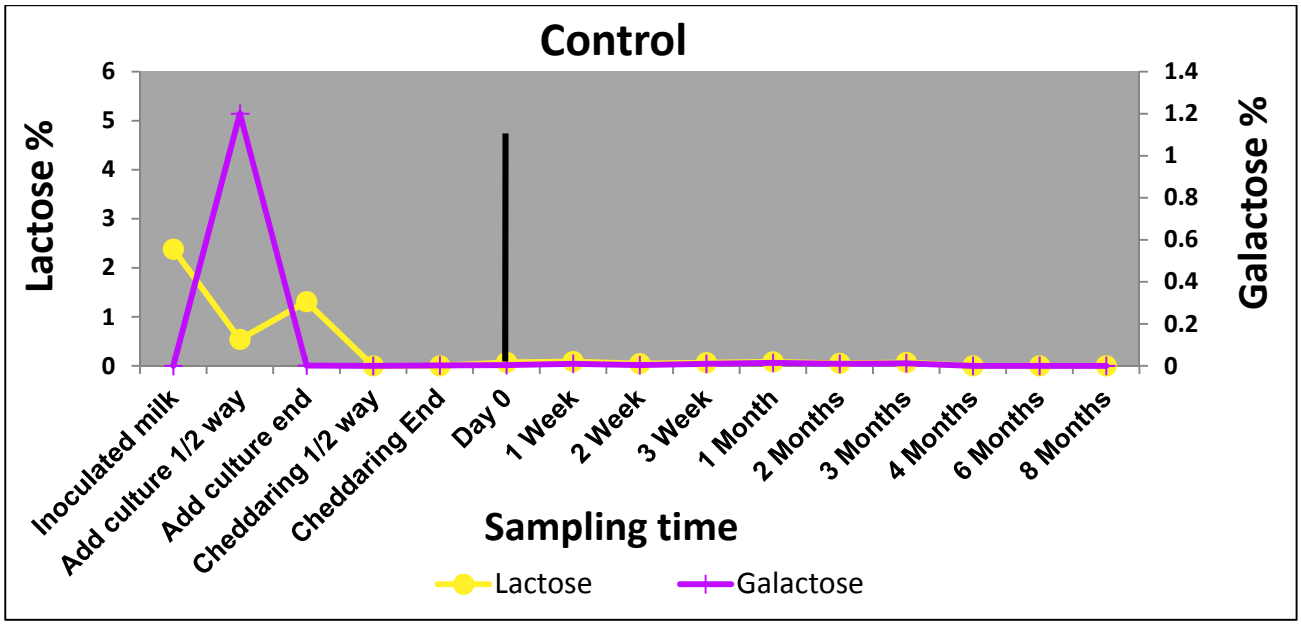
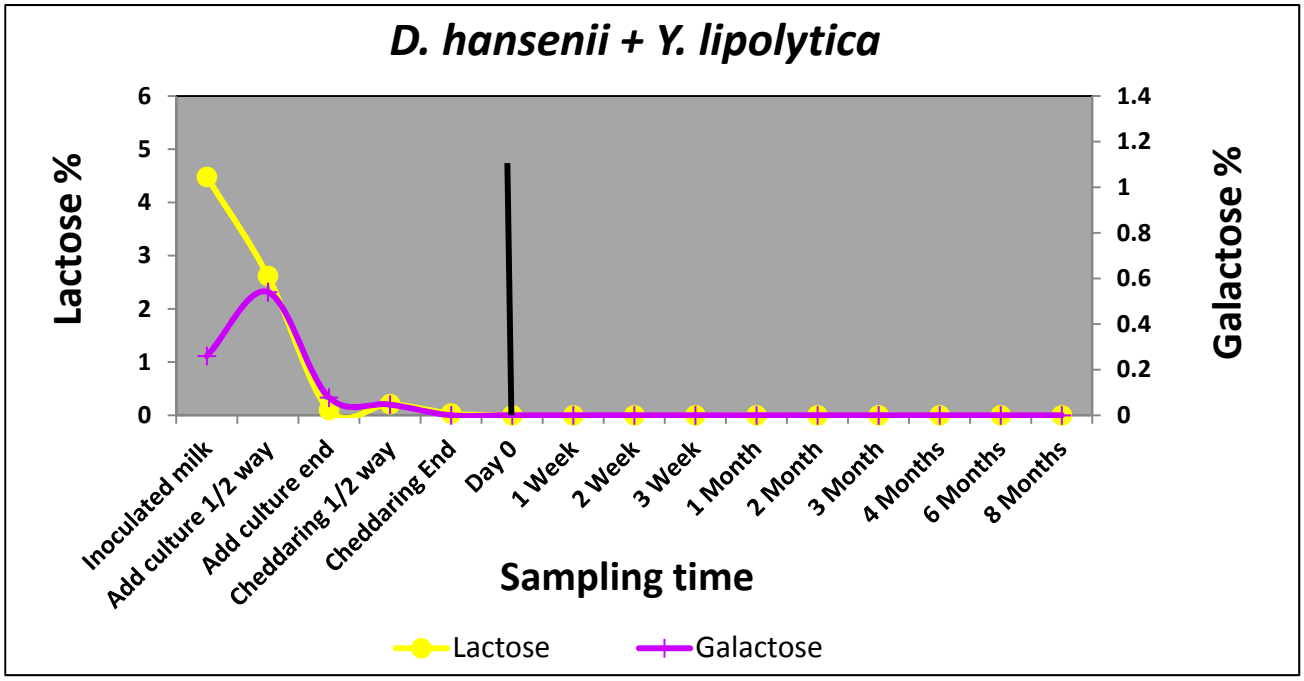


Figure 2: Sugar analysis during mafacturing and maturation of Cheddar cheese

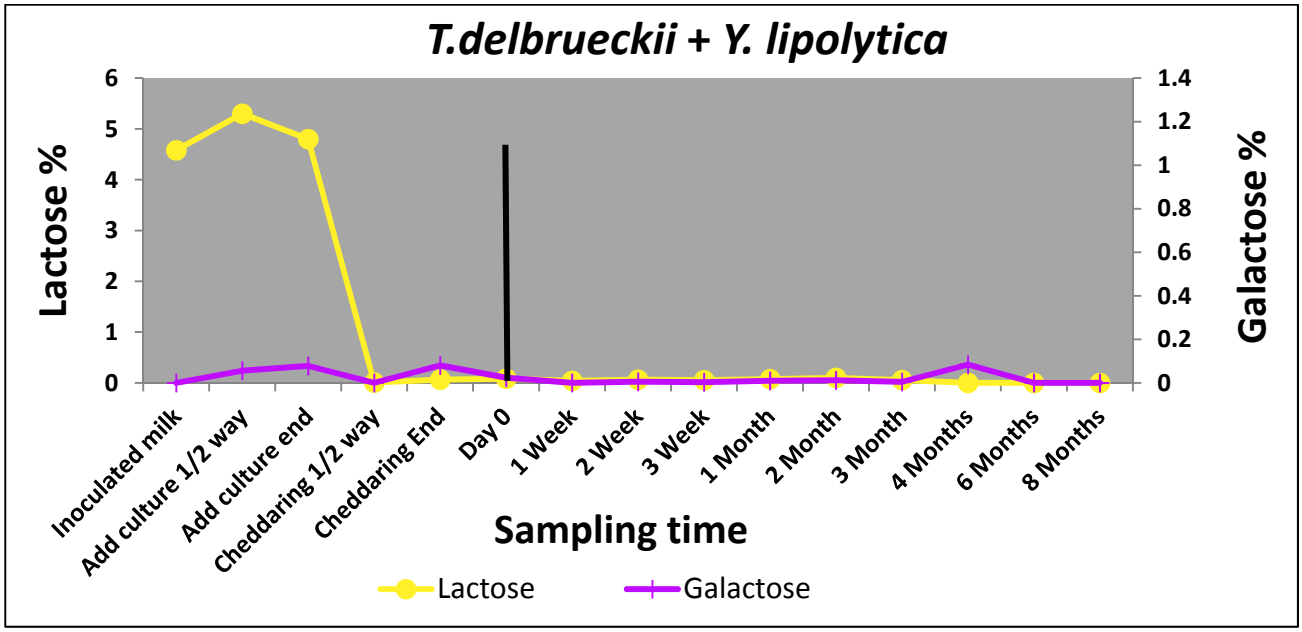
(a) Control (C)



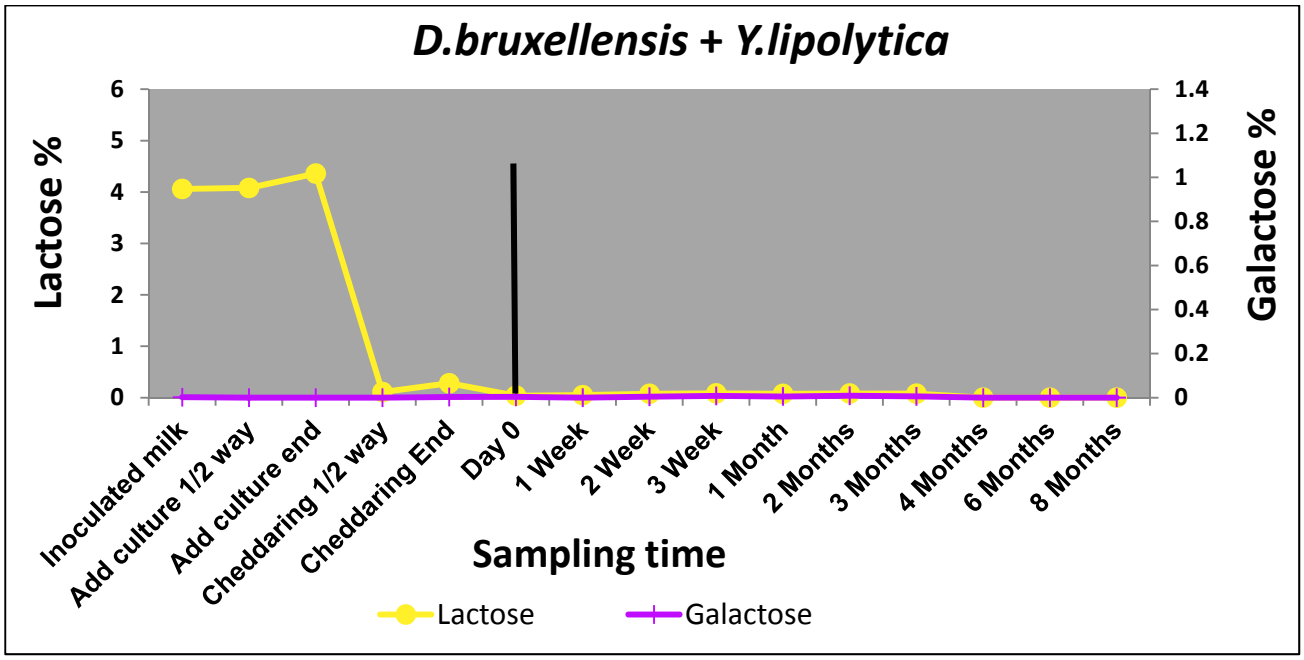
(b) *Debayromyces hansenii + Yarrowia lipolytica* (CC 1)



(c) *Torulaspora delbrueckii + Yarrowia lipolytica* (CC 2)



(d) *Dekkera bruxellensis + Yarrowia lipolytica* (CC 3)



3.5. REFERENCES

Addis, E., Fleet, G .H., Cox, J. M., Kolak, D. & Leung, T. (2001). The growth, properties and interactions of yeasts and bacteria associated with the maturation of Camembert and Blue-veined cheeses. International Journal of Food Microbiology, **69**, 25 – 36.

Álvarez-Martín, P., Flórez, A. B., Hernández-Barranco, A. & Mayo, B. (2008). Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation. Food Control, **19**, 62 - 70.

Amos, Lize-Mari (2007). Manufacture and sensory evaluation of model cheeses with and without yeast adjuncts in *Enzymes from yeast adjuncts in proteolysis during Cheddar cheese ripening*, Magister Scientiae, University of the Free State, Bloemfontein, South Africa, 71 – 83.

Arfi, K., Leclercq-Perlat, M. N., Spinnler, H. E. & Bonnarme (2005). Importance of curd-neutralising yeasts on the aromatic potential of *Brevibacterium linens* during cheese ripening. International Dairy Journal, **15**, 883 – 891.

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

Bockelmann, Wilhelm (2002). Development of defined surface starter cultures for the ripening of smear cheeses. International Dairy Journal, **12**, 123 – 131.

Bouzas, J., Kantt, A. C., Bodyfelt, F. & Torres, J. A. (1991). Simultaneous determination of sugars and organic acids in Cheddar cheese by High- Performance Liquid Chromatography. Journal of Food Science, **56**, 276 - 278.

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

Deiana, P., Fatichenti, F., Farris, G. A., Mocquot, G., Lodi R., Todesco, R. & Cecchi, L. (1984). Metabolization of lactic and acetic acids in Pecorino Romano cheese made with a combined starter of lactic acid bacteria and yeast. Le Lait, **64**, 380 – 394.

de Wit, M., Osthoff, G., Viljoen, B.C. & Hugo, A. (2005). A comparative study of proteolysis in Cheddar cheese and yeast-inoculated Cheddar cheese during ripening. Enzyme and Microbial Technology, **37**, 606 - 616.

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

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.

Irlinger, F. & Mounier, J. (2009). Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology, **20**, 142 - 148.

Jakobsen, M. & Narvhus, J. (1996). Yeasts and their possible beneficial and negative effects on the quality of dairy products. International Dairy Journal, **6**, 755 - 768.

McSweeney, P. L. H. & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In *Cheese: Chemistry, Physics and Microbiology, General Aspects Vol 1,3rd Edition.* Fox, P. F., McSweeney, P. L. H., Cogan, T. & Guinee, T. P. (Eds).pp 361 – 371. London: Chapman and Hall.

Mullin, W. J. & Emmons, D. B. (1997). Determination of organic acids and sugars in cheese, milk and whey by high performance liquid chromatography. Food Research International, **30** (2), 147 – 151.

Narvhus, J. A. & Gadaga, T. H. (2003). The role of interaction between yeasts and lactic acid bacteria in African fermented milks: A Review. International Journal of Food Microbiology, **86**, 51 - 60.

Prentice, G. A. & Brown, J. V. (1983). The microbiology of Cheddar cheese manufacture. Dairy Industries International, **48**, 23 - 26.

Roosista, R. & Fleet, G. H. (1996a). Growth of yeasts in milk and associated changes to milk composition. International Journal of Food Microbiology, **31**, 205 – 219.

Roosista, R. & Fleet, G. H. (1996b). The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. Food Microbiology, **28**, 393 – 404.

Rosalba, L., 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, **16**, 89 – 99.

Seiler, H. & Busse, M. (1990). The yeasts of cheese brines. International Journal of Food Microbiology, **11**, 289 – 304.

Sieuwerts, S., de Bok, F. A. M., Hugenholtz, J. & van Hylckama Vlieg, J. E. T. (2008). Unravelling microbial interactions in food fermentations from classical to genomics approaches. Applied and Environmental Microbiology, **74** (16), 4997 – 5007.

Singh, T. K., Drake, M. A. & Cadwallader, K. R. (2003). Flavour of Cheddar cheese: A Chemical and Sensory Perspective. Comprehensive Reviews in Food Science and Food Safety, **2**, 139 - 162.

Viljoen, B. C. (1996). The ecological diversity of yeasts in dairy products. In *Yeasts in the dairy industry: Positive and Negative Aspects*, Jakobsen, M., Narvhus, J., Viljoen, B.C. (Eds.), Copenhagen, Denmark. International Dairy Federation, Brussels, Belgium, 70 - 77.

Viljoen, B. C. (2001). The interaction between yeasts and bacteria in dairy environments. International Journal of Food Microbiology, **69**, 37 - 44.

Viljoen, B. C. & Greyling, T. (1995). Yeasts associated with Cheddar and Gouda making. Food Microbiology, **28**, 70 – 88

Welthagen, J. J. & Viljoen, B. C. (1996). The presence of yeasts in different cheese types. In *Yeasts in the dairy industry: positive and negative aspects*, Jakobsen, M., Narvhus, J., Viljoen, B.C. (Eds.), Copenhagen, Denmark. International Dairy Federation, Brussels, Belgium, 78 - 87.

Welthagen, J. J. & Viljoen, B. C. (1999). Isolation and identification of yeasts obtained during manufacture and ripening of Cheddar cheese. Food Microbiology, **16**, 63 – 73.

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, Marie-Therese & Puhan Zdenko (1999). Role of selected yeasts in cheese ripening: an evaluation in aseptic cheese curd slurries. International Dairy Journal, **9**,117 - 124.

CHAPTER 4

Free amino acids present in yeast inoculated Cheddar cheese during maturation

Abstract

The proteolytic activity of microorganisms involved during cheese maturation contributes to the proteolysis of the cheese through the extracellular, surface-bound, and intracellular enzymes. In literature, the flavour, aroma and texture development in the cheese is attributed to activity of these proteolytic enzymes. The accumulation of free amino acids (FAAs) in yeast co-inoculated and single inoculated Cheddar cheeses were investigated throughout the maturation stages.

Results obtained indicated an increase in the accumulation of free amino acids in the yeast inoculated cheeses. Concentrations of FAAs > 4 µmol/g were obtained in cheese inoculated with *Torulaspora delbrueckii* + *Yarrowia lipolytica* at 8 months of maturation compared to the control where concentrations < 2 µmol/g were found. The amino acids Leu and GABA were the dominant amino acids in all the cheeses investigated with concentrations > 4 µmol/g. Aromatic, sulfur and acidic amino acids were found in all the cheeses at various concentrations through the maturation stages of the cheeses. Highest concentrations for Lys (12.39 µmol/g), Glu (13.99 µmol/g), Leu (16.76 µmol/g) were obtained at 8 months of maturation. The amino acid Tyr was not detected throughout maturation except at 8 months in samples inoculated with *Torulaspora delbrueckii*, *Debaryomyces hansenii* + *Yarrowia lipolytica, Torulaspora delbrueckii* + *Yarrowia lipolytica.* Very low concentrations of the amino acids His, Arg, Ile were detected in all the cheeses throughout maturation. The overall specific FAA concentrations of the cheeses varied, however increased concentrations were still observed for the yeast inoculated cheeses. Furthermore it was observed that with an increased maturation period the concentration of the FAAs also increased.

4.1. INTRODUCTION

Proteins play an important role in the texture of cheese as they represent the only continuous solid phase of the cheese. Any modification of the nature or the amount of the protein present in the cheese will modify its texture. During cheese ripening, proteolysis modifies the textural and flavour properties of the curd (Adda *et al*., 1982). About 80 % of the milk proteins belong to the caseins, while the remaining 20 % consists of whey proteins. Caseins consist of two fractions: α- and β- caseins. The α-caseins are degradable by most proteolytic enzymes present in the cheese whether indigenous in the milk or exogenous from the coagulant or starter used during the manufacture of the cheese. For both α- and β- caseins the degradation during cheese ripening is characterized by being sequential. The first step is the splitting off of fairly large peptides from the proteins. These large peptides are further degraded into smaller peptides of which many have a bitter taste. The smaller peptides are degraded further into free amino acids which contribute to the taste of the cheese.

The main products of proteolysis which are essential in cheese flavour, aroma and texture are amino acids and peptides. Proteolysis contributes to cheese ripening in at least four ways: (i) direct contribution to flavour e.g. amino acids and peptides or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters (ii) greater release of sapid compounds during mastication (iii) changes in pH via formation of NH_3 (iv) changes in texture from breakdown of the protein network, increase in pH and greater water binding by the newly formed amino acid and carboxyl groups which contribute to the main characteristics of structure of the cheese and cheese aroma. Proteolysis in cheese can be divided into three phases: proteolysis in milk before cheese manufacture, the enzymatically induced coagulation of the milk, and proteolysis during cheese ripening.

In most cheese varieties, the initial hydrolysis of caseins is caused by the coagulant and to a lesser extent by plasmin, which results in the formation of large (water-insoluble) and intermediate-sized (water-soluble) peptides. These peptides are subsequently degraded by the coagulant and enzymes from the starter and non-starter enzymes of the microbial flora of the cheese. The production of small peptides

and free amino acids (FAAs) is caused by the action of microbial proteinases and peptidases (McSweeney and Sousa, 2000; Fox, 1989). Cell wall bound extracellular proteinases of most dairy LAB are responsible for the production of peptides and amino acids, however several LAB strains do not have these proteinases and are mainly dependent on other strains in the starter culture (Smit *et al*., 2002). LAB are nutritionally fastidious microorganisms, thus are able to hydrolyze milk peptides down to FAAs.

During ripening a multitude of chemical and biochemical changes occur in which the principal constituents of the cheese - proteins, lipids and residual lactose are degraded to primary products and later to secondary products (Fox, 1989). The final products of proteolysis are FAAs, the concentration of which has been used as indices of ripening for many years. Amino acids undergo various catabolic reactions, such as deamination, decarboxylation, transamination and side-chain modification, yielding NH_3, amines, aldehydes, acids or alcohols and are mainly responsible for flavour development in cheese. However, small peptides and some FAAs also contribute to the flavour development in some cheeses. High levels of Pro contribute to sweetness in Swiss cheese and to a sweet Swiss cheese-like flavour in experimental Cheddar. Arg has been associated with an unpleasant bittersweet taste.

The most abundant amino acids in Cheddar cheese during ripening are Glu, Leu, Val, Ile, Lys and Phe. Histidine and Alanine are also present at high concentrations. The concentration of total amino acids is not considered to be directly responsible for Cheddar flavour, but the release of certain amino acids, particularly Glu, Met and Leu, coincides with flavour development. Leucine and Met are considered to be the main contributors to cheesy flavour in the water-soluble extract of Cheddar cheese. The levels of peptides and FAAs soluble in 5 % phosphotungstic acid (PTA) in cheese are considered to be reliable indicators of the rate of flavour development, however the composition of the amino acid fraction and the relative proportions of individual amino acids are thought to be most important for the development of a typical characteristic flavour (Fox and Wallace, 1997; Wallace and Fox, 1997).

The levels of CdNR amino groups in the cheeses ripened with starter cultures of *Lactococcus lactis* subsp. *lactis* UC317, its protease – negative derivative FH041, and variants (JL3601, JL3602) of UC 317 modified in proteinase production, location and specificity were investigated by Law *et al*. (1993). Throughout ripening, the CdNR N was 30 - 40 % higher in the cheese made with strain UC317 than in those made with Prt^- (protease negative) cultures [strains FHO41 or FHMl (YE)]; suggesting that starter proteinase contributes to the formation of small peptides and free amino acids. The concentration of CdNR amino groups in cheeses made with strain UC317 and FHO41 harboring the cloned proteinase genes from UC317 (JL3601; CWAP (cell wall-associated proteinase) and JL3062; SP (secreted protease)) were compared and the concentration of CdNR N was significantly higher in cheeses made with *L. lactis* subsp. *lactis* UC317 than in those made with strains JL3601 and JL3602 at 2 and 4 months of ripening. Consistently more CdNR N in the cheeses occurred when the starter had a CWAP (strain JL3601) than when it had an SP (strain JL3602).

Lane and Fox, (1996) investigated the contribution of starter and adjunct lactobacilli on the proteolysis of Cheddar cheese during ripening and they found that the concentration of the FAAs was considerably higher in the STR (biologically acidified cheese with a mixed *Lactobacillus* culture) than in the GDL (glucono – δ – lactone) (chemically acidified cheese with GDL), suggesting that the peptidases of starter origin contribute significantly to the liberation of amino acids during maturation. The low level of FAAs in the GDL control cheese, even after 6 months of ripening confirmed the view that the coagulant (the main proteolytic agent in GDL cheese) contributes little to this level of proteolysis. The addition of defined strains of lactobacilli to the GDL cheese increased the FAA content two-fold, but it was still low compared to the two starter cheeses. The authors suggested that the peptidase system of the added lactobacilli is active in the GDL/NSLAB cheese, but contributes much less to the release of free amino acids than starter peptidase.

Yeasts are usually detected in high numbers in dairy products reflecting a good adaptation to a substrate rich on proteins, lipids, sugars and organic acids. This wide distribution is a consequence of the ability to ferment/assimilate lactose; utilize citric, lactic and succinic acids. In addition yeasts are able to grow in substrates with high

salt concentration, low temperatures, low pH, low a_w. Yeasts have an inherent trait of adapting to complex substrates, form precursor compounds due to the presence of proteolytic and lipolytic activity, excrete growth factors and have synergistic role they play with LAB in dairy products (Bánszky *et al*., 2006; Welthagen and Viljoen, 1999; Viljoen and Greyling, 1995).

Using β-casein-derived peptides, the hydrolytic activity of four yeast species, i.e. *Kluyveromyces lactis* (three strains), *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Pichia anomala* were compared with the activities of six bacterial strains, *Lactobacillus plantarum* (LMG 6907*)*, *Bifidobacterium bifidum* (CIP 567), *Leuconostoc lactis* (CNRZ 1091), *Pediococcus pentosaceus* (NCDO 559), *Brevibacterium linens* (550) including two strains (LRTL 735 & LBLH2) of *Lactobacillus helveticus* (a species known to have the highest peptidase activity among LAB). All four yeast species were able to degrade β-casein peptides at pH 5.7 and at 24°C. The bacterial species exhibited weaker hydrolytic activity as 1.7 mm FAAs was released after 168 h, except for *Lb. helvectus* which exhibited 3.3 –3.5 mm of FAAs compared to the yeasts which exhibited 2.5 – 4.2 mm of FAAs expressed as mm equivalent Met (Klein *et al*., 2002).

Fox, (1989) reported that a high correlation exists between the intensity of Cheddar cheese flavour and the concentration of FAAs. It is recognized that amino acids contribute to the background flavour of cheese and the concentration of amino acids in cheese at any stage of ripening is the net result of the liberation of amino acids from casein and their transformation to catabolic products. The concentration of amino acids generally increases during ripening, with the exception of Arg, which is reported to decrease later in ripening (Harper and Kristoffersen, 1956; McSweeney and Sousa, 2000). The comparison of FAAs profiles in different varieties is limited since cheeses are ripened at different rates even within a single variety. The relative proportions of individual amino acids appear to be similar in many varieties with Leu, Glu and Lys being the principal amino acids in Cheddar (Fox and Wallace, 1997).

Although amino acids are recognised as not the only compounds which generate flavour and aroma compounds in cheese, their contribution to the overall flavour and

aroma development in cheese cannot be denied as thus their concentration and catabolic breakdown products are associated with desirable changes in the cheese curd. The objective of the study was therefore to investigate the development of free amino acids in Cheddar cheese inoculated with yeasts as co-inoculum and as sole inoculum during a maturation period of 8 months.

4.2. MATERIALS AND METHODS

Cheddar cheese samples single inoculated (Table 1) and co-inoculated with yeast cultures (Table 2) prepared by Mai Nguyen, were analysed for the release or accumulation of free amino acids during maturation stages of Day 0, 2, 4 and 8 months. 1.00 g cheese was weighed in a 25 ml tube, Nagle (New York, USA). 5 mL of 0.1 N HCl containing 0.4 umol/ml L-norvalin as internal standard was added. The mixture was then homogenized on Ultra-Turrax for 5 min at 20 000 rpm and the tube was put in an ultrasonic bath for 30 min. Branson (Soest, Netherland). This was followed by centrifugation for 40 min at 3400 rpm (3000 g), 4°C, Beckman J2-MC (GMI INC, Minnesota, USA). 1 mL of the supernatant was taken and to it 1 mL of 4 % trichloroacetic acid (Merck) was added. The suspension was mixed on a Vortex mixer, Gene 2 (New York) and kept in an ice bath for 30 min, followed by centrifugation for 5 min at 13000 rpm (20 000g) Eppendorf 5415D (Hamburg, Germany). The solution was filtered through a 0.2 μm membrane, MFS-13 mm CA filter (California, USA). The amino acids were derivatized by mixing 50 µL of sample solution with 350 µL borate buffer (Agilent Tecnologies). 5 µl from the sample-borate buffer solution was mixed with 5 µl OPA, for 15 s. Because the OPA derivates are not very stable, inject the solution directly into the HPLC-system. The samples were kept at 5°C. The derivatized amino acids were separated by a stepwise linear gradient from 3.3 to 20.7c % B over 12 min and 20.7 to 30 % B over 12 min and from 30 to 100 % B over 4 min. Column clean-up with 100 % B and re-equilibration required 29 min. Flow rate was 1.0 mL/min. and column temperature 42 °C. The detector's parameters were set to detect OPA derivates at excitation 340 nm and emission 455 nm. The analysis were done using a fluorescence detector – 1200 series (Agilent Technologies) and a column - XTerra RP 18, 150 x 4,6 mm id, particle size 3.5 μm (Waters Massachusetts, USA).

4.3. RESULTS AND DISCUSSION

Cheese is a food with very important nutritional aspects, contributing to high efficiency in the metabolism. The substances are quickly and easily absorbed by the body. Cheese is easily digestible and constitutes an important source of vitamins, but also calcium, magnesium, phosphorus, and other trace elements as well as amino acids and proteins. The presence of amino acids, vitamin B (especially B6 and folic acid) and vitamins A and E, minerals (in particular calcium and magnesium), and protein derivates make cheese a unique food. The amounts and patterns of free amino acids in cheeses are dependent on many factors. These comprise the quantities of proteins in the raw materials used for the production, activity of proteolytic enzymes in the dairy procedures, and microorganisms involved in this process. The release of amino acids (proteolysis) during cheese ripening was shown already 100 years ago (Kabelová, 2009).

The concentration of FAAs correlates significantly with flavour development in cheese and is considered a reliable indicator of the rate of flavour production. Amino acids increase with ripening time, and flavour development coincides with the appearance of certain specific amino acids, primarily Glu, Met and Leu. The kinetics of amino acids in cheese during ripening is of particular interest because of their role in cheese flavour development and their significance as quantitative indicators of proteolytic activity during the ripening process (Puchades, 1989).

Investigation of the FAAs in the cheese samples resulted in an increase in the FAA concentration with maturation time (Fig. 1 (a – e) and Fig 2 (a – d)). The control sample (C), resulted in an increase in the concentration of the amino acids with maturation time however the concentration never exceeded 1.0 μmol/g in some of the amino acids detected. The amino acids His, Arg, Asp, Gln, Cit, GABA, Orn were not detected at day 0 of maturation. The concentration of the amino acids Val, Phe, Leu, Asp, Gln, Cit, GABA, Orn were > 1.0 μmol/g at 8 months of maturation compared to the other amino acids which were below 1.0 μmol/g. From the data the concentration of Phe and Leu was enhanced from day 0 compared to the rest of the amino acids and GABA and Leu were the amino acids with the greatest concentrations from 2 to 8

months of maturation. The amino acid Tyr was not detected in the sample during all the maturation stages.

Investigation of the single yeast inoculated cheeses (Fig. 1 (a – e)) yielded increasing accumulation of the amino acids with maturation time. The accumulated amino acids in SC 1 were greater at day 0 than at 2 and 4 months of maturation except for GABA. The amino acids Ser, Ala, Val, Phe, Leu, Lys, Asp, Gln, GABA and Orn resulted in concentrations equal or greater than 1.0 µmol/g at 8 months of maturation. The amino acids Gly, Val, Phe, Leu, Lys, Asp, Gln, GABA, Orn increased from day 0 to 8 months of maturation to reach higher concentrations > 1.0 µmol/g in SC 2. No amino acids were detected at day 0 and 4 months in sample cheeses SC 3 and low concentrations were detected at 2 months except for Leu, GABA, Asp, and Phe which were high at 8 months of maturation. A progressive increase in amino acids Phe, Leu, GABA to reach high concentrations at 8 months in cheese sample SC 4 was observed. In all the single inoculated cheeses (SC1, SC 2, SC 3 & SC 4) the amino acid Tyr was not detected. Very low amino acid concentrations < 1.0 µmol/g were detected for the cheese samples SC 1 (His); SC 2 (His, Arg, Ile); SC 3 (His, Arg, Trp); SC 4 (His, Thr, Arg, Ile) throughout the maturation stages.

Co-inoculation of the yeasts in cheese resulted in increasing accumulation of the amino acids (Fig. 2 (a – d)). No amino acids were detected in the CC 1 and CC 2 samples at day 0. The amino acids Phe, Leu, Asp, GABA, and Orn were > 1.0 µmol/g from day 0 to 8 months in the sample CC 1. The rest of the detected amino acids increased throughout the maturation stages, however did not reach > 1.0 µmol/g amino acid concentration. At 2 and 4 months of maturation the amino acid concentration ranged between 0 and 0.39 µmol/g for the sample CC 2. Within this sample very high amino acid concentrations were observed at 8 months for Glu (13.99 µmol/g), Val (8.39 µmol/g), Leu (16.76 µmol/g), Lys (12.39 µmol/g) and Gln (8.52 µmol/g). A progressive accumulation of the amino acids was observed for CC 3 and high concentrations of Phe, Leu, and GABA were observed. In the co-inoculated cheese samples CC 1 and CC 2, Tyr was 0.15 µmol/g and 1.05 µmol/g respectively at 8 months of maturation, and Trp was not detected in CC 3. Arginine, Ile and Trp were

detected at very low concentrations, < 1.0 µmol/g in CC 1 and His, Arg, Ile for the samples CC 2 and CC 3.

The dominant FAAs in Cheddar cheese are Leu, Glu and Lys (Tavaria *et al*., 2002). The determination of amino acids in cheese resulted in Ala, Gly, Thr, Val, Leu, Ile, Pro, Asp, Phe, Glu, Lys, and Tyr increasing in quantity from the control at day 0 to day 14, and a decrease in quantity in the matured cheese at 30 days. In cheese samples with proteolytic enzymes the amino acids Ala, Thr, Val, Leu, Ile, Glu, and Tyr were predominantly dominant at 30 days of maturation with an increase in quantity from 14 days of maturation while Asp, Gly and Phe decreased in quantity at 30 days of maturation. Ser, Pro and Lys increased in quantity during the 14 days of maturation to reach Ser (1400 µg/g), Pro (472 µg/g) and Lys (667 µg/g) at 30 days of maturation with Met appearing only at the end of maturation – 30 days (Mirela *et al*., 2007).

From the experimental results obtained in all the cheeses the most abundant amino acids were Leu, GABA and to a certain extent varied concentrations of Phe, Asp, Orn, Val, Glu, Gly, Ser, Ala, Lys among SC 1, SC 2, CC 1 and CC 2 yeast cultured cheeses. The accumulation of the amino acids is attributed to the proteolytic activity of the yeasts as well the proteolytic system of the LAB population present in the cheeses. Viljoen, (2001) reported on yeasts contributing to desirable biochemical changes e.g. the production of aromatic and flavour compounds through proteolytic and lipolytic activities as well as providing essential growth metabolites such as amino acids. Klein *et al*. (2002) reported that the yeasts released 25 – 50 % of the total amount of Pro after 168 h, while *Lb. helveticus* released 70 – 75 % of Pro and that the yeasts were more efficient in releasing Asp (70 – 90 %) compared with *Lb. helveticus (*40 – 60 %). Within the yeasts investigated, efficiency varied depending on the species and the amino acid. Regarding aromatic, branched chain and sulfur-containing amino acids, which are very important for subsequent flavour development, a 2-fold variation was observed at 168 h, whereas for His, Glu, Gly, Thr and Asn, a 3-fold and for Gln a 10-fold variation was observed and this was suggested to be inter- and intra-species differences in the peptidase specificity and activity of the yeasts investigated. This supports the experimental results as different amino acid concentrations were obtained for the culture combinations e.g. the sample CC 2

compared to the other samples. As reported in literature that LAB are able to degrade caseins to amino acids and peptides. This was evidenced by the accumulation of amino acids in the control cheese with increasing maturation time, and this was favourable compared to the other samples.

4.3. CONCLUSION

The FAAs were among the first compounds investigated for their contribution to cheese flavour as precursors of flavour and aroma compounds as well as providing the background for cheese flavour (Aston and Dulley, 1982; Yvon and Rijnen, 2001). The presence of yeast proteolytic activity during the ripening of cheese has been acknowledged by many authors (Ferreira and Viljoen, 2003; Viljoen, 2001, Yvon and Rijnen, 2001; Fleet, 1990; Fleet and Mian, 1987; Suzzi *et al*., 2001). Smit *et al*. (2002) reported that although LAB (*Lactococcus*, *Lactobacillus*, *Streptococcus*) are weakly proteolytic, they possess a very comprehensive proteinase/peptidase system and are able to hydrolyze milk peptides down to FAAs, supporting the findings of Law and Haandrikman, (1997) of the proteolytic system of LAB.

From the present study the evolution of FAAs was reported and greater concentrations were observed in yeast inoculated cheeses. This was attributed to the enhanced proteolytic activities of the yeasts. The proteolytic activity of LAB was also investigated with special attention to the accumulation of FFAs in the control cheese through the maturation stages. Results indicated the presence of effective proteolytic enzyme activity of the starter culture as indicated by the presence of the FAAs during the ripening stages. Free amino acid evolution in cheese co-inoculated or single inoculated with yeast cultures did not seem to have a greater deviation in the accumulation of the FAAs. The yeast inoculated cheeses resulted in uniform FAA concentrations except for the samples SC 3, CC 2 and CC 3 which differed compared to the other cheeses. From the investigation the amino acids Glu and Met as well as the other amino acids present were detected at low concentrations compared to Leu and GABA. It is thus deduced that the amino acids were catabolised to secondary flavour and aroma compounds via reactions of decarboxylation, deamination,

desulfuration, transamination and side chain modification to yield amines, acids, thiols, thioesters, aldehydes, alcohols and volatile sulfur compounds as reported in literature.

Based on the varied concentrations of the FAAs throughout the maturation stages, the conclusion is that the accumulation of amino acids during maturation forms the basis of cheese flavour and aroma, and that presence of amino acids is dependent on the proteolytic activities of the microbial community present in the cheese during maturation. The specific amounts of the released FAAs is therefore not an indicator of maturation of the cheese rather the presence of the amino acids and their subsequent secondary catabolism will determine whether they indeed contribute to the flavour and aroma profile of cheese .

Table 1: Yeast cultures single inoculated in cheese for analysis of amino acids

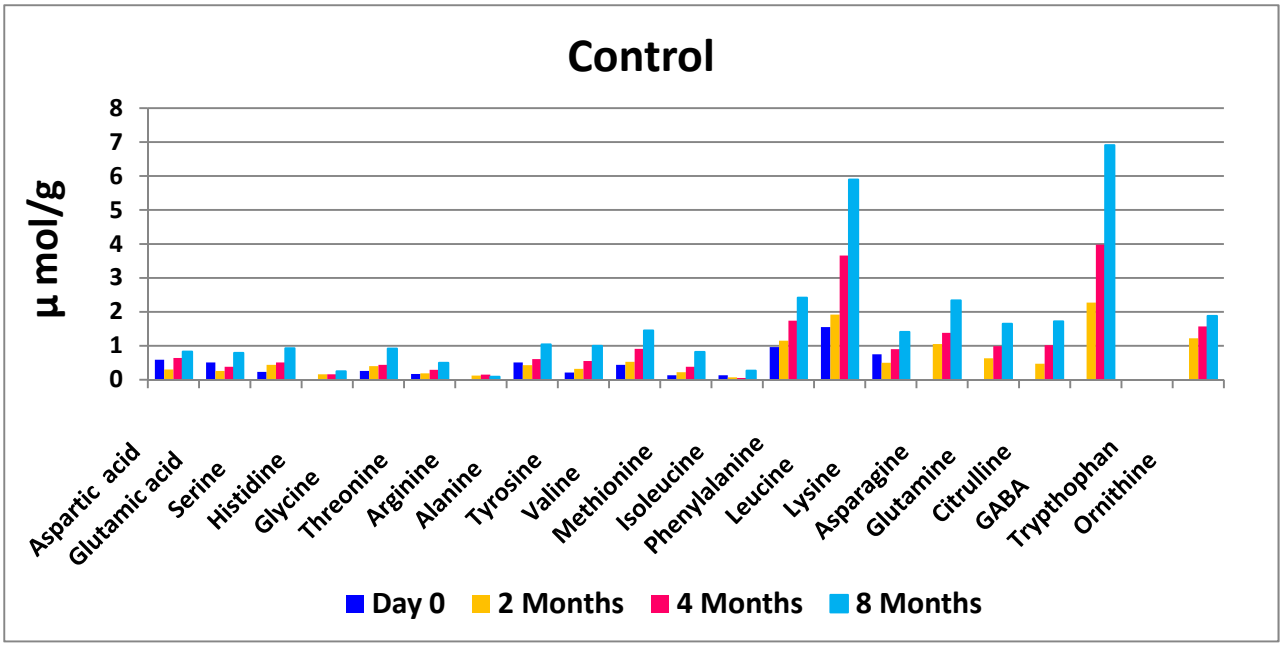
Cheese sample	**Cultures inoculated in cheese**
C	Control (*Lactococcus lactis* subsp. *cremoris* + *Lactococcus lactis* subsp. *lactis*)
SC 1	*Yarrowia lipolytica*
SC 2	*Debaryomyces hansenii*
SC 3	*Torulaspora delbrueckii*
SC 4	*Dekkera bruxellensis*

Table 2: Yeast cultures co-inoculated in cheese for analysis of amino acids

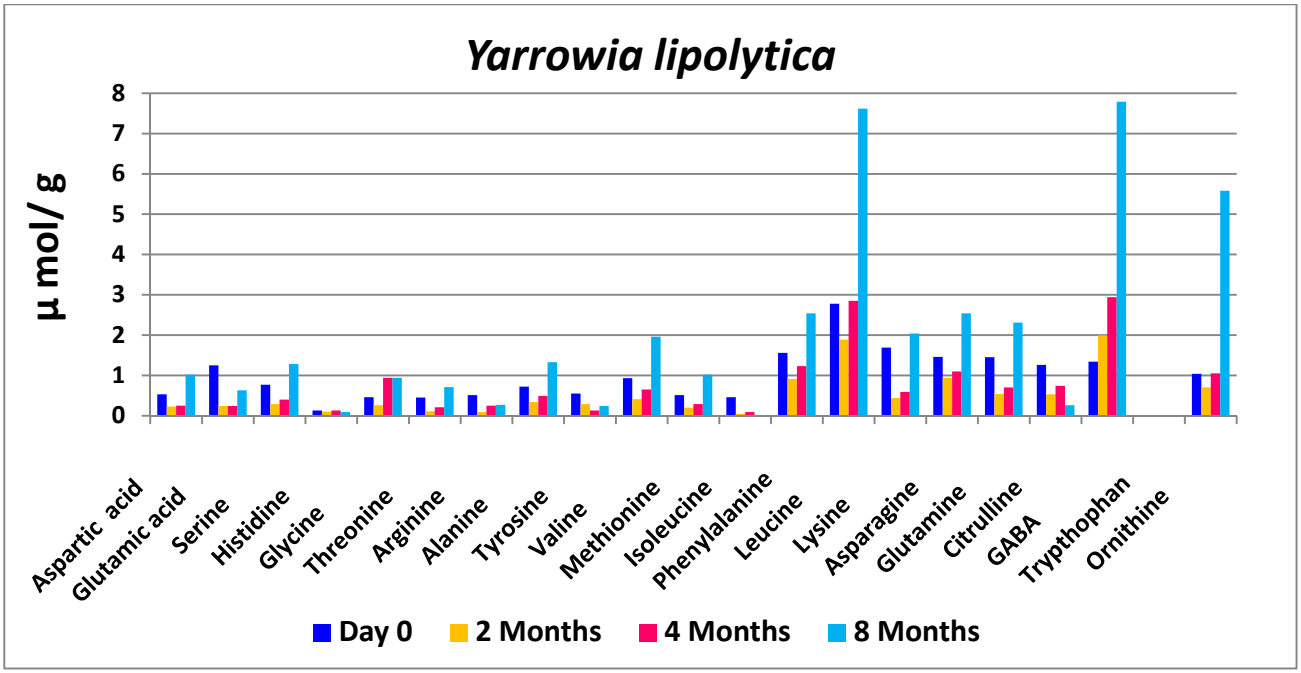
Cheese sample	**Cultures inoculated in cheese**
C	Control (*Lactococcus lactis* subsp. *cremoris* + *Lactococcus lactis* subsp. *lactis*)
CC 1	*Debaryomyces hansenii* + *Yarrowia lipolytica*
CC 2	*Torulaspora delbrueckii* + *Yarrowia lipolytica*
CC 3	*Dekkera bruxellensis* + *Yarrowia lipolytica*

Figure 1: Free amino acids present in single yeast inoculated cheeses

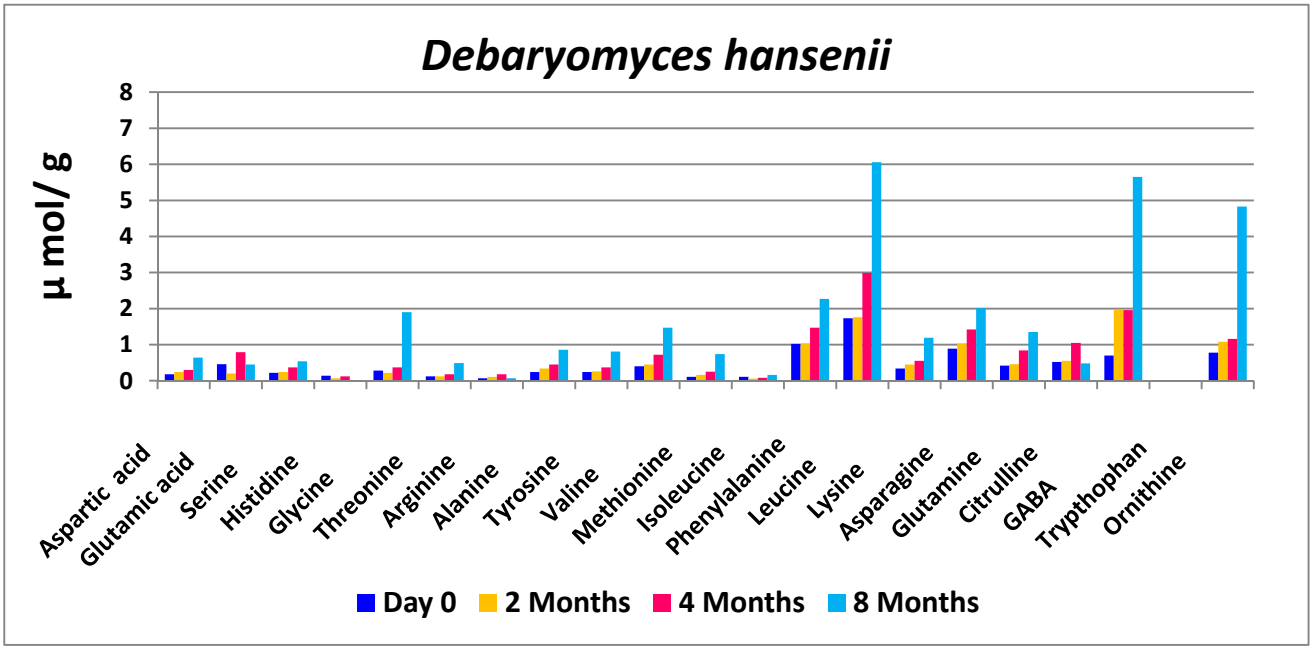
(a) Control (C)



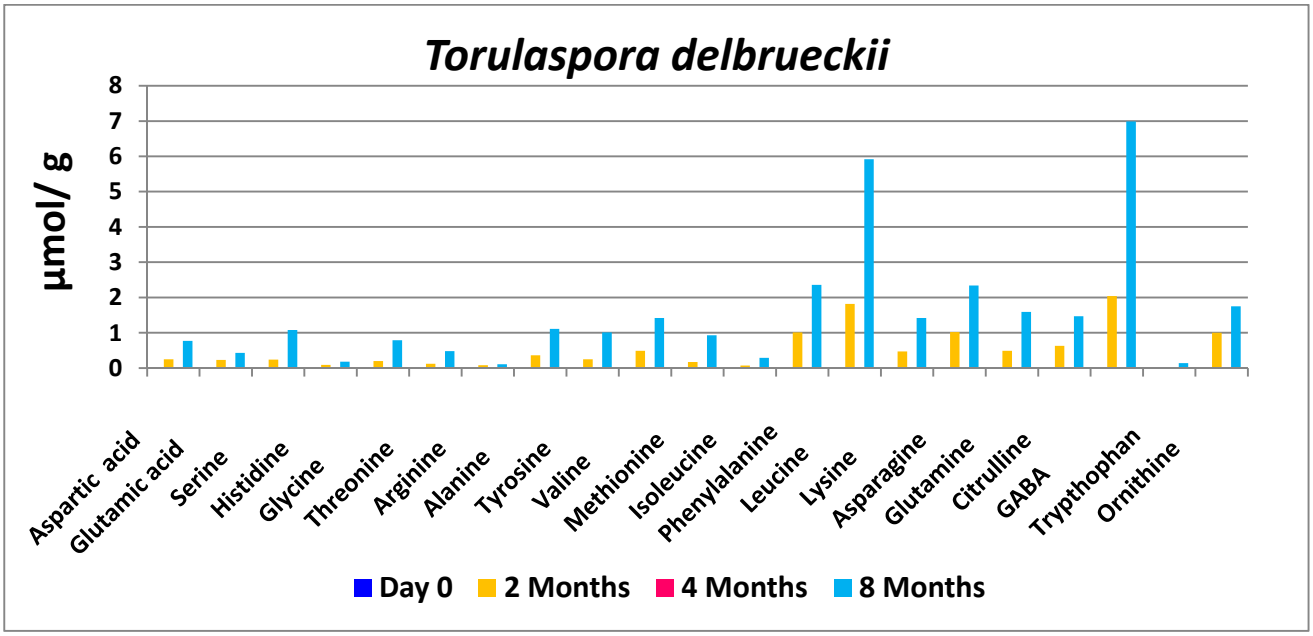
(b) *Yarrowia lipolytica* (SC 1)



(c) *Debaryomyces hansenii* (SC 2)



(d) *Torulaspora delbrueckii* (SC 3)



(e) *Dekkera bruxellensis* (SC 4)

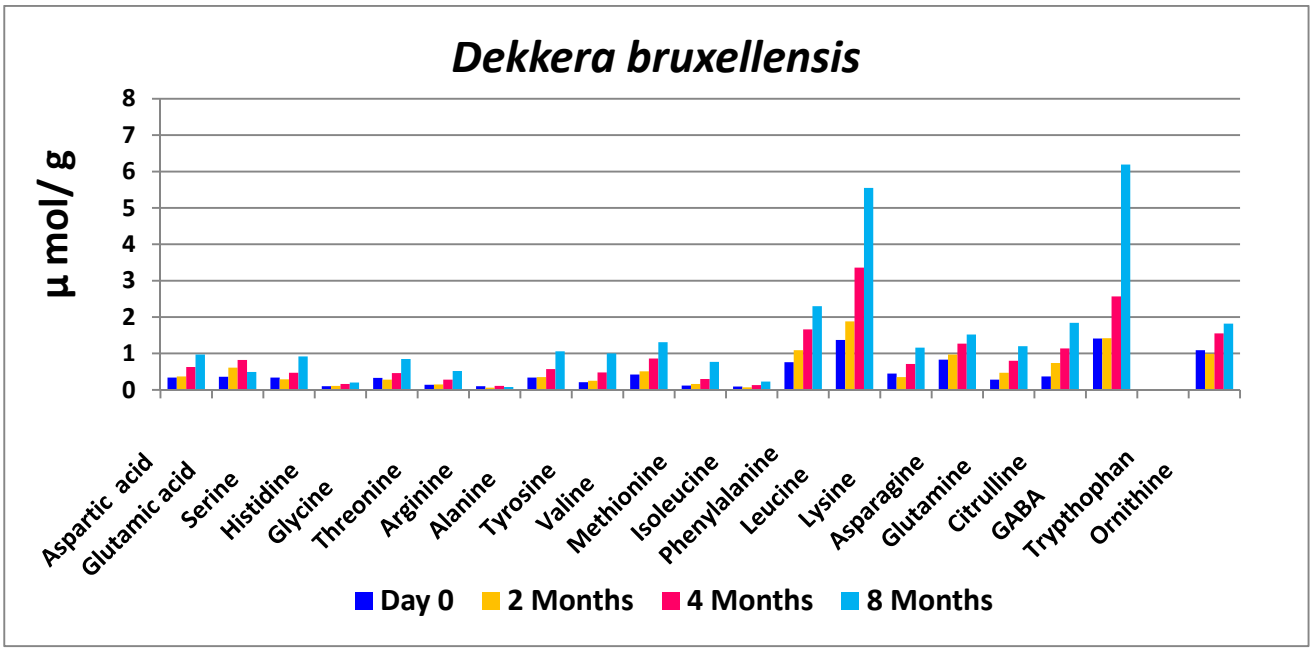
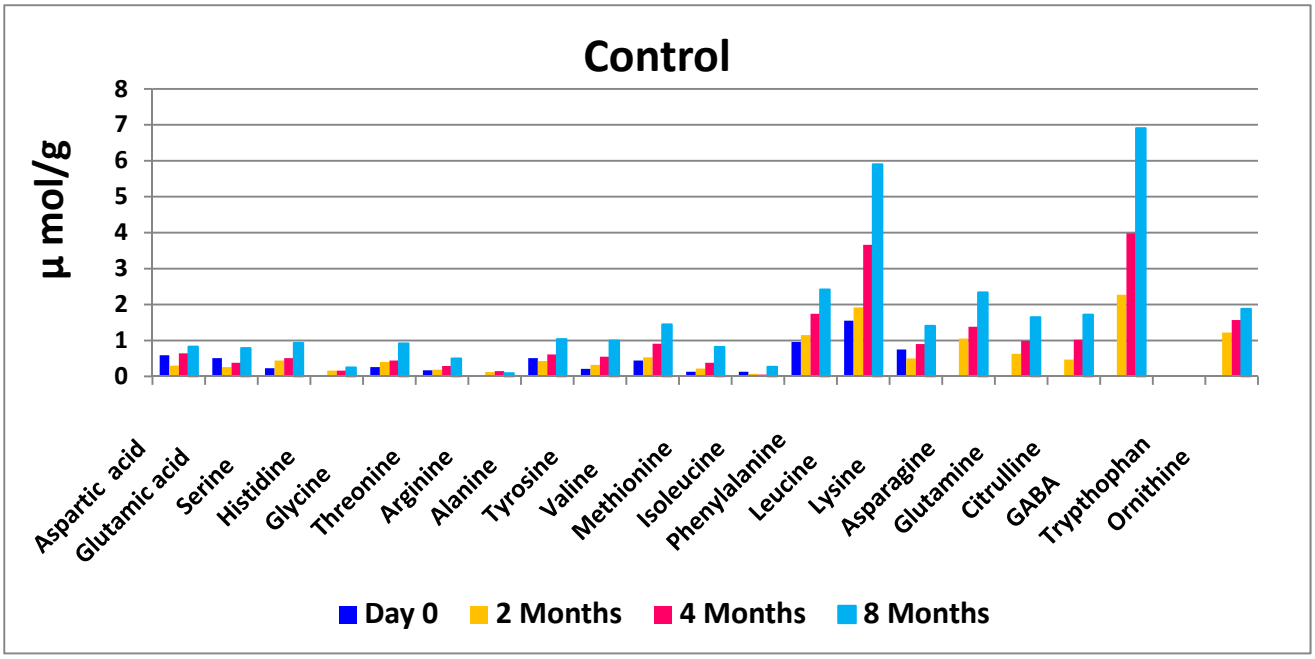
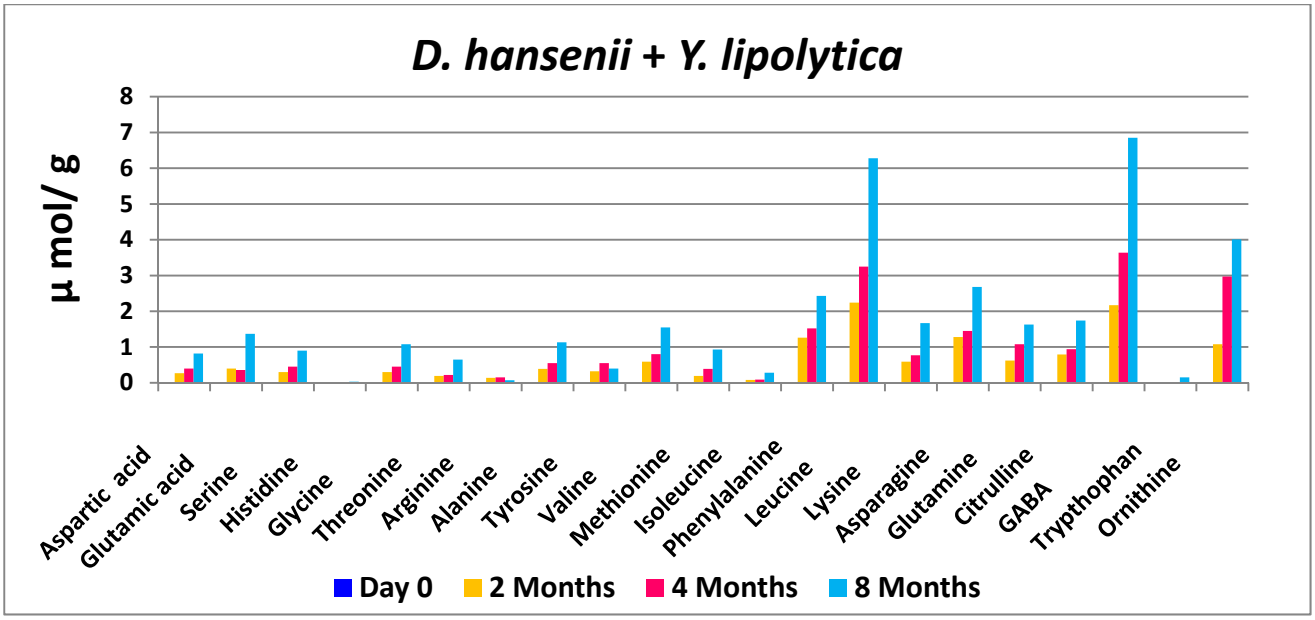


Figure 2: Free amino acids in yeast co-inoculated cheeses

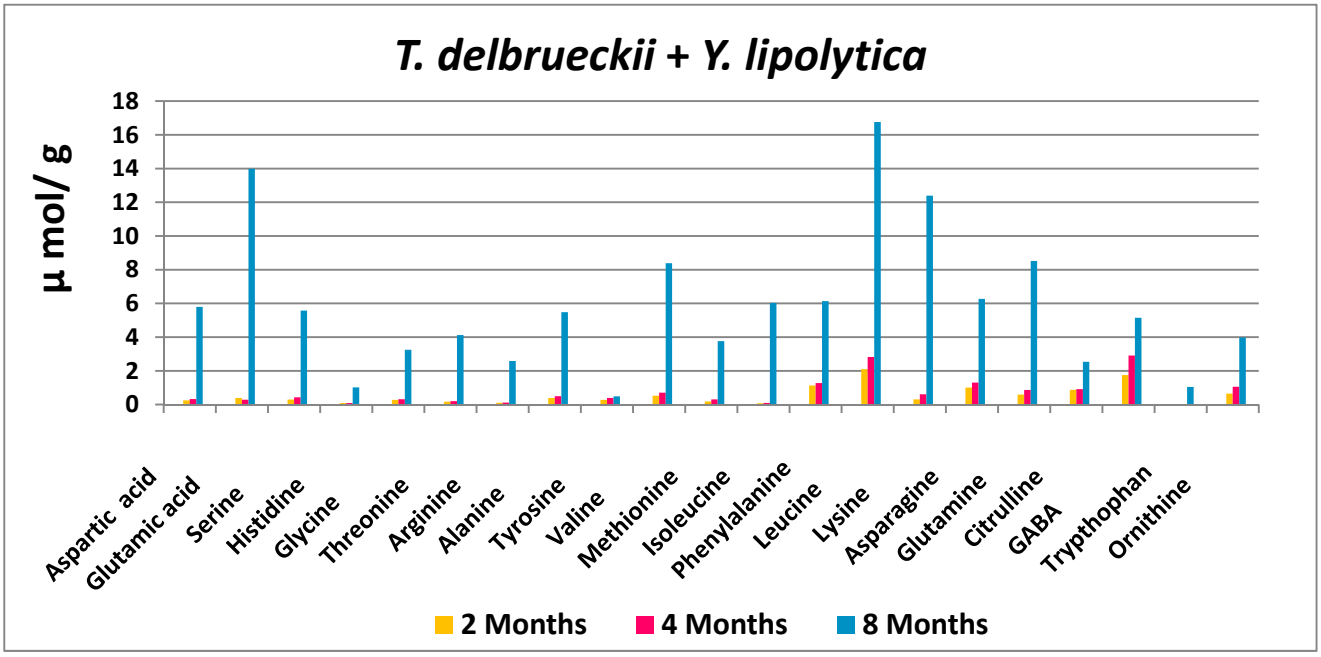
(a) Control (C)



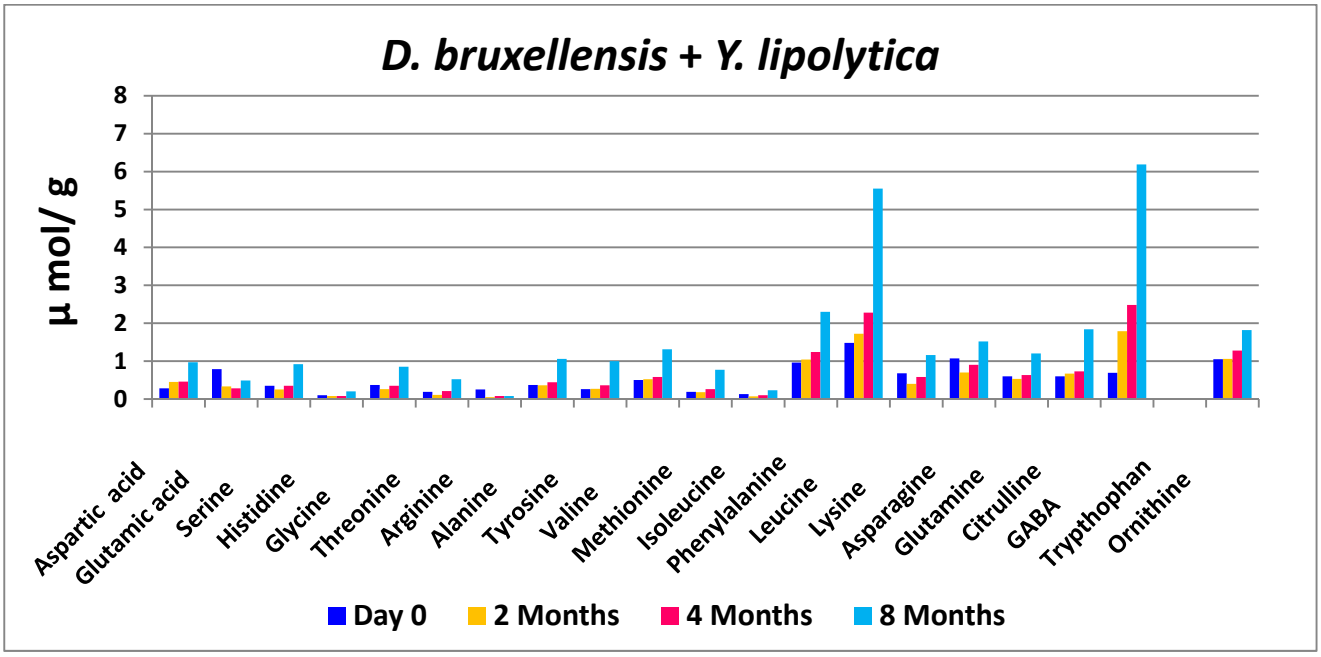
(b) *Debaryomyces hansenii* + *Yarrowia lipolytica* (CC 1)



(c) *Torulaspora delbrueckii + Yarrowia lipolytica* (CC2)



(d) *Dekkera bruxellensis + Yarrowia lipolytica* (CC 3)



4.5. REFERENCES

Adda, J. Gripon, J. C. & Vassal, L. (1982). The chemistry of flavour and texture generation in cheese. Food Chemistry, **9,** 115 – 129.

Aston, J. W. & Dulley, J. R. (1982). Cheddar cheese flavour. The Australian Journal of Dairy Technology, **37**, 59 – 64.

Bánszky, L. K., Eliskases - Lechner, F. & Prillinger, H. (2006). Identification of yeasts associated with milk products using traditional and molecular techniques. Food Microbiology, **23**, 341 – 350.

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.

Fleet , G. H. & Mian, M. A. (1987). The occurrence and growth of yeasts in dairy products. International Journal of Food Microbiology, **4**, 145 – 155.

Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. Journal of Dairy Science, **72** (6), 1379 – 1400.

Fox, P. F. & Wallace, J. M. (1997). Formation of flavour compounds in cheese. In *Advances in Applied Microbiology*, Vol 45, Academic Press, New York, 17 – 75.

Harper, W. J. & Kristoffersen, T. (1956). Biochemical aspects of cheese ripening. Journal of Dairy Science, **39**, 1172 - 1775.

Kabelová, I., Dvořáková, M., Č□žkova, H., Dostálek, P. & Melzoch, K. (2009). Determination of free amino acids n cheeses from the Czech market. Czech Journal of Food Science, **27**, 143 – 150.

Klein, N., Zourari, A. & Lortal, S. (2002). Peptidase activity of four yeast species frequently encountered in dairy products- comparison with several dairy bacteria. International Dairy Journal, **12**, 853 – 861.

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, J., Fitzgerald, G. F., Uniacke - Lowe, T., Daly, C. & Fox, P. F. (1993). The contribution of lactococcal starter proteinases to proteolysis in Cheddar cheese. Journal of Dairy Science, **76** (9), 2455 – 2467.

Law, J. & Haandrikman, A. (1997). Proteolytic enzymes of lactic acid bacteria. International Dairy Journal, **7**, 1 – 11.

McSweeney, P. L. H. & Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: A Review. Lait, **80**, 293 – 324.

Mirela, J., Laslo, C., Ţibulcă, D., Culea, M., Adela, P. & Ştețca, G. (2007). Amino acids determination in cheese. Bulletin of University of Agricultural Sciences and Veterinary Medicine, Cluj - Napoca, **63**, 569 – 574.

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 (4**), 885 – 888.

Smit, G., van Hyclckama Vlieg, J. E. T., Smit, B. A., Ayad, E. H. E. & Engels, W. J. M. (2002). Fermentative formation of flavour compounds by lactic acid bacteria. The Australian Journal of Dairy Technology, **57**, 61 – 67.

Suzzi, G., Lanorte, M. T., Galgano, F., Andrighetta, C., Lombardi, A., Lanciotti, R. & Guerzoni, M. E. (2001). Proteolytic, lipolytic and molecular characterization of *Yarrowia lipolytica* isolated from cheese. International Journal of Food Microbiology, **69**, 69 - 77.

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

Viljoen, B. C. & T. Greyling (1995). Yeasts associated with Cheddar and Gouda making. International Journal of Food Microbiology, **28**, 79 – 88.

Viljoen, B. C. (2001). The interaction between yeasts and bacteria in dairy environments. International Journal of Food Microbiology **69**, 37 - 44.

Wallace, J. M. & Fox, P. F. (1997). Effect of adding free amino acids to Cheddar cheese curd on proteolysis, flavour and texture development. International Dairy Journal, **7**, 157 – 167.

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.

Yvon, M. & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism. International Dairy Journal, **11,** 185 – 201.

Chapter 5

General results and discussion

The fermentative and spoilage activities of yeasts are well known, and over the past decades literature on the occurrence and significance of yeasts in dairy has been investigated. The presence, ability, inherent characteristics, interactions, influence and growth of the yeasts in cheeses have been studied by many authors (Welthagen and Viljoen, 1996; Viljoen and Greyling, 1995; Roostita and Fleet, 1996; Seiler and Busse, 1990; Ferreira and Viljoen, 2003; Arfi *et al*., 2005; Corsetti *et al*., 2001; Sohier *et al*., 2009; Deiana *et al*., 1984; Mounier *et al*., 2008; Irlinger and Mounier, 2009). Cheese curd is a complex and favourable habitat for yeast growth and thus the presence of yeasts in cheeses is thought to contribute to desired biochemical activities in cheeses.

Yeast cultures were inoculated in cheeses for the investigation of the yeasts as adjunct starter cultures in single and co-inoculation, the fermentation of the carbon substrate - lactose and the accumulation of free amino acids.

5.1. SURVIVAL OF YEASTS AS ADJUNCT CULTURES IN MATURED CHEDDAR CHEESE

Starter culture of LAB in the inoculated milk were > 10 log cfu/g in all cheeses except for SC 4 and CC 3 (*D. bruxellensis* - 9.8 log cfu/g and *D. bruxellensis* + *Y. lipolytica* – 9 log cfu/g respectively) at the beginning of manufacture. The overall initial population decreased throughout manufacturing to reach a population between 8.7 – 10.3 log cfu/g at the beginning of maturation except for the CC 3 which increased to 10.5 log cfu/g. The cheeses indicated an overall LAB population between 7.1 – 10.5 log cfu/g for all the cheese samples during maturation except for the sample CC 3 which showed a cessation of survival of the LAB at 6 and 8 months of maturation. LAB are known to attain densities of 10^8 cfu/g within hours of the beginning of manufacture and 10^9 - 10^7 cfu/g at 2 – 12 months of maturation, respectively in Cheddar cheese (Beresford *et al*., 2001; Lues *et al*., 1999) or 10^7 colonies/g in 10 - 60 days (Prentice and Brown, 1983) and that viable starter cell counts are sometimes undetectable (< 10 cfu/g) after about 3 to 4 months although this is dependent on starter strain used (Swearingen *et al*., 2001).

The highest LAB populations were observed for the combinations SC 1 (*Y. lipolytica*) and CC1 (*D. hansenii* + *Y. lipolytica*) between 10.3 – 7.7 log cfu/g and 10.2 – 7.1 log cfu/g respectively at the beginning to the end of maturation. The population of SC 1 was similar to that of the sample C (control). The lowest LAB population were those of the sample SC 2 (*D. hansenii*) at 8.7 – 6.9 log cfu/g from the beginning to the end of maturation respectively.

Yeasts are generally assumed to be post-pasteurisation contaminants and in cheese can rise to levels as high as 10^5 cfu/g without any deterious effect on the quality of the product (Prentice and Brown, 1983). The occurrence of yeasts in retail cheese where counts are often 10^5 - 10^6 cfu/g and in some cheese varieties as high as 10^7 - 10^8 cfu/g, play a significant role in later stages of cheese maturation as they are considered insignificant at the beginning (Welthagen and Viljoen, 1999).

When the yeast cultures were co-inoculated and single inoculated in the cheeses they did not show any inhibition of the starter culture as evidenced by the high LAB counts between 6.9 – 10.5 log cfu/g when the yeast species were present at populations between 4 – 7.5 log cfu/g during the maturation of the cheeses. The ability of *Y. lipolytica* to grow and compete with other naturally occurring microbial groups or yeasts was reported not to show any inhibition of both inoculated and naturally occurring LAB; on the contrary, the strain *Y. lipolytica* RO21 seemed to stimulate the proliferation of the starter culture evaluated in a cheese making trial by Guerzoni *et al*., (1996).

In the beginning of manufacturing the yeast counts in the single inoculated cheeses SC 3 and SC 1 were low at 3.8 log cfu/g compared to those of SC 2 and SC 4. These cheeses reached yeast counts between 5.1 log cfu/g – 6.0 log cfu/g at the end of maturation from a population between 4.1 log cfu/g – 7.5 log cfu/g at the start of cheese maturation. Co-inoculation of the yeast species *D. hansenii, T. delbrueckii* and *D. bruxellensis* with *Y. lipolytica* (samples CC 1, CC 2 and CC 3 respectively) resulted in constant yeast counts (6.1 – 7.3 log cfu/g) at the beginning of maturation. A decrease in the counts to between 4.2 log cfu/g – 6.2 log cfu/g was observed in the cheeses at the end of maturation. In all the cheeses the survival of both the yeast and

LAB population was attributed to the mutualistic interactions between the species as well as the assimilating capabilities of the yeasts and the fermentation activity of the LAB.

5.2. LACTOSE SUGAR FERMENTATION AND ORGANIC ACID ACCUMULATION

Although yeasts are traditionally associated with fermentation, nearly half of the presently known species lack the ability to ferment sugars (Ferreira and Viljoen, 2003) and the yeast cultures used in this study do not ferment lactose with only *D. hansenii* weakly assimilating lactose. According to literature the monosaccharide glucose is readily utilised directly by the starter cultures during lactose fermentation (Ferreira and Viljoen, 2003; Fagen *et al.*, 1952) while the accumulation of galactose is important for the growth of the lactose-negative yeasts as reported by Álvarez- Martín *et al*. (2008).

In the experimental cheeses the utilization of lactose was similar to that of the control, where an initial concentration was observed followed by a decrease at the beginning of cheddaring and the complete depletion of lactose after 4 months. Lactose concentration was initially present between 2 – 6.0 % and galactose concentration remained below 2 % during the inoculation of the milk and none or trace amounts of the sugars were detected after cheddaring. Fagen *et al.* (1952) found that there was a strong positive test for lactose up to the time the cheese was pressed.

Organic acid concentrations of all the cheeses indicated varied concentrations and no organic acids were detected after 2 months for the control as well as for SC 2 at day 0 and SC 3 at 4 months. Concentrations for orotic, uric, formic and propionic acid were detected to be less than 600 µg/g in all the cheese samples during maturation. Bouzas *et al. (*1991) reported on low concentrations (< 2 mg/g cheese) of these acids during a period from day 0 to 60 days. Concentration of pyruvic and succinic acid in the cheeses resulted in concentrations as low as 23.67 µg/g but not exceeding 1123.42 µg/g in the later stages of maturation. Citric and lactic acid concentrations in the samples were between 403.76 – 1401.39 µg/g and 5216.84 – 14173.28 µg/g respectively, during maturation. The organic acids pyruvic and acetic increased throughout maturation. Due to the accumulation of organic acids, cheese curd post manufacture has a pH ranging between 4.5 and 5.3, which does not allow for the

survival of acid sensitive species (Beresford *et al.*, 2001). From the experimental data it was observed that in both single and co-inoculated cheeses the pH decreased at 2 and 4 months of maturation however the pH increased at 8 months of maturation except for samples SC 2 and CC 1 which decreased. The decrease in pH was attributed to the accumulation of the organic acids and the increase at the end of maturation to the deacidification/organic acid assimilating activities of the yeasts which were present at significant counts.

5.3. AMINO ACID ACCUMULATION IN YEAST CULTURED CHEDDAR CHEESE

Yeasts are known to have enhanced proteolytic and lipolytic activity compared to LAB. In the present study the investigation of the amino acids in the yeast inoculated cheeses resulted in the increased accumulation of the amino acids with maturation time. The amino acids Leu and GABA were the dominant amino acids. Amino acids such as Phe, Asp, Orn, Val, Lys, Gln, Cit also increased, however the concentrations never exceeded 2.0 µmol/g. The amino acid Met is reported to be transaminated to form volatile sulfur compounds in cheese which contribute to flavour of the cheese (Seefeldt and Weimer, 2000). In all the samples Met was detected at low concentrations, below 1.0 µmol/g. The non- proteinogenic amino acid GABA was one of the dominant FAAs, and this amino acid is formed during metabolism and may serve as an early indicator of quality defects as a consequence of undesired fermentation and may inhibit further proteolysis (Krause *et al*., 1997).

From the study no inhibition of proteolysis, was observed as the amino acids accumulated throughout maturation, although casein breakdown studies are advised to be conducted in order to confirm the conclusion. However the concentrations of the amino acids were low and this may also be attributed to the activities of the yeasts further catabolising the amino acids to flavour and aroma compounds. Thus a study on the overall flavour compounds in yeast inoculated cheeses is proposed. Of the yeast cultures investigated the combination of *T. delbrueckii* + *Y. lipolytica* showed greater amounts of the amino acids at 8 months of maturation compared to the other yeast cultures inoculated in the cheeses. The samples SC 2, CC 1 showed progressive increases in the amino acids.

The dominant amino acids in Cheddar cheese are reported to be Leu, Glu and Lys (Tavaria *et al*., 2002) and that the release of certain amino acids, particularly Glu, Met and Leu, coincides with flavour development (Fox and Wallace, 1997). The amino acid Leu was detected > 1.0 µmol/g in all the cheese samples except for samples SC 1 and CC 2 where it was > 2.0 µmol/g during maturation. Glutamic acid was detected < 1.0 µmol/g during maturation and Lys was > 1.0 µmol/g in all the cheese samples at 8 months of maturation. In samples SC 1 and CC 2, Lys concentrations of 2.04 µmol/g and 12.39 µmol/g were detected at 8 months of maturation respectively. In all the samples the amino acids Arg, His, Trp, Thr were detected at low concentrations < 1.0 µmol/g, except in CC 2 were the amino acids were (Arg – 2.59 µmol/g, His – 1.02 µmol/g, Trp – 1.05 µmol/g, Thr – 4.12 µmol/g at 8 months of maturation).

5.4. SENSORY ANALYSIS

Sensory analysis of the cheeses was done on 4 months matured cheese by Mai Nguyen at the Department of Microbial, Biochemical and Food Biotechnology. These data were included in the manuscript to further support our conclusions based on the relevance of yeasts in Cheddar cheese. The sample cheeses SC 4 and CC 3 had scores higher than 5 which were regarded as desired Cheddar cheese character based on the comparison with the control cheese. The samples SC 1 and CC 2 scored scores below 5 and these were not the desired Cheddar cheese scores as determined by the evaluating method. The high scores of the cheese samples SC 4 and CC 3 were attributed to the constant LAB and yeast population observed during maturation as well as the accumulation of the amino acid Leu which was reported to coincide with flavour development. The amino acid GABA is reported to be an indicator of quality defects, and thus its high accumulation in the cheese CC 2 was attributed to the low sensory scores of the cheese. The presence of yeasts in the cheeses was also attributed to the undesired and desired scores, as the yeasts are known to catabolise carbon substrates within the cheese curd to form flavour and aroma compounds as well as that excessive proteolysis due to the yeasts' proteolytic activity that can lead to the formation of bitter taste in cheese and spoilage. From the results obtained with the sensory analysis, a more descriptive method of sensory

analysis is proposed in conjunction with studies on the flavour and aroma compounds of yeast inoculated cheeses.

5.5. REFERENCES

Álvarez-Martín, P., Flórez, A. B., Hernández-Barranco, A. & Mayo, B. (2008). Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation. Food Control, **19**, 62 - 70.

Arfi, K., Leclercq-Perlat, M. N., Spinnler, H. E. & Bonnarme (2005). Importance of curd-neutralising yeasts on the aromatic potential of Brevibacterium linens during cheese ripening. International Dairy Journal, **15**, 883 – 891.

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

Bouzas, J., Kantt, A. C., Bodyfelt, F. & Torres, J. A. (1991). Simultaneous determination of sugars and organic acids in Cheddar cheese by High- Performance Liquid Chromatography, Journal of Food Science, **56**, 276 - 278.

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

Deiana, P., Fatichenti, F., Farris, G. A., Mocquot, G., Lodi R., Todesco, R. & Cecchi, L. (1984). Metabolization of lactic and acetic acids in Pecorino Romano cheese made with a combined starter of lactic acid bacteria and yeast. Le Lait, **64**, 380 – 394.

Fagen, H. P., Stine, J. B. & Hussong, R. V. (1952). Identification of reducing sugars in Cheddar cheese during the early stages of ripening. Journal of Dairy Science, **35**, 779 - 782.

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

Fox, P. F. & Wallace, J. M. (1997). Formation of flavour compounds in cheese. In *Advances in Applied Microbiology*, Vol 45, Academic Press, New York, pp 17 – 75.

Guerzoni, M. E., Gobbetti, M., Lanciotti, R., Vannini, L. & Lòpez, Chaves C. (1996). *Yarrowia lipolytica* as a potential ripening agent in milk products. In Yeasts in the dairy industry: positive and negative aspects, 70 - 77.

Irlinger, F. & Mounier, J. (2009). Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology, **20**, 142 - 148.

Krause, I., Bockhardt, A. & Klostermeyer, H. (1997). Characterization of cheese ripening by free amino acids and biogenic amines and influence of bactofugation and heat-treatment of milk. Lait, **77**, 101 – 108.

Lues, J. F. R., Smit, E. J. & van Zyl, J. M. (1999). Patterns of non-starter microflora during ripening of selected South African Cheddar cheeses manufactured by the open vat procedure. Food Microbiology, **16**, 645 - 651.

Mounier, J., Monnet, C., Vallaeys, T., Arditi, R., Sarthou, A. S.., Hélias, A. & Irlinger, F. (2008). Microbial interactions within a cheese microbial community. Applied and Environmental Microbiology, **74** (1), 172 – 181.

Prentice, G. A. & Brown, J. V. (1983). The microbiology of Cheddar cheese manufacture. Dairy Industries International, **48**, 23 - 26.

Roosista, R. & Fleet, G. H. (1996). The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. Food Microbiology, **28**, 393 – 404.

Seefeldt, K. E. & Weimer, B. C. (2000). Diversity of sulphur compound production in lactic acid bacteria. Journal of Dairy Science, **83**, 2740 – 2746.

Seiler, H. & Busse, M. (1990). The yeasts of cheese brines. International Journal of Food Microbiology, **11**, 289 – 304.

Sohier, D., Le Dizes, A. S., Thuault, D., Neuveglise, C., Coton, E. & Casaregola, S. (2009). Important genetic diversity revealed by inter-LTR PCR fingerprinting of *Kluveromyces marxianus* and *Debaryomyces hansenii* strains from French traditional cheeses. Dairy Science Technology, **89**, 569 – 581.

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.

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

Viljoen, B. C. & Greyling, T. (1995). Yeasts associated with Cheddar and Gouda making. International Journal of Food Microbiology, **28**, 79 – 88.

Welthagen, J. J. & Viljoen, B. C. (1996). The presence of yeasts in different cheese types. In *Yeasts in the dairy industry: positive and negative aspects*, Jakobsen, M., Narvhus, J., Viljoen, B.C. (Eds.), Copenhagen, Denmark. International Dairy Federation, Brussels, Belgium, 78 - 87.

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.

SUMMARY

A literature review on the role and presence of microorganisms in cheese was reviewed. The biochemical pathways involved in the cheese manufacture from the milk to the resultant cheese curd at the end of manufacturing were also reviewed. The activity of microorganisms used in cheese manufacture and microorganisms isolated from cheeses were also discussed and their role in the cheese curd formation. Yeasts, one of the microorganisms isolated from cheeses, were reviewed in detail. The use of yeasts as adjunct starter cultures in matured Cheddar cheese was investigated. The yeast cultures (*Yarrowia lipolytica*, *Debaryomyces hansenii*, *Torulaspora delbrueckii* and *Dekkera bruxellensis*) were inoculated in milk for the manufacture of matured Cheddar cheese as adjunct starter cultures. The yeast cultures supported the role of the starter culture (LAB) – lactose fermentation, and assimilated the organic acids present and inhibited spoilage microorganisms. The growth of the yeast and LAB was mutualistic in all the cheeses and no defects were detected in the cheeses as observed by the favourable sensory scores for the yeast inoculated cheeses.

Co-inoculation of yeasts in the making of matured Cheddar cheese resulted in enhanced survival of the yeasts and the LAB population in the cheeses. The yeasts exhibited increased growth, without suppressing the viability and activity of LAB. Organic acids which are associated with aroma and flavour compound production were increased in the cheeses. The cheese inoculated with *Dekkera bruxellensis* + *Yarrowia lipolytica* had superior Cheddar cheese scores which were greater than 5 as well as the cheese single inoculated with *Dekkera bruxellensis*. The pH measurements of the cheeses indicated the deacidification abilities of the yeasts and the spoilage inhibiting acidity in the cheeses. Free amino acid accumulation in cheeses was also investigated. It was observed that the yeast inoculated cheeses had greater free amino acid accumulation compared to the control cheese. The dominant amino acids were Leu and GABA amino acids in all the cheese samples and low concentrations were observed for the other amino acids.

(Keywords: Yeasts, LAB, Organic acids, Amino acids, Cheddar cheese, Growth, Inoculated, Co-inoculated)