THE SURVIVAL OF YEASTS AND PROBIOTICS AS ADJUNCT STARTERS IN CHEESE

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by

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"I will live more true to life,

I will remember all I can,

I will grow."

Deela Khan

Dedicated to my husband,

Floris Kotzé, and my parents, Oubaas and Miemie Ferreira

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LIST OF ABBREVIATIONS

a_w:	water activity
cfu:	colony forming units
CRM:	Callichia *et al*'s resuspension medium
d:	day(s)
Fig(s):	Figure(s)
g:	gram
h:	hour
HPLC	high-performance liquid chromatography
hrs:	hours
l:	litres
min:	minute(s)
ml:	millilitre
M-MRS:	Maltose-MRS
pH	hydrogen ion concentration
rpm:	revolutions per minute
sign.	Significance
UHT	ultra-high temperature
μm:	micrometer
UV	ultraviolet

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CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

Since biblical times, mankind has included cultured food products in his diet. Starter cultures today, comprise of those bacteria that predominated in the historical fermented foods (Gilliland, 1985). During the past decades, several research efforts have been attempted to improve starter culture technology for Cheddar cheese making (Cogan *et al*., 1991). A world-wide market increase in the demand for, and consequent production of cheese and cheese products evolved since the early 1900's. Methods of cheese production have progressed towards an increasing mechanisation, consolidation of smaller factories and increases in factory sizes, shorter cheese "make times" and increased milk throughput. This has increased the demand on starter cultures to remain active for the production of larger amounts of cheese under intensified manufacturing conditions. Numerous studies have been conducted to accelerate cheese ripening, urged by the economic advantages of a rapid development of stronger cheese flavour in a shorter time (Law, 1984). Almost all of these attempts fall into one of four categories, which include the use of elevated temperatures, the addition of enzymes for speeding up flavour producing reactions, the use of modified starter cultures and liquid slurry methods.

Lactic acid bacteria usually cause fermentation of milk products and are therefore considered to be of major importance during the making of cheese (Cousin, 1982). These bacteria furthermore thrive in immature acid cheese and a number of chemical reactions responsible for the development of flavour and aroma are brought about. Apart from lactose breakdown, maturing mainly involves the breakdown of protein and fat (Fox and Cameron, 1982).

The growth of the lactic acid bacteria starters is, however, inhibited by unfavourable environmental conditions such as low pH-values (McSweeney *et al*., 1994) and high salt concentrations (McSweeney *et al*., 1994; Laubscher and Viljoen, 1999). Yeasts,

in contrast, can grow under conditions unfavourable to many bacteria and therefore play a significant role in the ripening of some cheese varieties as well as the spoilage of dairy products (Fleet and Mian, 1987; Seiler and Busse, 1990; Fleet, 1992). The proteolytic and lipolytic activity of certain yeast species, possible microbial interactions, their inhibitory activity against spoilage organisms (Loretan *et al*., 1998), their ability to produce vitamins (Purko *et al*., 1951) and amino acids (Wyder *et al*., 1999) and the utilisation of lactic acid with a resulting higher pH (Lenoir, 1984; Wyder *et al*., 1999) all make yeasts potentially viable organisms for use as starter cultures in the dairy industry. Although yeasts were always considered as contaminants in dairy products, causing spoilage during the fermentation process (Fleet, 1990; 1992), the above mentioned characteristics of the yeasts, indicate that they could be included as part of the starter cultures for cheese manufacture with possible accelerated ripening of the cheese. This could be of considerable economic importance for the dairy industry.

Consequently it has been considered relevant to study the potential of applying different yeast species as agents for accelerated ripening of matured Cheddar cheese. The interaction between the yeasts and the lactic acid bacteria as well as the physical and chemical properties of the cheese were determined.

Probiotics are a mono- or mixed culture of live microorganisms which, applied to human or animal (e.g. as dried cells or as a fermented product), beneficially effects the host by improving the properties of the indigenous microflora (Huis in't Veld and Havenaar, 1991). This concept originated at the end of the last century with the work of the Russian bacteriologist, Eli Metchnikoff (1907), who postulated that lactic acid bacteria could help restore the balance of intestinal flora and therefore improve health and prolong life. In the century that has elapsed since Metchnikoff's work, scientists and consumers throughout the world have accepted the probiotic concept.

Yoghurt and fermented milks have received considerable attention as carriers of live probiotic cultures. Numerous studies, however, have shown poor survival of probiotic organisms in the market preparations of yoghurt (Gilliland and Speck, 1977b; Klaver *et al*., 1993; Rybka and Kailasapathy, 1995; Dave and Shah, 1997; Lourens and Viljoen, 2002). With the growing consumer awareness of the

importance of balanced and varied diets for the maintenance of good health, a demand for new food products with proven health claims was established.

Cheese may offer certain advantages as a carrier system for live probiotics to the gastro-intestinal tract of humans. The development of a probiotic cheese will expand the probiotic product range and could lead to a major economic advantage.

2. STARTER CULTURES

Microbial starter cultures are pre-requisites for the production of safe products of uniform quality in the modern cheese industry (Petterson, 1988). Selected starter cultures were obtainable from special laboratories as early as the end of the 19th century (Petterson, 1988). During the past decades several research efforts have been attempted to improve starter culture technology for Cheddar cheese making (Cogan *et al.*, 1991). Several microorganisms (bacteria, yeasts, moulds or combinations of these) are employed in the fermentation process of milk during the manufacturing of cheese, mainly to produce lactic acid from lactose (Robinson, 1981). The most important factor in controlling cheese quality is the acid production in the vat (Heap, 1998). An acceptable dairy starter strain must be capable of rapid acid production, contribute to the desired flavour of cheese (especially when ageing Cheddar), and be relatively insensitive to bacteriophage. These requirements have led to necessary refinements in the process of selecting new strains (Huggins, 1984).

Important criteria to recognise in optimising starter culture performance in a cheese plant include the characterisation of the starter strains, the number of strains used at any time, factory design and process equipment layout, the preparation of bulk starter, plant hygiene, plant effluent and whey stream handling, as well as the availability of starter expertise and technical back-up to solve starter-related problems (Heap, 1998). Starter bacteria are inhibited by antibiotics (Robinson, 1981), bacteriophage (Cogan and Accolas, 1990), detergents and disinfectant residues (Robinson, 1981).

Defined strain starter systems were originally developed in New Zealand (Limsowtin *et al.*, 1977; Lawrence *et al.*, 1978). Based on the reduced number of strains, it was possible to compose starters by using a small number of strains which could survive

the onslaught of phages (Limsowtin *et al.*, 1977; Lawrence *et al.*, 1978). The presence of strains with known characteristics caused the starters to be more stable and the manufacturing times and cheese quality between batches more predictable (Reddy *et al.*, 1972).

The most important characteristic of an individual strain is insensitivity to a wide variety of phages. Other important characteristics include acid-producing activity in milk and their effect on flavour development. Strains selected for starter cultures have to be as unrelated as possible with regard to all known tests in terms of phage sensitivity (Heap, 1998).

2.1 Selection of strains

The following criteria are used for strain selection (Heap, 1998):

2.1.1 Phages

Strains resistant to phages are selected by exposing the candidate strains to a mixture of phages. This method of selection reduces the probability of selected strains being attacked by a phage in the cheese plant (Heap, 1998).

2.1.2 Genetically based selection criteria

DNA analysis is applied to differentiate starter strains into groups. Relationships are suggested by similarity of plasmid content and are undesirable in combined strains in a starter culture. Strains can also be differentiated on the basis of similarity of the total DNA content. The presence/absence of specific genes furthermore indicates on differences between strains (Heap, 1998).

2.1.3 Temperature sensitivity

In the New Zealand starter system a key feature is the use of temperature-sensitive strains. A triplet starter consists of two temperature-sensitive and one temperature-resistant strain. A temperature-sensitive strain stops growing at 38°C, but continues

to produce acid and because of the inability to reach high cell densities in the cheese curd, the number of potential host cells is limited. Therefore the use of temperature-sensitive strains helps to reduce the phage level in the plant (Heap, 1998).

2.1.4 Proteolytic activity

A proteinase negative starter included as one of the three strains of a triplet starter decreases the proteinase concentration of a starter (Heap, 1998). Consequently acid production is not compromised whereas bitterness in susceptible cheese types is controlled (Heap, 1998).

2.1.5 Maintenance

Desirable strains have to be maintained in a manner that minimises the genetic variability as a result of plasmid loss. This can be achieved by limiting the number of sub-cultures in the media and by freezing the culture and storing it at -75°C or lower (Heap, 1998).

Lactic acid bacteria transform food into new products and exert an antagonistic effect on harmful microorganisms. Much development took place in the production of fermented milk by using natural or selected lactic acid bacteria (Dellaglio, 1988).

Reasons why lactic acid bacteria are used in the production of fermented milks include the following (Dellaglio, 1988):

(i) their ability to produce lactic and acetic acid, aroma compounds and polysaccharides,

(ii) their antibiotic, antitumor and antileukemic activities contribute to human health, and,

(iii) the production of a large variety of products are facilitated by their growth over a wide temperature range in many types of milk products.

Fresh cheese curd contains between 10^8 and 10^9 viable starter bacteria/g. These die off after 4-6 months at a rate broadly dependent on the species (Law *et al*., 1973).

2.2 Taxonomy

The streptococci can be placed in one of four groups, namely the pyogenic, viridans, enterococci and lactic streptococci (Sandine, 1985). All but the lactic group (*Lactococcus*) contain potentially pathogenic organisms and it is from this group that isolates are selected and used as starter cultures in the preparation of fermented dairy products (Dellaglio, 1988).

2.2.1 Mesophilic lactic cultures

These cultures grow in the temperature range of 10 to 40°C with optimum growth around 30°C. They contain group N lactococci and/or leuconostocs. These mesophilic microorganisms form part of starter cultures which are used in the production of many cheese varieties, comprising important characteristics like acid producing activity, gas formation, and the production of enzymes for cheese ripening. These enzymes include active proteases and peptidases (Petterson, 1988).

Genus *Lactococcus*

Streptococcus lactis is divided into three subspecies: *S. lactis* subsp. *lactis*; *S. lactis* subsp. *diacetylactis* and *S. lactis* subsp. *cremoris* (Jarvis and Jarvis, 1981). Taxonomic changes have resulted in a reclassification of these streptococci as lactococci (Schleifer *et al.*, 1985).

Lactococcus lactis subsp. *cremoris* is less competitive in nature than the enterococci, *L. lactis* or *L. diacetylactis* and is therefore present in smaller numbers. Strains of *L. cremoris* are used in starter cultures for the production of fermented dairy products and are the most desirable organisms from a flavour point of view (Sandine, 1985).

Genus *Leuconostoc*

Garvie (1960) divided the genus *Leuconostoc* into four species: *L. cremoris, L. lactis, L. dextranicum* and *L. mesenteroides.* Two additional species, *L. paramesenteroides* and *L. oenos* have since been added (Garvie 1967a and 1967b). DNA-DNA

hybridisation studies, however, suggested the maintaining of four species, *L. oenos*, *L. lactis*, *L. paramesenteroides* and *L. mesenteroides*, indicating that *L. cremoris* and *L. dextranicum* are subspecies of *L. mesenteroides* (Garvie, 1976).

According to Garvie (1976) and Galesloot and Hassing (1961), *L. cremoris* is regarded as the main *Leuconostoc* species. Research on Dutch cultures in 1936 and 1948, showed that *Leuconostoc* species (generally *L. cremoris*) are the sole flavour producers, while Dutch starters examined in 1954 contained both *L. cremoris* and *Lactococcus diacetylactis* as flavour producers (Galesloot and Hassing, 1961).

Types of starters

Lactococcus lactis and *Lactococcus cremoris* produce lactic acid from lactose and are referred to as acid producers. The flavour producers are *Lactococcus diacetylactis* and *Leuconostoc* species, capable of fermenting citric acid and producing important metabolites such as CO_2, acetaldehyde and diacetyl (Petterson, 1988).

Mesophilic starters are often divided into various types depending on their species and strain composition (Huggins, 1984).

Table 1. Composition of starter cultures (Huggins, 1984).

Type	Species	Characteristics and method of use
Single-strain starter	*Lactococcus lactis* *L. cremoris* *L. diacetylactis*	Single or paired
Multiple-strain starter	*L. lactis* *L. cremoris* *L. diacetylactis* *Leuconostoc* species	Defined mixtures of two or more strains (may be used in pairs)
Mixed-strain starter	*L. lactis* *L. cremoris* *L. diacetylactis* *Leuconostoc* species	Unknown proportions of different strains which can vary upon subculture (may be used in pairs)

Enhanced control of cheese flavour and phage infections is accomplished when characterised single strain starters are used (Limsowtin *et al.*, 1977; Thunnel *et al.*, 1981; Heap, 1998). These strains have been successfully used singularly, in paired rotations and in multiple-strain blends. The use of a multiple starter is advantageous to the cheesemaker in that the same starter can be used continuously. A lower inoculum is needed in the cheese vats compared with paired starters, whilst the starter activity will be consistent despite the changes in milk composition that probably occurred over the season. Further advantages include flavour uniformity, known composition of the starter for easy identification of affected strains and an increase in the rate of acid production when strains are grown in association (Limsowtin *et al.*, 1977; Thunell *et al.*, 1981; Heap, 1998).

Mesophilic starters are further divided into various types, depending on the identity of the flavour-producing bacteria. The "flavour starters" are composed in the following way (Daly, 1983):

(i) B- or L-type: *Lactococcus lactis, Lactococcus cremoris* + *Leuconostoc* species as flavour producer.

(ii) D-type: *Lactococcus lactis, Lactococcus cremoris* + *Lactococcus diacetylactis* as flavour producer.

(iii) BD- or LD-type: *Lactococcus lactis, Lactococcus cremoris* + *Leuconostoc* species and *Lactococcus diacetylactis* as flavour producers.

(iv) N- or O-type: *Lactococcus lactis, Lactococcus cremoris* + no flavour producers.

2.2.2 Thermophilic starter cultures

Because of the domination of cheese production by Cheddar cheese and its varieties, dairy culture research has focussed on mesophilic lactococci. Increased demands by consumers for Italian cheeses, particularly Mozzarella, over the past decade resulted in increased production, demanding thermophilic starter cultures (Oberg and Broadbent, 1993). A unique characteristic of thermophilic lactic cultures, is the microbial interaction between lactobacilli and streptococci that are cultured together. These cultures which include the group I lactobacilli and *Streptococcus salivarius* subsp. *thermophilus* are not true thermophiles, but they are nevertheless described as

the thermophilic lactic bacteria exhibiting similar problems as those encountered by mesophilic starter systems (Oberg and Broadbent, 1993).

The key property that differentiates thermophilic starter cultures from mesophilic starters is their ability to grow at higher temperatures. Their optimal growth temperature ranges between 40 and 50°C in comparison to the 22 to 30°C of mesophilic cultures. *Lactobacillus, Streptococcus* and *Pediococcus* are typical lactic acid bacteria comprising of species capable to grow at 45°C (Oberg and Broadbent, 1993). Thermophilic lactobacilli used for dairy fermentations include *L. delbrueckii* ssp. *bulgaricus, L. delbrueckii* spp. *lactis, L. helveticus and L. acidophilus.* Among the streptococci, *S. salivarius* ssp. *thermophilus* is the only dairy starter that remains in this genus. Pediococci able to grow at temperatures above 45°C, include *P. acidilactic* with an optimal growth temperature of 40-52°C (Oberg and Broadbent, 1993).

Thermophilic starter cultures are generally composed of two different genera and therefore symbiosis and competition occur simultaneously during growth. This yields a particular ratio of streptococci to lactobacilli in a thermophilic starter (Oberg and Broadbent, 1993). The rate of acid production as well as proteolytic activity in such a mixed culture is greater than the sum of the acid production and proteolytic activity of the two single cultures (Oberg and Broadbent, 1993)

Although lactobacilli and streptococci are both classified within the thermophilic group, they exhibit different optimal growth temperatures. Lactobacilli are also more acid-tolerant than streptococci. Because of this difference in pH tolerance, streptococci initially predominate in a starter culture, but are succeeded by lactobacilli as the pH declines below five. As a result, an approximate 1:1 ratio of streptococci to lactobacilli is obtained (Oberg and Broadbent, 1993).

Thermophilic cultures can also be used as adjunct strains. In association with mesophilic cultures in a cheese vat they contribute particular functions, i.e. accelerated cheese ripening and improved cheese texture and body. Thermophiles are sometimes used to ensure acid production in cheeses in case of attack of mesophilic cultures by bacteriophages (Oberg and Broadbent, 1993).

3. THE BIOCHEMICAL AND MICROBIOLOGICAL ASPECTS OF MATURED CHEDDAR CHEESE RIPENING

During the cheese ripening process changes occur that alter the cheese from a bland, hard, rubbery mass to a smooth-bodied and full flavoured product. Cheese ripening is a complex system that involves numerous chemical, physical and bacteriological changes, occurring in a temperature and humidity controlled cold store (Harper and Kristoffersen, 1956).

Lactic acid bacteria thrive in immature acid cheese and a number of chemical reactions responsible for the development of flavour and aroma are brought about. Apart from lactose breakdown, maturing mainly involves breakdown of protein and fat (Fox and Cameron, 1982). According to Harper and Kristoffersen (1956), the changes taking place during cheese ripening may be divided into two general stages. The first stage includes changes that occur in carbohydrate, fat and protein, which result in the accumulation of lactic acid, fatty acids and free amino acids (the primary compounds). The second stage involves the formation of compounds brought about by the action of enzymes primarily from microorganisms on the primary compounds. According to Law and Sharpe (1977), flavour compounds are formed by non-enzymatic or at least non-microbial reactions. Intracellular starter enzymes play no direct role in flavour formation, but produce breakdown products from which Cheddar flavour compounds may be formed (Law *et al*., 1976). Starter enzymes contribute to the development of typical flavour in Cheddar cheese by producing the correct chemical conditions (e.g. acidity and redox potential) in cheese. In addition, starters produce low molecular weight precursors of aroma compounds (e.g. free amino acids) from cheese proteins (Law *et al*., 1976).

3.1 Protein

Protein breakdown represents the primary phenomenon of the ripening process of cheese, since it results in a suppleness of the cheese-body and in changes in its appearance (Desmazeaud and Gripon, 1977). Proteins are progressively broken down into smaller molecules such as peptones and ultimately into amino acids. Such soluble and low molecular weight nitrogen compounds contribute to cheese flavour

and bring about physical changes in the cheese, causing it to become softer and creamier (Fox and Cameron, 1982). Two major types of proteolytic agents exist in cheeses: (Desmazeaud and Gripon, 1977)

(i) coagulating enzymes: rennet or rennet substitutes, and

(ii) proteolytic enzymes of the starter cultures: mesophilic and thermophilic lactic acid bacteria and fungal starters.

3.1.1 Role of rennet

Rennet is the first proteolytic agent involved in the overall mechanism of casein breakdown in cheeses. During the coagulation process, rennet attacks the Phe_{105} – Met_{196} bond of casein by solubilising a fraction of this protein (Mercier *et al*., 1973; Visser *et al*., 1976). The enzyme is very bond specific, but contributes to gross proteolysis in cheese, producing a large proportion of the larger peptides (Desmazeaud and Gripon, 1977; Visser, 1977). Rennet does not induce the release of free amino acids in the curd (Desmazeaud and Gripon, 1977). Following this process, a portion of the rennet remains in the curd and is therefore involved in the breakdown of protein during cheese ripening (Harper and Kristoffersen, 1956; Holmes *et al*., 1977; Stadhouders *et al*., 1977).

3.1.2 Role of proteolytic enzymes

Proteolytic enzymes from lactic acid bacteria are mainly aminopeptidases and endopeptidases (Exterkate, 1975; Castberg and Morris, 1976). These starter proteinases function in cheese by degrading those peptides released from casein by rennin, releasing small peptides and amino acids (Davies and Law, 1984). This is in contrast to the conclusion of Green and Foster (1974) who showed that rennet and proteases from lactic acid bacteria exhibit similar patterns of protein breakdown in cheeses.

Amino acids contribute to the background flavour of cheese (Harper and Kristoffersen, 1956). The catabolism of amino acids by surface flora yields a variety of flavour compounds and precursors, which are important to the development of the subtle, distinct flavour and aroma of cheese. Ammonia results from amino acid

deamination by contaminating yeast microflora and contributes to the aroma profile of cheese (Greenburg and Ledford, 1979; Hemme *et al*., 1982). Volatile sulphur compounds make up another important group in soft cheese flavour and aroma. H_2S, dimethylsulphide and methanediol are produced from methionine by a combination of oxidative deamination and demethiolation (Tsugo and Matsuoko, 1962; Sharpe *et al*., 1977). The actual pathways and subsequent accumulation products involved in each cheese are varied and depend on the combination of enzyme systems that are present and active in the cheese system (Harper and Kristoffersen, 1956).

3.2 Fat

Fat hydrolysis occurs to some extent in all cheese varieties (Harper and Kristoffersen, 1956). Fat, like protein, is broken down by enzymatic hydrolysis and is converted into glycerol and free fatty acids. Milk fat is relatively rich in low molecular weight fatty acids such as butyric, caproic and capric, which are released on hydrolysis and, being volatile and strong smelling, contribute to cheese flavour (Fox and Cameron, 1982). Lactic acid bacteria produce only small amounts of lipase, if any, and these microorganisms have no influence on fat hydrolysis in cheese ripening (Stadhouders and Mulder, 1958). Starter bacteria have a very limited ability to hydrolyse the triglycerides of fat during cheese ripening. Free fatty acids and mono- and diglycerides are formed by the hydrolysation of milk fat or, during ripening, cheese fat, by the natural lipases of milk and/or the lipases of Gram-negative rods. Starter bacteria are able to produce free fatty acids from these mono- and diglycerides (Stadhouders and Veringa, 1973). Fatty acids may be further broken down by enzymes, yielding low molecular weight molecules such as ketones, secondary alcohols, lactones and esters (Fox and Cameron, 1982; Choisy *et al*., 1986; Schrödter, 1990; Ha and Lindsay, 1991; Molimard and Spinnler, 1995). Both primary and secondary degradation products of fat are powerful flavour and aroma components of cheese (Harper and Kristoffersen, 1956).

3.3 Lactose

The conversion of lactose to lactic acid during and after the manufacturing of cheese is essential in all cheese varieties (Harper and Kristoffersen, 1956). Lactose

breakdown leads mainly to lactic acid production by the hexose diphosphate pathway, but heterofermentative bacteria convert lactose into lactic acid, acetic acid, ethanol and CO_2 (Devoyod *et al*., 1968; Devoyod and Muller, 1969; Davies and Law, 1984; Gripon, 1987). Lactic acid has a stabilising effect on cheese by virtue of its antibacterial properties (Babel, 1977) and its effect in lowering the cheese redox potential and pH (Davies and Law, 1984). The CO_2 contributes to the formation of openings in the curd (Gripon, 1987). Cheese with clean, uniform quality is more likely to result from manufacturing conditions which allow the starter streptococci to utilise all the lactose (Davies and Law, 1984).

3.4 The effect of psychrotrophic bacteria on cheese ripening

Psychrotrophic bacteria are defined as being able to grow at or below 7°C, irrespective of their optimal growth temperatures (Eddy, 1960; Thomas and Druce, 1969). Keeping milk at refrigeration temperatures has created new problems due to the selection of psychrotrophic bacteria (Thomas, 1974; Cousins *et al*., 1977). Many psychrotrophs are active producers of heat resistant extracellular enzymes such as lipases and proteinases (Cousins *et al*., 1977). Overcast (1968) showed that actively lipolytic psychrotroph enzymes in milk pasteurised after 2 days at 4°C, could produce rancidity in milk, increasing the free fatty acids 3-5 fold. The proteinases of gram negative bacteria are very heat resistant (Cousins *et al*., 1977). These proteinases lead to the development of gelation, bitterness or clearing of UHT milks on long term storage (Law *et al*. 1977). Casein in cheese milks may be broken down into more soluble constituents such as polypeptides. Some of these could be lost into the whey instead of forming part of the curd, therefore reducing the yield of cheese (Cousins *et al*., 1977). According to Law *et al*. (1979a), proteolytic psychrotrophs are unlikely to have an adverse effect either on the manufacture of Cheddar cheese, or on its maturation. Lipolytic psychrotrophs, however, are a confirmed source of off-flavour, and is likely to be of far greater commercial significance than the proteinase activity (Law *et al*., 1979a).

4. THE ACCELERATED RIPENING OF CHEESE

Cheddar cheese requires a long period of time to develop the full flavour and texture of ripened cheese. Therefore, the operating and capitol costs for aging cheese represent a significant portion of the production cost for Cheddar cheese manufacture (Raksakulthai *et al*., 2002). While maturation time for cheese is inevitable, some aspects of cheese ripening have led to much experimentation into means of shortening it by speeding up the reactions which generate flavour and modify texture. Attempts to accelerate cheese ripening fall into one of four categories, namely the use of elevated temperatures, enzyme addition to the cheese, the use of modified starters and slurry methods (Law, 1984).

4.1 Elevated temperatures

Most reports on the use of temperature control to accelerate cheese ripening involved hard and semi-hard cheeses with relatively simple microfloras (Law, 1984). In low fat Gouda cheese, the flavour balance was easily impaired. At 16°C, proteolysis was accelerated more than lipolysis, causing a bitter defect (International Dairy Federation, 1983). Forced ripening of Edam cheese at high temperatures caused microbiologically induced texture and flavour spoilage (International Dairy Federation, 1984). Some success has however being met with the application of elevated temperatures for the accelerated ripening of Cheddar cheese. Law *et al*. (1979b) noted that ripening temperature had the greatest influence on the flavour intensity of cheeses after 6 and 9 months storage. In a study by Klantschitsch *et al*. (2000), it was found that accelerated ripening of Raclette cheese was achieved with the use of increased ripening temperature. The higher ripening temperature led to a higher concentration of free short chain fatty acids, accelerated proteolysis, higher aroma intensity, decrease in water content and higher firmness. In a different study, acceleration of ripening of Zamorano cheese through raised temperatures was proposed by Ferazza *et al*. (2003). While this approach may give satisfactory results with the highest quality cheese, with a pH, moisture, salt concentration and bacteriological quality close to ideal, a high storage temperature would probably exaggerate any tendency towards lower standards. The economic loss resulting from down-grading or complete rejection of a proportion of a factories output could easily

outweigh any savings in storage costs for those cheeses which were suitable for flavour acceleration (Law, 1984).

4.2 Enzyme additions

A more specific alternative method for accelerating flavour-producing reactions, is the addition of enzymes. Indigenous enzymes are responsible for the maturation of many cheese varieties and it seems therefore logical to induce accelerating cheese maturation by artificially increasing the concentration of these enzymes in the cheese. Proteolysis and lipolysis are important processes in the maturation of most cheese varieties (Law, 1984). Strong flavoured cheese is produced in a short time by the addition of commercial food grade proteinases and the consequent accelerated production of low molecular weight peptones and amino acids. Careful choice of enzymes is, however, necessary to avoid flavour imbalance and bitter defects (Sood and Kosikowski, 1979). Excessive breakdown of β-casein by exogenous proteinases is responsible for textural defects, causing soft-bodied, crumbly cheese (Law and Wigmore, 1982). Legal barriers, difficult incorporation and limited sources of useful enzymes are other difficulties associated with the use of enzymes which have yet to be overcome (Law, 1984). These problems can be overcome by combining the predominantly endopeptidase activity of neutral proteinase with an exopeptidase preparation, yielding a high proportion of low molecular weight N with low degrees of gross proteolysis (a high amino acid : peptide ratio) (Law and Wigmore, 1982). Kheadr *et al.* (2000) successfully used proteases encapsulated in liposomes to accelerate cheese ripening and to avoid drawbacks resulting from the use of free enzymes. Liposome-entrapped lipases were also successfully used to accelerate cheese ripening (Kheadr *et al*., 2002). The use of this system is considered as a way to avoid the flavour defects that usually result from the addition of free lipases to either cheese milk or curd. Since the characteristic aroma, flavour and texture of a cheese is the result of the action of numerous enzymes (El Soda, 1993), the use of a single enzyme to accelerate ripening is likely to disturb the flavour component equilibrium and cause flavour defects. Therefore Cheddar cheese proteolysis and lipolysis were accelerated using liposome-encapsulated enzyme cocktails (Kheadr *et al*., 2003). Preference was given to a mixture of bacterial protease and lipase, which

resulted in the accelerated development of a strong Cheddar flavour and did not exhibit any off-flavours. Bikash *et al*. (2000) reported that in the acceleration of the cheese ripening process, it is possible to improve flavour and eliminate bitterness with the use of proteolytic enzymes from the organism *Brevibacterium linens*, alone, or in combination with commercially available enzymes. A thermostable proteinase from *Bacillus licheniformis* was used successfully for improving and accelerating Domiati cheese ripening (El-Sawah and El-Din, 2000).

The acceleration of lipolysis by the addition of either animal of microbial lipases has been successfully applied to the relatively strong flavoured cheeses. It is noted, however, that the 'typical mature flavour' has changed as this type of cheese has been made and consumed in more and more countries (Law, 1984). Attempts to accelerate the development of typical flavour in English Cheddar cheese using commercial lipases have failed (Law and Wigmore, 1985). Long-chain fatty acids (C12-C16) released by a lipase, produced an unpleasant 'soapy' flavour defect, while the short chain acids released by animal esterases produced an unclean flavour. Many levels of addition were investigated but these enzymes either produced no flavour effect at all or they produced defects.

4.3 Modified starters

The use of modified starters falls into two main categories. In the first, the starter bacteria remain unchanged, but the preparation process for cheesemaking is changed, allowing the starter culture to produce more metabolites which contribute towards cheese with desirable properties (Law, 1984). The enzyme Rulactine from *Micrococcus caseolyticus* is used to treat a small volume of milk prior to cheesemaking in order to liberate peptides and amino acids which serve as growth stimulants for the starter bacteria (Vassal *et al*., 1982). The digest is added to the cheese vat during filling and results in the starter growing and producing more acid in the vat, which consequently speeds up processing. Aroma-producing bacteria in cheese can also be stimulated by treating the culture in a small volume of milk with rennet powder prior to inoculating the cheesemilk. The breakdown of milk proteins to low molecular weight starter nutrients (amino acids and peptides), stimulates the growth of starter bacteria with a resulting accelerated ripening of the cheese

(Dilanian, 1980). Yeasts have also been used in combination with starter bacteria to speed up cheese ripening (Dilanian, 1980). The growth of the starter bacteria is stimulated by means of free amino acids and vitamins produced by the yeasts.

The second category involves possible modifications like genetic manipulations to introduce new enzyme producing capabilities or overall changes in culture composition (Law, 1984). Methods in this category involve lysozyme treatment and heat shock, since both prevent the bacteria from producing acid, while having a minimal effect on their proteolytic enzymes (Law, 1980). Exterkate (1979) showed that the prevention of acid production by starters had the effect of activating some peptidases in the cell by up to 10 times their normal levels. Other genetic modifications to starter bacteria which enhance their proteolytic and lipolytic activity, can be obtained by treating them with n-butanol, X-rays and UV-light (Dilanian *et al*., 1976; Exterkate, 1979; Singh *et al*., 1981a,b).

The proteolytic system of lactococci and lactobacilli consists of an extracellular proteinase and a range of intracellular peptidases (Christensen *et al*., 1999). Cell lysis is therefore necessary to release the cytoplasmic peptidases into the cheese curd. According to Law (2001), the quality and rate of development of flavour in Cheddar cheese is positively linked to the rate and extent of starter culture lysis in young cheese. The highly autolytic strain of *Lactobacillus helveticus* DPC4571 was used as a starter adjunct in different studies for cheese production, with resulting accelerated flavour formation (Kiernan *et al*., 2000; Fenelon *et al*., 2002; Hannon *et al*., 2003). Furthermore, *Lb. helveticus* WSU19 increased proteolysis and significantly enhanced flavour scores and reduced bitterness after 3 and 6 months of ripening of reduced-fat Cheddar cheese (Drake *et al*., 1996; Drake *et al*., 1997).

In the complex environment of a bacterial cell, many processes are influenced by high pressure (HP), resulting in the inability of the bacteria to survive (Tewari *et al*., 1999). Changes in the enzymatic activity (Saldo *et al*., 1999) or the release of starter enzymes by HP-treatment (Saldo *et al*., 1999; Messens *et al*., 2000) may reduce ripening time. There have been several reports on the effects of HP-treatment on acceleration of proteolysis and flavour development in different cheese varieties, including Cheddar (Yokoyama *et al*., 1992; O'Reilly *et al*., 2000), Gouda (Messens *et*

al., 1999), goats cheese (Saldo *et al*., 2000), Camembert (Kolakowski *et al*., 1998), smear-ripened cheese (Maher, 2000) and caprine milk cheese (Saldo *et al*., 2002). Some peptides may impart a bitter flavour to cheese if proteolysis is not well balanced (Habibi Najafi and Lee, 1996), which present a restriction to the HP-treatment method for accelerated ripening.

4.4 Slurry methods

Slurried curd can be used to accelerate cheese ripening (Dulley, 1976). Slurries are prepared from normally manufactured curd by aseptically blending it with 5 % NaCl and 3 % sorbate until a smooth semi-liquid paste is obtained. After storage in closed containers at 30°C for 7 days, this slurried matured curd is incorporated into cheese by addition either to the cheesemilk, the curd before Cheddaring or the salted curd before pressing. This results in accelerated flavour development in the cheese, but a high incidence of off-flavours occurs. The high numbers of lactobacilli could be responsible for accelerating ripening (Dulley, 1976). In similar work done by Baky *et al*. (1982), it was assumed that the slurry contained high numbers of proteolytic and lipolytic bacteria which had developed from the natural flora of the slurried curd, before being added to the curd for normal cheesemaking. This reduced the ripening time significantly. In a more recent study, Ras cheese slurries were prepared from 24-h old Ras cheese curd and from ripened Ras cheese. Each cheese slurry was added to ultrafiltered Ras cheese curd at a ratio of 2 %. The development of cheese flavour was accelerated and there was an improvement in cheese body and texture (Mostafa *et al*., 2000). Liquid slurry methods therefore have the main advantage of rapid flavour development. It has, however, high microbial spoilage potential and the final product requires processing (Law, 1984).

5. YEASTS ASSOCIATED WITH DAIRY PRODUCTS

Yeasts originate as natural contaminants of the cheesemaking process and are therefore associated with the secondary flora of many different types of cheese, making a significant contribution to the process of maturation (Wyder *et al*., 1999). Depending on the species, yeasts grow to populations as high as 10^6 – 10^9 cfu/g during the maturation phase of production (Lenoir, 1984; de Boer and Kuik, 1987;

Besançon *et al.*, 1992). Contaminating yeasts contribute to the overall microbial ecology of Cheddar cheese, despite being produced from pasteurised milk (Welthagen and Viljoen, 1999).

Although great variance amongst dairy products exists, each offers a special ecological niche that selects for the occurrence and activity of specific yeast species. Because of the characteristic nutritional composition of dairy products, a specific association of yeasts is expected. The composition of the nutrients available determines the origin, development and succession of this association (Deàk and Beuchat, 1996).

A large number of yeasts of different origin are frequently found in dairy products. Despite this fact, two distinct groups can be identified: The first group is resident yeasts with characteristics that enable them to survive and reproduce. The second group of yeasts lacks these characteristics and is dependent on dissemination for survival. An accurate understanding of the ecological diversity of yeasts in dairy products would therefore demand a quantitative description of all the yeast species present in the product at continuous intervals before and during processing, as well as storage and the retail points (Deàk and Beuchat, 1996).

Reviews on the microbial organisms present in milk and dairy products (Cousin, 1982; Bishop and White, 1986) deal mainly with bacteria and only refer to yeasts. Milk is fermented by bacteria and therefore bacteria are considered to be of major importance during cheese manufacture (Cousin, 1982). Yeasts, however, can grow under conditions unfavourable to many bacteria and therefore play a significant role in the ripening of some cheese varieties as well as the spoilage of dairy products (Fleet and Mian, 1987; Seiler and Busse, 1990; Fleet, 1992).

5.1 The occurrence of yeasts in dairy products

During the processing of milk into dairy products, the growth of lactic acid bacteria causes the pH to decrease, favouring the growth of spoilage yeasts (Walker and Ayres, 1970). Due to conditions such as low pH, low moisture content, elevated salt concentration and refrigerated storage of cheese, the occurrence of yeasts in dairy

products is not unexpected (Fleet, 1990; Wyder and Puhan, 1999a). In general, yeasts occur at low numbers in raw and pasteurised milks (Fleet, 1992), but yeast counts of approximately 10^5 - 10^6 cells/g have been reported in some cheese varieties (Fleet, 1992). After the inoculation of sterilised milk, several strains of *Candida*, *Kluyveromyces marxianus*, *Cryptococcus flavus* and *Saccharomyces cerevisiae* can grow to populations of 10^8 to 10^9 cells/ml (Fleet and Mian, 1987). Roostita and Fleet (1996) frequently isolated the species *Candida famata*, *Candida diffluens*, *Candida blankii*, *C. flavus* and *K. marxianus* from milk samples. Lipolytic yeast species, especially species of the genus *Rhodotorula*, have been reported to cause pink spots when it grows on the surface of butter (Walker and Ayres, 1970). Cream can be spoiled by *Geotrichum candidum* when machinery on farms are not cleaned properly (Marth, 1978).

Species encountered most frequently in cheeses comprise of *Debaryomyces hansenii*, *S. cerevisiae*, *Yarrowia lipolytica*, *K. marxianus*, *Torulaspora delbrueckii*, *R. glutinis*, *Cryptococcus albidus*, *Candida catenulata* and *R. minuta* (Welthagen and Viljoen, 1998a; Welthagen and Viljoen, 1999; Viljoen *et al*., 2003). Lenoir (1984) identified more than 10 yeast species associated with Camembert cheese from different regions and stated that the basic yeasts flora consists of *Kluyveromyces*, *D. hansenii*, *S. cerevisiae* and *Zygosaccharomyces rouxii*.

Predominating yeast species found during various studies are representatives of *D. hansenii*, *Y. lipolytica*, *K. marxianus* and several *Candida* species (Lenoir, 1984; de Boer and Kuik, 1987; Nooitgedagt and Hartog, 1988; Besançon *et al*. 1992; Welthagen and Viljoen, 1998a; Welthagen and Viljoen, 1999) with *D. hansenii* being the most dominant (Eliskases-Lechner and Ginzinger, 1995; Eliskases-Lechner, 1998; Welthagen and Viljoen, 1998a,b; Welthagen and Viljoen, 1999; Wyder and Puhan, 1999a,b; Addis *et al*., 2001; Petersen *et al*., 2002; Vasdinyei and Deàk, 2003).

5.2 Characteristics of yeasts associated with dairy products

In a study on the occurrence and growth of yeasts in dairy products, *Candida famata*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* exhibited best growth at 20ºC. At 5ºC, *Candida diffluens*, *Cryptococcus flavus* and *Rhodotorula glutinis*

exhibited the best growth. Fermentation and assimilation of lactose and sucrose, hydrolysis of casein and fat, and the resistance to benzoate are key properties exhibited by these yeasts (Fleet and Mian, 1987). *K. marxianus* and *C. famata*, furthermore, are able to ferment and assimilate lactose, the major sugar of milk, lactic acid and citric acid, and produce proteases and lipases that can hydrolyse milk casein and fat (Fleet and Mian, 1987). The growth of oxidative yeast species *R. glutinis, C. diffluens* and *C. flavus* in dairy products is attributed to their abilities to assimilate milk sugars, organic acids and the hydrolisation of milk casein and fat (Ahearn *et al.*, 1968; Fleet and Mian, 1987).

Debaryomyces hansenii, the perfect form of *Candida famata*, predominated in most studies on yeasts associated with dairy products (Fleet, 1992). This is due to its ability to grow at extreme high salt concentrations (Mrak and Bonar, 1939), low water activity levels, low temperatures and its lipolytic and proteolytic activity (Walker and Ayres, 1970; Lenoir, 1984; Seiler and Busse, 1990).

Yarrowia lipolytica strains possess strong proteolytic and lipolytic activities at temperatures below 0°C (Alford and Pierce, 1961). The yeast is considered as the most predominant species contributing to lipolytic activity (Choisy *et al*., 1987) and was also indicated as an exceptional inducer of proteolytic activity (Wyder and Puhan, 1999b; Lucia *et al*., 2001). *Y. lipolytica* and *Candida laurentii* are thought to be the most common spoilage species during the ripening of cheeses (Deàk and Beuchat, 1996). *D. hansenii* and *Y. lipolytica* have, however, been regarded as good candidates for ripening agents in cheese (Guerzoni *et al*., 1998) fulfilling specific criteria to be regarded as co-starters for cheesemaking (Van den Tempel and Jakobsen, 2000; Guerzoni *et al*., 2001; Suzzi *et al*., 2001).

S. cerevisiae is a widespread and natural occurring species' in different foods, causing spoilage in the form of gas production and yeasty or fruity flavours in dairy products (Walker, 1988). Strains of *S. cerevisiae* are sensitive to high salt concentrations (Roostita and Fleet, 1996). This species also lacks the ability to utilise lactose and citric acid, and to produce lipase or protease. It only weakly utilises lactic acid (Fleet and Mian, 1987).

Yeast counts may vary between dairy plants and even between consecutive days in the same plant due to the variation in salt concentration (Seiler and Busse, 1990), temperature (Davenport, 1980), accidental occurrences of contaminating yeasts (Fleet, 1990) or the varying standards of hygiene during cheese making and the efficiency of pasteurisation (Fleet and Mian, 1987).

6. THE CONTRIBUTION OF YEASTS IN THE DAIRY INDUSTRY

The occurrence of yeasts in dairy products is significant because they can cause spoilage, effect desirable biochemical changes and adversely affect public health (Seiler and Busse, 1990). Depending on the strain properties and the contamination level, the yeasts affect the ripening process positively (de-acidification, production of aroma substances) or negatively (smell and taste defects) (Eliskases-Lechner, 1998). According to Marth (1978) yeasts play an important role in dairy products in:

(i) the processing of certain fermented products and in the ripening of certain cheeses;

(ii) the spoilage of milk and dairy products; and

(iii) the usage of yeasts to ferment whey, a major by-product of cheese-making.

Milk and cheese are the two most important dairy products in which yeast activity plays a major role (Fleet, 1992). The particular type of cheese determines the presence and significance of yeasts. In some cheeses, yeasts contribute to the flavour, texture and aroma during the ripening stage of the cheese, or cause spoilage (Fleet, 1990).

6.1 Yeast spoilage of dairy products

Spoilage yeasts are defined as microorganisms that produce undesirable changes in food during the fermentation process (Fleet, 1990; Fleet, 1992; Deàk and Beuchat, 1996). They are generally heat sensitive and therefore can be assumed to be post-pasteurisation contaminants (Walker, 1988). The role of yeasts as spoilage organisms are linked to their nutritional requirements, growth at low temperatures, low pH values, low water activities and high salt concentration (Davenport, 1980; Seiler and Busse, 1990). For this reason, yeast spoilage is recognised as a potential problem in cheese (Fleet, 1990). Yeast spoilage may appear in the form of a pellicle or turbidity

in liquids, or as a slimy or powdery coating on solid surfaces (Fleet, 1992). Other undesirable changes due to the production of metabolic products include the formation of unnatural odours or flavours (Walker, 1988). Some yeast species containing the decarboxylase enzyme, produce biogenic amines from amino acids, which result in bitter flavours (Wyder *et al*., 1999). The metabolic activity of yeasts can also cause an increase in pH due to the utilisation of organic acids such as lactic, citric or acetic acids added as food preservatives (Walker, 1988). The removal or reduction in concentration of these compounds by yeasts can encourage spoilage bacteria to develop (Walker and Ayres, 1970; Warth, 1991).

Food spoilage by yeasts becomes a specific problem in those foods where bacterial growth is restricted, rendering yeasts a competitive advantage (Ingram, 1958). Sweetened condensed milks with their high sugar contents and low water activity, and cream and butter with their low water content and high fat concentration are restrictive environments that can select for the growth of yeasts (Walker and Ayres, 1970; Fleet, 1990). Products such as yoghurt, cheese, processed fruits and vegetables, foods with high sugar or salt concentrations as well as beer and wine may be contaminated with spoilage yeasts if hygiene standards are neglected during production, manufacture or handling (Garcia *et al*., 2002). Excessive growth of *Candida albicans*, *Geotrichum candidum*, *Kluyveromyces marxianus*, *Pichia membranaefaciens*, *Yarrowia lipolytica*, *Debaryomyces hansenii*, *Candida zeylanoides*, *Cryptococcus albidus* and *Cryptococcus laurentii* can cause undesirable sensory changes, softening of structure, slime formation and blowing of cheese (Ingram, 1958; Walker and Ayres, 1970; El-Bassiony *et al*., 1980; Lenoir, 1984; Brocklehurst and Lund, 1985; Pitt and Hocking, 1985; Engel, 1986; Romano *et al*., 1989; Rohm *et al*., 1990; Seiler and Busse, 1990; Tudor and Board, 1993).

The question of whether yeast activity during cheese maturation is detrimental or beneficial to product quality complicates the assessment of cheese spoilage by yeasts. Over-ripening during maturation could be interpreted as spoilage, whereas continued lactose fermentation by yeasts at the later stages of cheese ripening, could lead to an increase in acidity, gassiness and fruity flavours (Fleet, 1990). When slurries were incubated with fermentative yeast species (Wyder and Puhan, 1999b) they were described to be acidic, fermented, cidery, alcoholic and fruity, which could be a result

of volatile fermentation products such as formic or acetic acids. Martin *et al*. (2002) evidenced the role of yeasts in the generation of fruity notes, probably through the generation of alcohols, aldehydes and esters. In association with *Geotrichum candidum*, yeasts were able to produce different sulfur compounds and appeared to be potential generators of sulfury and cheesy notes (Martin *et al*., 2002). Continued hydrolysis of protein and fat contribute to bitter and rancid flavours as well as a softening of product texture (Fleet, 1990). Based on a viewpoint concerning public health and product spoilage, losses caused by yeast spoilage are generally considered to be minor compared with those caused by bacteria and fungi. (Marth, 1978; Fleet, 1990). Yeasts, in this sense, grow much slower than bacteria and are easily overgrown by the indigenous bacteria present in foods, therefore causing less spoilage (Fleet, 1990; Fleet,1992; Deàk and Beuchat, 1996).

The presence of spoilage yeasts in food has never resulted in food poisoning phenomena (Fleet and Mian, 1987; Fleet, 1990; Fleet, 1992). The metabolic products of yeast are not considered toxic, and the yeasts themselves, even though some pathogenic species exist, are not known to be responsible for infections or poisoning, as is the case with a number of bacterial and fungal species (Peppler, 1976; Fleet, 1992; Deàk, 1994).

6.2 The positive contribution of yeasts

Yeasts also contribute positively to the fermentation and maturation process of cheeses by inhibiting undesired microorganisms present (Kaminarides and Laskos, 1992), supporting the function of the starter culture (Kalle *et al.*, 1976) and due to proteolytic and lipolytic activity, which contribute directly to the ripening process (Coghill, 1979; Lenoir, 1984; Siewert, 1986; Fleet, 1990; Jakobsen and Narvhus, 1996). When lactic acid is metabolised by yeasts during the maturation process of the cheese an increase in pH results encouraging the growth of bacteria (Fleet, 1990). The formation of alkaline metabolism products, such as ammonia from amino acid deamination also lead to the deacidification of cheese (Eliskases-Lechner and Ginzinger, 1995; Smacchi *et al*., 1999; Wyder *et al*., 1999). The yeasts furthermore excrete growth factors like B-vitamins, pantothenic acid, niacin, riboflavin and biotin (Purko *et al*., 1951; Lenoir, 1984; Fleet, 1990; Jakobsen and Narvhus, 1996) that

promote the growth of lactic acid bacteria. Gas production by the yeasts leads to curd openness (Choisy *et al*., 1987). The predominance of *Debaryomyces hansenii* in some cheeses may reduce the risk of cheese spoilage by clostridial species through the production of antibacterial metabolites (Fatichenti *et al*., 1983). Yeasts may ferment lactose, metabolise lactate, influence flavour formation by producing volatile acids and carbonyl compounds (Fleet and Mian, 1987) and prevent cheese surfaces from forming a "toad skin" (Schmidt *et al*., 1979; Baroiller and Schmidt, 1984). Yeasts have a definite effect on the sensory properties of the final product in model cheese media (Martin *et al*., 1999) or in cheeses (Molimard *et al*., 1997). They have capabilities to produce sulfur flavour compounds essential for cheese flavour (Spinnler *et al*., 2001; Arfi *et al*., 2002).

In certain milk products, yeasts contribute to the fermentation process. Liquid milk products like kefir and koumiss derive some of their characteristic flavour from the activity of fermenting yeasts (Prillinger *et al*., 1999). The fermentation of lactose results in the formation of carbon dioxide but also in flavouring compounds such as ethanol and acetaldehyde (Lenoir *et al*., 1985; Devoyod, 1990).

Yeasts have been mentioned for their ability to improve the quality of numerous cheeses (Proks *et al*., 1959; Mahmoud *et al*., 1979; Masek and Zak, 1981; Choisy *et al*., 1987), mainly by their lipolytic activity. Lipases produced by yeasts contribute to the maturation of cheese through the breakdown of fat during ripening. This accumulation of fatty acids is responsible for flavour development in cheese, whereas proteases are responsible for protein breakdown in the maturing process. The resulting peptides and amino acids are important for the development of background flavour. Proteolysis is also necessary for proper texture development (Coghill, 1979). Amino acids are further catabolised by the surface flora of cheese, yielding a variety of flavour compounds and precursors. Ammonia, for example, contributes to the aroma profile and results from amino acid deamination by the contaminating yeast microflora, particularly species of *Geotrichum* (Greenburg and Ledford, 1979; Hemme *et al*., 1982). In a comparison study, yeasts exhibited higher peptidase activity than the dairy bacterial species tested, and therefore significantly influence proteolysis in cheese (Klein *et al*., 2002). Furthermore, the intracellular proteolytic activity of *D. hansenii* and *Saccharomyces cerevisiae* was found to be

higher than that of lactobacilli and therefore contributed to flavour development in Feta cheese (Bintsis *et al*., 2003).

It can be concluded that yeasts are important in the maturation process of cheese. Little is, however, known concerning the direct contribution of yeasts to cheese ripening and flavour formation.

7. BIOLOGICAL CONTROL EXHIBITED BY YEASTS

7.1 Killer yeasts

Many yeast species produce and secrete toxins which inhibit the growth of other yeast strains, but to which they are immune. Two species of yeast killer toxins exist: mycocins are large (glyco) protein molecules with molecular masses of 10-20 kDa or higher (Golubev and Shabalin, 1994). They are toxic to closely related organisms and are inactivated at elevated temperatures and by proteolytic enzymes. Microcins are (glyco) oligopeptides with molecular masses of 1 kDa or less (Golubev and Shabalin, 1994). They have a much broader range of action and are thermostable and often resistant to proteases (Golubev and Shabalin, 1994). Yeast killer toxins are pH and temperature dependent, usually active and stable at pH 4-5 and at 20-25°C (Izgü *et al.*, 1997). They cause membrane permeability in sensitive cells (Kagan, 1983) and in some cases inhibit DNA replication (Schmitt *et al.*, 1989), or stop cell division at the G1-phase (Stark *et al.*, 1990). Based on the killer factor, yeasts are grouped as follows:

(i) killers - producers of a lethal toxin and resistant to it;
(ii) neutrals - non-producers, but resistant; and
(iii) sensitives - killed by the killer factor (Giovanni *et al.*, 1991).

Killer yeasts and their toxins have found several applications. In the food and fermentation industries, contamination of starter culture strains with undesirable yeast species, can dramatically decrease the quality of the product (Izgü *et al.*, 1997; Valentino *et al.*, 1991). Contamination with killer toxin-producing yeast species is in particular a potential problem in yeast fermentations. Although killer strains are immune to their own toxins, they are susceptible to the effect of the toxins of other

immunity groups. For this reason, the killer phenomenon can be used against contaminating yeast to protect fermentation (Izgü *et al.*, 1997).

Killer yeasts are also useful in the biological control of undesirable yeasts in the preservation of foods (Walker *et al.*, 1995). In the medical field, killer yeasts have found applications and potential therapeutic effects of killer yeast toxins have been reported (Polonelli *et al.*, 1986). It has also been suggested that yeast killer toxins have potential as novel antimycotic agents in the treatment of human and animal fungal infections (Walker *et al.*, 1995).

Killer toxins of certain yeast strains showed growth inhibitory and killing activity against a range of fungal pathogens of agronomic, environmental and clinical significance (Walker *et al.*, 1995). Gram-positive pathogenic bacteria were also found to be inhibited by yeast killer toxins (Izgü and Altinbay, 1997). Furthermore, the killer phenomenon was confined to a few strains of *Debaryomyces hansenii* that inhibited another strain of the same species (Addis *et al*., 2001).

According to Pfeiffer *et al.* (1988), the yeast killer toxins do not deserve the frightening name that has made them so well known. Because of its instability, the killer toxin is rapidly inactivated at body temperature. Even if it survives the action of the protease in the stomach, it would be inactivated at the elevated pH of the duodenum. It is therfore unlikely to cause any action when consumed orally.

7.2 Antibiotics

A fraction containing a protein with anti-infectious properties was isolated from aqueous extracts of brewer's and baker's yeast. This fraction, called malucidin, differs from other antibiotics in that it has a much broader spectrum and produces a longer lasting effect. Malucidin protected laboratory animals against a number of infections caused by Gram positive and Gram negative bacteria, including pathogens such as *Proteus, Salmonella, Pseudomonas* and *Brucella.* It also inhibits certain fungi (Parfentjev, 1957). Robinson *et al.* (1958) reported that a decrease in the numbers of *Staphylococcus aureus, Escherchia coli* and mixed pre-ferment cultures in yeast fermentations was due to the activity of antibiotic substances elaborated by *S.*

cerevisiae. Unsaturated fatty acids from brewer's and baker's yeasts, *Debaryomyces mucosus* and *Torulopsis utilis*, and succinic acid or other acidic substances from *Torulopsis utilis* var. *major* also possess antibacterial properties. The growth of certain strains of bacteria is inhibited by cyclic peptides from baker's yeast, certain proteins from brewer's and baker's yeast, the crystalline carotenoid lusomycin from *Rhodotorula glutinis* var. *basitarica* and unidentified substances from *Saccharomyces cerevisiae.* *Candida pulcherrima* produces pulcherriminic acid, which inhibits the growth of certain *Mucor, Fusarium* and *Penicillium* spp. The yeasts *Brettanomyces bruxellensis, Schizosaccharomyces pombé* and *Saccharomyces carlsbergensis* were found to inhibit the growth of *Bacillus subtilis* and the pediococci (MacWilliam, 1959).

7.3 Yeasts as biocontrol agents

Microbial antagonists have been reported to control several rot pathogens on diverse commodities. Of particular interest among these antagonists are yeasts and yeast like organisms (Wisniewski and Wilson, 1992). This may be true for several reasons:

(i) yeasts can colonise the surface for long periods of time under dry conditions;
(ii) they produce extracellular polysaccharides that enhance their survivability and may restrict colonisation sites and the flow of germination cues to fungal propagules;
(iii) they rapidly use available nutrients and proliferate; and
(iv) are minimally impacted by pesticides (Janisiewicz, 1988).

McLaughlin *et al.* (1990) reported that yeasts are a major component of the epiphytic microbial community on the surfaces of fruits and vegetables and therefore may act as an effective biocontrol agent being phenotypically adapted to this niche, and consequently able to more effectively colonise and compete for nutrients and space on the surfaces. Examples are *Pichia guilliermondii* that control postharvest rots of citrus and other fruits, and *Acremonium breve* and seven species of *Cryptococcus* that control postharvest rots of apple and pear (Wisniewski and Wilson, 1992). *Candida* spp. have been found to be highly effective against different fungal pathogens (McLaughlin *et al.*, 1990).

7.4 Predacious yeasts

Haustorium-mediated predation was observed in seven yeast species. *Arthroascus javanensis, Botryoascus synnaedendrus, Guilliermondella selenospora, Saccharomycopsis fibuligera* and three unknown species penetrate and kill other yeasts. *Candida* sp. strain 95-697.4 produces small feeding appendages (haustoria) that penetrate and kill other yeast cells (Lachance and Pang, 1997).

8. PROBIOTIC MICROORGANISMS

8.1 Background on probiotic microorganisms

8.1.1 History

The history of the probiotic effect dates backs many centuries and has been well-documented many times previously (Bibel, 1982; Fuller, 1992). The consumption of fermented milks dates from pre-biblical times with known scientists in early ages, such as Hippocrates considering fermented milk not only a food product but a medicine as well (Oberman, 1985). The probiotic concept was born at the end of the last century with the work of the Russian bacteriologist Eli Metchnikoff at the Pasteur Institute in Paris (Hughes and Hoover, 1991; O' Sullivan *et al*., 1992). Metchnikoff (1907) postulated that lactic acid bacteria could help restore the balance of intestinal flora and therefore improve health and prolong life. The principle of his theory was based on the displacement of toxin producing bacteria normally present in the intestine by the lactic acid bacteria. He explained that owing to lactic acid and other products produced by lactic acid bacteria in sour milks, the growth and toxicity of anaerobic, spore-forming bacteria in the large intestine are inhibited. During that time, in 1899, Tissier (Pasteur institute, France) recommended the administration of Bifidobacteria to infants suffering from diarrhoea (O' Sullivan *et al*., 1992). He also believed that the Bifidobacteria would displace putrefactive bacteria responsible for gastric upsets and re-establish themselves as the dominant intestinal microorganisms.

In the century that has elapsed since Metchnikoff's work, scientists and consumers throughout the world have accepted the probiotic concept. Attempts to refine the

practice from the use of traditional soured milks to preparations containing specific microorganisms have occupied the thoughts of scientists in many different countries. Earlier work dealt with the use of fermented milk to treat intestinal infections. More recent studies focused on other aspects of health benefits that might be derived from these organisms, as well as strain selectivity to ensure survival of these bacteria in the gastrointestinal tract and the carrier food.

8.1.2 Definition

Probiotics are a mono- or mixed culture of live microorganisms which, applied to man or animal (e.g. as dried cells or as a fermented product), beneficially effects the host by improving the properties of the indigenous microflora (Huis in't Veld and Havenaar, 1991). This definition implies that probiotic products contain live microorganisms and promote good health of the host by exerting beneficial effects on intestinal function.

8.2 Selection criteria for probiotics

While there is not a basis for conclusive and optimal selection of probiotic bacteria, certain key criteria have been established (Havenaar *et al.*, 1992). These can be devided into three main categories:

8.2.1 General microbiological criteria

- The organism must be safe (i.e. non-pathogenic).
- It should survive initial attacks of human defence systems (saliva, gastric and bile juice).
- The microbe should presumably originate from humans.
- The strain should be genetically stable.

8.2.2 Technological aspects

- It should be possible to culture the organism on an industrial scale.

- A suitable carrier or fermenting substance (e.g. milk) should be available.
- The final product should have acceptable shelflife and sensory attributes – colour, taste, aroma and texture.

8.2.3 Proven functional effects

- Stimulation of the immune response.
- Promotion of colonisation.
- Resistance or functional effects on the intestinal flora e.g. modification of bacterial counts and/or their metabolic activity.

In addition, the organism must be capable of colonising in the intestine, being metabolically active to produce beneficial effects and must remain viable in the food carrier up to consumption (Rasic and Kurmann, 1983).

8.3 Therapeutic value

Yoghurt containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* is rapidly gaining popularity on the world market (Shah *et al*., 1995). The beneficial effects of consuming large numbers of viable cells (> 10^6 cells/ml) of *L. acidophilus* and *B. bifidum* have been well established (Rybka and Kailasapathy, 1995). The potential health benefits of probiotics include the following:

8.3.1 Maintenance and restoration of normal intestinal balance

A normal intestinal balance can be achieved by the reduction of the intestinal pH, due to organic acid production (Sandine, 1979), the destruction of toxic substances and the production of antimicrobials to inhibit pathogens (Richardson, 1996). Mechanisms for the inhibition of pathogens ascribed to lactobacilli and bifidobacteria include the production of antimicrobial substances such as organic acids especially acetic acid, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids, their acting as competitive antagonists i.e. competition for adhesion sites and nutrients and their stimulation of the immune system (Rasic and Kurmann, 1983; Gorbach *et al*., 1987;

Gilliland, 1989). The severity and duration of traveller's diarrhoea as well as rotavirus and diarrhoea in children can be reduced or prevented by probiotic lactic acid bacteria, while the adverse effects of antibiotic therapy can be minimised (Richardson, 1996; Gorbach, 2002).

8.3.2 Improvement of lactose intolerance and digestibility of milk products

Some people have reduced ability to digest lactose into its component sugars, glucose and galactose. This is due to the absence of β-D-galactosidase in the human intestine and leads to various degrees of abdominal discomfort (Kim and Gilliland, 1983). Probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* and lactic acid bacteria used as starter cultures in milk and fermentation produce β-D-galactosidase. This enzyme hydrolyses lactose, which results in increased tolerance for dairy products (Kim and Gilliland, 1983; De Vrese *et al*., 2001).

8.3.3 Antitumorigenic activities and decreased risk of cancer

Probiotics assist in the inactivation of dietary and intestinally generated mutagenic compounds such as azo dyes and nitrosamines (Richardson, 1996). Faecal bacterial enzymes such as nitroreductase, azoreductase and β-glucuronidase are reduced with an enhanced resistance to tumours (Goldin and Gorbach, 1977; Goldin and Gorbach, 1984; Richardson, 1996). Kailasapathy and Rybka (1997) reported on several animal studies confirming that the intake of fermented milk products containing probiotic bacteria inhibit tumour formation and proliferation.

8.3.4 Activation of the host's immune system

Some species and strains have the potential to enhance the human immune system by non-specific anti-infective mechanisms of defence (Richardson, 1996), like the phagocyte activity in peripheral blood. Lactobacilli and bifidobacteria have a major part in the maintenance and restoration of health in children and adults (Reid, 2002a). This includes their ability to decrease risk of infection and stone disease as well as possible positive effects on preventing and managing inflammation (Reid, 2002a).

Furthermore, there is growing in vitro and human data proving probiotics to be effective against urogenital infections (Reid, 2002b). Gorbach (2002) reported promising results in patients with inflammatory bowel disease, cystic fibrosis, dental caries and irritable bowel syndrome.

8.3.5 Reduction of serum cholesterol levels

Elevated serum cholesterol is a risk factor for ischaemic heart disease and therefore significant reduction in plasma cholesterol levels is associated with a significant reduction in the risk of heart attacks. Claims are strong that certain *Lactobacillus acidophilus* strains and some bifidobacteria species are able to lower cholesterol levels within the intestine, but the mechanisms are still uncertain. Cholesterol co-precipitates with deconjugated bile salts at lower pH values due to lactic acid production by the lactic acid bacteria, with resulting reduction of the cholesterol levels (Marshall, 1996). Another theory is that *L. acidophilus* deconjugates bile acids into free acids, which are excreted more rapidly from the intestinal tract than conjugated bile acids. This excretion of bile acids can reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977a). Wagner (2001) confirmed the bile salt hydrolase theory, but also suggested that the part of cholesterol that is bound to the microorganisms at pH<9 may be removed from the gastrointestinal tract together with the microorganisms.

8.3.6 Production of vitamins

Bifidobacteria are capable of synthesising some B-vitamins and digestive enzymes such as casein phosphatase and lysozyme (Richardson, 1996).

All these beneficial effects of probiotic cultures grant them with enough credit to be introduced into cheese as part of the starter culture to develop another probiotic carrier food with therapeutic effects.

8.4. Level and survival of *Lactobacillus acidophilus* and Bifidobacteria in Bio-Yoghurt

To achieve the optimal potential therapeutic effects, the minimum number of viable cells in a probiotic product should be > 10^6 cfu/ml until the expiry date – the so-called "therapeutic minimum" (Davis *et al*., 1971; Kurmann and Rasic, 1991; Rybka and Kailasapathy, 1995). It is imperative that bio-yoghurt, claiming "with live AB cultures", meet the criteria of 10^6 cfu/ml viable cells of probiotic bacteria at the expiry date. If the product does not comply with the required criteria, it can be considered misleading to the consumers and limited health properties are conferred.

8.4.1 Factors affecting the viability of *L. acidophilus* and bifidobacteria species in bio-products

8.4.1.1 *Product acidity*

One of the most constraining drawbacks associated with the use of dietary cultures in fermented milk products is the lack of acid tolerance of some species and strains (Klaver *et al*., 1993; Lourens and Viljoen, 2002). When the lactic acid content increases, pH levels correspondingly decrease during fermentation. The survival of microorganisms is affected by the low pH of the environment. According to Hood and Zotolla (1988) *Lactobacillus acidophilus* grows and survives better at pH 4.0 than at pH 2.0. Martin and Chou (1992) reported that a pH of 5.5 –5.6 was determined as the minimum pH for survival of some species/strains of bifidobacteria. Overall, most strains of bifidobacteria are sensitive to pH values below 4.6. Therefore, for practical application, the pH value of the final product must be maintained above 4.6 to prevent decline of bifidobacteria populations (Tamime and Robinson, 1985; Modler *et al*., 1990; Laroia and Martin, 1991).

8.4.1.2 *Species/strains*

Various species of both *Lactobacillus* and *Bifidobacterium* diminish markedly during refrigerated storage at low pH levels while the main requirement in selecting bifidobacteria for use in a yoghurt product is the ability to grow in milk. Many

Bifidobacterium strains are unable to grow in milk due to their lack of proteolytic activity. Careful strain selection and monitoring are therefore necessary to ensure high quality fermented bioproducts (Gilliland and Lara, 1988; Klaver *et al*., 1990; Klaver *et al*., 1993; Hughes and Hoover, 1995; Shah *et al*., 1995).

8.4.1.3 *Co-culture and species interaction*

The composition of the species participating in fermentation has been found to affect the survival of *Lactobacillus acidophilus* and *Bifidobacterium* species. A potential growth medium contains metabolic products secreted by other microorganisms, which influence the viability of *L. acidophilus* and *B. bifidum* (Gilliland and Speck, 1977b). Hydrogen peroxide produced during the manufacture and storage of yoghurt seems to be the main substance responsible for the antagonism towards *L. acidophilus* (Gilliland and Speck, 1977b). Most strains of *B. bifidum* lack proteolytic activity (Klaver *et al*., 1993). The proteolytic *L. acidophilus* species, therefore lives in excellent symbiosis with bifidobacteria providing the necessary growth stimulants (Hansen, 1985). Aerobic microorganisms act as oxygen scavengers in dairy products and are therefore beneficial to the growth of the anaerobic *Bifidobacterium* species (Shankar and Davies, 1976; Ishibashi and Shimamura, 1993; Van den Tempel *et al*., 2002).

8.4.1.4 *Inoculation size*

An important key factor to ensure sufficient viable cells in the final food product is the inoculum size of probiotic bacteria. It is imperative that manufacturers of probiotic foods ensure that at least one million viable cells/g of *Bifidobacterium* are present at the end of fermentation (Samona and Robinson, 1994). Fermented products should contain more than 10^6 cfu/ml of viable probiotic cells at the time of consumption to be effective (Robinson, 1987; Rybka and Kailasapathy, 1995; Dave and Shah, 1997). According to Dave and Shah (1997), however, an increased inoculum size did not improve the viability of bifidobacteria in yoghurt.

8.4.1.5 *Temperature*

According to Kneifel *et al*. (1993), storage temperature substantially influences the production of lactic acid, related to the growth and survival of the starter cultures at higher temperatures. The storage temperature furthermore plays an important role in the control of excessive growth of microorganisms responsible for over-acidification of the products (Kneifel *et al*., 1993). Bacteria most likely die off in yoghurt during storage due to postacidification by yoghurt culture organisms. According to Hughes and Hoover (1995), Bifidobacteria are substantially less tolerant to low temperature storage when compared to *Lactobacillus acidophilus*.

8.4.1.6 *Dissolved oxygen*

Since *Bifidobacterium* is strictly anaerobic, oxygen toxicity is an important and critical problem (Klaver *et* al., 1993). During yoghurt production, oxygen easily penetrates and dissolves in milk (Ishibashi and Shimamura, 1993). This obstacle may be overcome if Bifidobacteria are introduced at a later stage during cheese making, such as milling or salting (Dinakar and Mistry, 1994). Oxygen, however, also permeates through packages during storage. To avoid the oxygen problem it has been suggested to inoculate microorganisms with high oxygen utilisation ability together with *Bifidobacterium* (Ishibashi and Shimamura, 1993). This will result in the depletion of dissolved oxygen in the fermentation product and an enhancement in the viability of Bifidobacteria.

8.5 The expansion of the probiotic product range: efforts to incorporate probiotic cultures into cheese and other dairy products

To date, the most popular food delivery systems for probiotic cultures have been freshly fermented dairy foods, such as yoghurt and fermented milk, as well as unfermented milk with cultures added (Fernandes *et al*., 1987; Bourlioux and Pochart, 1988; de Simone *et al*., 1989; Alm, 1991; Sanders *et al*., 1996). In an effort to expand the probiotic product range a small number of researchers and companies have endeavoured to manufacture cheeses that sustain a high viable count of probiotic cultures. The incorporation of such health-enhancing cultures into cheese during

manufacture would only result in a functional food if the culture(s) are to maintain viability during ripening and if the added culture(s) do not adversely affect composition, texture or sensory criteria of the product (Stanton *et al*., 1998).

8.5.1 Probiotics in yoghurt

Yoghurt and fermented milks have received most attention as carriers of live cultures. A study by Dave and Shah (1997) has shown poor viability of probiotic organisms in the market preparations of yoghurt. In a study on the survival of probiotic bacteria in South African commercial bio-yoghurt (Lourens and Viljoen, 2002) it was found that probiotic cultures in yoghurt survive for less than thirty days after the date of manufacture. The numbers didn't meet the therapeutic minimum at the end of the yoghurt's shelflife, with the consumer not receiving sufficient numbers of the probiotic cultures. The bacteria most likely died off in yoghurt during storage due to post-acidification by the yoghurt culture organisms with a resulting decrease in the pH of the yoghurt to a value below 4. Martin and Chou (1992) reported that a pH of 5.5 – 5.6 was determined as the minimum pH for survival of some species/strains of bifidobacteria. According to Gilliland (1979) their growth is retarded below pH 5. The acid tolerance of *Bifidobacterium* is, however, strain specific. Growth of *Lactobacillus acidophilus* is inhibited below pH 4 (Playne, 1994; Hood and Zotolla, 1988). Bifidobacteria also died off due to dissolved oxygen in the yoghurt (Klaver *et al*., 1993).

8.5.2 Survival of probiotic cultures in other dairy foods

Gomes *et al*. (1995) used bifidobacteria in combination with *Lactobacillus acidophilus* strain Ki as the starter in Gouda cheese manufacture. Both species survived relatively well in the cheese, and their application as starters in a Gouda-type cheese was suggested. There was, however, a significant effect on cheese flavour after nine weeks of ripening, probably due to acetic acid production by the bifidobacteria. Crescenza is a soft, rindless, Italian cheese with a short ripening time that has been produced in Lombardy for many decades. Bifidobacteria incorporated during the production of Crescenza cheese (Gobbetti *et al*., 1998) remained viable during the ripening period of the cheese and had no effect on the flavour, appearance

and the microbial and physicochemical features of the cheese. In a study by Blanchette *et al.* (1996), cottage cheese did not sustain high viability of *Bifidobacterium infantis* that had been introduced during manufacture. *B. infantis* reached levels of approximately 10^7 cfu/g^{-1} cheese after 1 day of storage. Large losses in viability, however, were observed after 15 days at 4°C, the normal shelflife of the product. In addition, consumers showed a reduced preference for the product manufactured with added bifidobacteria over the control cheese (Blanchette *et al.*, 1996). *L. reuteri*, *L. rhamnosus*, *L. acidophilus* and *B. bifidum* were used for the manufacture of a soft, fresh cheese (Nayra *et al.*, 2002). The organisms remained above the therapeutic minimum for 2 months. A fresh cheese of very good sensory quality with a shelf life of 15 days and good viability of the organisms was obtained when *L. casei* and *B. bifidum* was added to the starter culture (Suarez-Solis *et al.*, 2002). During the ripening of white brined cheese, *B. bifidum* survived in higher numbers than *B. adolescentis* (Ghoddusi and Robinson, 1996) and declined only one log cycle (to 5,5 x 10^5 cfu/g^{-1} cheese) following 60 days of ripening. Different combinations of *Bifidobacterium* and *Lactobacillus* species showed satisfactory viability in Argentinean Fresco cheese during storage for 60 days (Vinderola *et al.*, 2000).

Hekmat and McMahon (1992) demonstrated ice cream being a suitable vehicle for delivering beneficial microorganisms such as *L. acidophilus* and *B. bifidum* to consumers. The bacteria could be grown to high numbers in ice cream mix and remained viable for more than 17 weeks of frozen storage at -29°C. Christiansen *et al*. (1996) produced probiotic ice cream by mixing commercial *L. acidophilus* and *B. bifidum* cultured milks with unfermented ice cream mix. After 16 weeks of frozen storage at -20°C the ice cream still contained high levels of viable probiotic microorganisms. During a study by Hagen and Narvhus (1999) viable numbers of the studied probiotic strain remained above 10^6 cfu/g during 52 weeks storage of ice cream at -20ºC.

In a study on the growth and survival of bifidobacteria in milk (Klaver *et al.*, 1993) it was found that only a few *Bifidobacterium* strains seemed suitable to obtain sufficiently large numbers of viable cells at the time of consumption of the milk. The inability to grow in milk was mostly due to the lack of proteolytic activity in most

Bifidobacterium strains. Ziajka and Zbikowski (1986) also suggested the lack of proteolytic properties to cause failure of growth in milk. This problem can be overcome by adding proteolytic products (peptide compounds and amino acids) to the milk and by co-culturing *Bifidobacterium* strains with proteolytic lactobacilli like *L. acidophilus* (Klaver *et al*., 1993). Growth of bifidobacteria in milk can therefore be considered as commensal with proteolytic species.

8.5.3 Probiotics in Cheddar cheese

Cheddar cheese may offer certain advantages as a delivery system for live probiotics to the gastro-intestinal tract of humans. Having a higher pH than the more traditional probiotic foods, it may provide a more stable milieu to support the long-term survival of probiotic organisms. Furthermore, the matrix and high fat content of the cheese may offer protection to the organisms during passage through the gastro-intestinal tract (Stanton *et al*., 1998). The oxygen toxicity problem may be overcome by introducing *Bifidobacterium* during a later stage of cheese making, such as milling or salting (Dinakar and Mistry, 1994). Furthermore, the metabolism of the microorganisms within the cheese results in an almost anaerobic environment within a few weeks of ripening favouring the survival of bifidobacteria and other anaerobic microorganisms (Van den Tempel *et al*., 2002).

Bifidobacterium bifidum was incorporated into Cheddar cheese as a starter adjunct (Dinakar and Mistry, 1994). The strain survived well in the cheese and retained a viability of approximately 2 x 10^7 cfu/g of cheese even after 6 months of ripening without adversely affecting the flavour, texture or appearance. This suggests that Cheddar could provide a suitable environment for the maintenance of probiotic organisms at high levels over long periods. Studies by Stanton *et al*. (1998) as well as Gardiner *et al*. (1998) showed that Cheddar cheese can be an effective vehicle for the delivery of some *Lactobacillus paracasei* strains to the consumer without any negative impact on cheese quality, including aroma, flavour and texture. McBrearty *et al*. (2001) also demonstrated Cheddar cheese to be a suitable food system for the delivery of some commercially available strains of probiotic bifidobacteria to the consumer. *L. paracasei* NFBC 338 Rif^r remained highly viable during the 3 month

ripening period of cheddar cheese, without adversely affecting cheese quality (Gardiner *et al*., 2002).

In many parts of the world, cheeses are frequently consumed at least once a day, making cheese an excellent carrier for probiotic organisms because of the high daily consumption (Boylston *et al*., 2003). If probiotic cheese can be manufactured with little or no alteration to the cheese making technology, it would make the development of probiotic cheese attractive for commercial exploitation. The successful incorporation of probiotic cultures into cheese will expand the probiotic product range with cheese industries benefiting from marketing advantage such as value-added probiotic containing cheeses.

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CHAPTER 2

THE ROLE OF *DEBARYOMYCES HANSENII* IN THE RIPENING OF MATURED CHEDDAR CHEESE

ABSTRACT

Debaryomyces hansenii predominated in most studies of yeasts associated with dairy products attributed to the species adaptation to the environmental conditions prevailing in dairy products. The potential of this species as an agent for accelerated ripening of matured Cheddar cheese was evaluated. The interaction between the yeast species and lactic acid bacteria as well as the physical and chemical properties of the cheese was determined. The yeast species remained viable throughout the ripening period and grew in association with the lactic acid bacteria without any inhibition of the starter culture. Despite the yeasts contribution to the development of a very good texture and body of the cheese, a major constraint, however, was the development of a slightly bitter taste. The inclusion of yeasts as adjunct starter cultures remains uncertain and more yeasts need to be evaluated.

INTRODUCTION

Reports on the occurrence of yeasts in cheeses date back to the early part of this century, but it is still not widely appreciated that yeasts can be an important component of many, if not all, cheese varieties (Fleet and Mian, 1987; Walker, 1988; Devoyod, 1990; Fleet, 1990). Yeasts are frequently found within the microflora of a wide variety of cheeses (Lenoir, 1984; Fleet and Mian, 1987; Devoyod, 1990; Fleet, 1990; Seiler and Busse, 1990; Viljoen and Greyling, 1995; Roostita and Fleet, 1996) and may cause spoilage of products (Walker and Ayres, 1970; Lenoir, 1984; Seiler and Busse, 1990; Westall and Filtenborg, 1998), but their presence is, however, also responsible for desirable biochemical changes (Fleet and Mian, 1987).

Yeasts contribute positively to the fermentation and maturation process of cheeses by inhibiting undesired microorganisms present (Kaminarides and Laskos, 1992), supporting the function of the starter culture (Kalle *et al*., 1976) and by metabolising lactic acid leading to an increase in pH (Fleet, 1990). Yeasts also have proteolytic and lipolytic activity (Coghill, 1979; Wyder and Puhan, 1999a,b), excrete growth factors (Jakobsen and Narvhus, 1996) and produce gas that leads to curd openness (Jakobsen and Narvhus, 1996).

The yeast species primarily found during maturation comprise of *Debaryomyces hansenii*, *Kluyveromyces marxianus, Yarrowia lipolytica* and species of *Candida* (Lenoir, 1984; Nooitgedagt and Hartog, 1988; Devoyod, 1990; Fleet, 1990; Roostita and Fleet, 1996). Laubscher and Viljoen (1999a) reported that yeast species isolated from matured Cheddar cheese, comprised of *Trichosporon beigelii*, *D. hansenii*, *Dekkera custersiana*, *Candida versatilis*, *Rhodotorula minuta*, *Rhodotorula mucilaginosa, Candida rugosa, Dekkera bruxellensis*, *Dekkera anomala*, *Torulaspora delbrueckii, Rhodotorula glutinis* and strains of *Debaryomyces vanrijae*. Predominant species were *T. beigelii* and *D. hansenii*. *D. hansenii,* the perfect state of *Candida famata*, predominated in most studies of yeasts associated with dairy products (Walker and Ayres, 1970; Seiler and Busse, 1990; Welthagen and Viljoen, 1998). Characteristic properties governing its growth and survival in dairy products, include its ability to grow at high salt concentrations (Mrak and Bonar, 1939), low a_w values

(Tilbury, 1980), low temperatures (Davenport, 1980) and the ability to produce extracellular proteases and lipases (Fleet and Mian, 1987).

The possibility of using *D. hansenii* and *Y. lipolytica* as starter cultures for cheese production were proposed, since both species stimulate ripening of cheese by means of lipolytic and proteolytic activities (Devoyod, 1990; Fleet, 1990), and control the pH by the utilisation of the lactic and acetic acid present (Devoyod, 1990; Fleet, 1990). The inclusion of *D. hansenii* as a starter culture was supported by Yamauchi *et al*. (1976) based on the species proteolytic activity encouraging the survival and growth of lactic acid bacteria. The inclusion of the species as part of the starter culture, has a dual role by also inhibiting the germination of undesired microorganisms, like *Clostridium butyricum* and *Clostridium tyrobutyricum* in cheese brines (Fatichenti *et al.,* 1983). Based on its inhibitory effect on the growth of spoilage microorganisms and the species proteolytic activity, Fatichenti *et al*. (1983) and Deiana *et al*. (1984) proposed the incorporation of *D. hansenii* as part of the starter culture for the making of Romano cheese. Welthagen and Viljoen (1998) and Laubscher and Viljoen (1999b) suggested further research on the possibility of including *D. hansenii* as part of cheese starter cultures, due to its great resistance against high salt concentrations, low temperatures and proliferating activities.

In this study, *D. hansenii* was included as part of the starter culture for the production of matured Cheddar cheese. The interaction between the yeast and the lactic acid bacteria as well as the physical and chemical properties of the cheese was determined.

MATERIALS AND METHODS

Starter cultures

Lactic acid bacteria starter strains (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* spp. *thermophilus*), RAO 21 (Rhodia Foods, France) were used as starter cultures for the production of matured Cheddar cheese. The LAF 3 starter strains of *Debaryomyces hansenii* were obtained from Chr. Hansen (Hørsholm, Denmark) and incorporated into the cheese as part of the starter culture. The RAO 21 lactic acid starter strains were also applied for the production of a traditional matured Cheddar cheese as control at the same conditions.

Matured Cheddar Cheese Manufacture

Pasteurised milk (400 l) was used to manufacture matured Cheddar cheese at a commercial cheese factory in the Free State in South Africa. The procedure for cheese making was carried out as described by Kosikowski (1970a).

Sampling description

Samples were taken at selected points during the processing of matured Cheddar cheese – from the cheese vat, during and after the renneting process, after cutting and during and after cheddaring. The cheese was kept under controlled conditions (8-12ºC) and sampled after 24 hrs, 48 hrs, 12 d, 30 d, followed by consecutive intervals of 30 d for 6 months. Similar samples were collected from the model cheese and the control cheese.

Sampling procedure

Duplicate cheese samples were prepared for microbial analysis by opening the cheeses aseptically and cutting portions with a sterile knife. For each sample, 10 g cheese were aseptically weighed and added to 90 ml of sterile peptone water into Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.). Further decimal dilutions were carried out as required for

microbial assays in 9 ml sterile peptone water and plated in duplicate by the spread plate technique onto selective media. DeMann, Rugosa and Sharpe (MRS) plates (Oxoid, Basingstoke, U.K.) which are selective for lactic acid bacteria were incubated under aerobic conditions for 48 hrs at 28ºC whereas Chloramphenicol-Agar plates (Oxoid, Basingstoke, U.K.), selective for yeasts, were incubated under aerobic conditions for 96 hrs at 28ºC.

Sample analysis

All plates containing between 25 and 150 colony forming units (cfu) on the highest dilution (or the highest number if below 25) were counted and mean values were determined from duplicate plates. Results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions (2x2x3).

Physical and chemical analysis

For cheese samples taken on each occasion, an additional 20 g of cheese were weighed into 20 ml of distilled water in Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.).

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Bouzas *et al*., 1991) whereas the organic acid contents were measured 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 24ºC with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1970b). The moisture content was determined throughout the ripening period according to the method described by Marshall (1992). The fat content of the cheese samples was determined based on the Schmid-Bondzynski-Ratzlaff method (IDF 5B, 1986). The salt content was determined as described by Marshall (1992).

RESULTS AND DISCUSSION

Microbial populations

After inoculation of the pasteurised milk, the lactic acid bacterial counts increased rapidly from 6.4 x 10^5 cfu/g at 0 h in the cheese vat to 1.5 x 10^9 cfu/g after 2 hrs (after renneting) (Figs. 1 and 2). The initial increase was followed by stable counts ranging between 10^7 – 10^8 cfu/g for the remaining period of processing and ripening. The lactic acid bacterial numbers of the model cheese, however, remained substantially higher compared to the traditional matured Cheddar cheese produced as a control under similar circumstances (Fig. 2). This remarkable survival of the lactic acid bacteria throughout the ripening period may be attributed to the enhanced number of yeasts, providing growth factors to the starter bacteria (Riviere, 1969). The numbers of *Debaryomyces hansenii* decreased from 7.5 x 10^4 cfu/g at the beginning of processing to 3.6 x 10^3 cfu/g after 4 hrs (during cutting) (Figs. 1 and 2). The initial decrease in the yeast counts continued until a cell population of 4.25 x 10^2 cfu/g was obtained after 6 months of ripening. The decrease in yeast counts may be attributed to competitive interaction between the lactic acid bacteria and the yeast, with the latter being overgrown. The yeast, however, remained viable throughout the ripening period. Their survival might be attributed to the utilisation of organic acids produced by the lactic acid bacteria, the utilisation of available sugars and their proteolytic and lipolytic activity (Fleet and Mian, 1987; Kreger-van Rij, 1984). Yeasts and lactic acid bacteria occur simultaneously in many natural food habitats sharing many common ecological determinants (Deàk and Beuchat, 1996). Generally yeasts utilise by-products of bacterial metabolism as energy sources, while bacteria depend on several growth factors supplied by yeasts (Riviere, 1969) like the liberation of vitamins through autolysis or excretion (Fleet, 1990). In addition, end products derived from reactions of these microorganisms in milk can be used as energy sources by others. On the other hand, accumulation of these end products of metabolism to high levels may inhibit the growth of undesired microorganisms (Kosikowski, 1970a).

Physical and chemical analysis

The initial pH of the raw milk was 6.53. It remained stable during renneting, but decreased to 6.04 at the end of the cutting process (Table 1; Fig. 3). The decrease in pH was due to starter cultures producing organic acids (Welthagen and Viljoen, 1999). A further decrease took place to a value of 5.21 at the end of cheddaring (just before press). The pH remained stable for the remaining period of maturation (Table 1; Fig. 3). The pH of the control cheese also decreased during the manufacturing process and reached a value of 5.48 at the end of cheddaring (Table 1). The pH decreased further to a value of 5.28 after 24 hours of ripening and remained at a higher value than those of the model cheese throughout the ripening period. This can be attributed to the stimulating action of the yeasts on the lactic acid bacteria, resulting in the production of an enhanced quantity of organic acids, especially towards the end of the maturation period.

No substantial changes were observed in the cheese moisture content during the study. The retention of the moisture values is attributed to the wrapping of the cheese in impermeable plastic film. Moisture values varied between 35.44% and 36.76% during the ripening period (Results not shown). Laubscher and Viljoen (1999b) reported values between 36.45% and 37.0%. Salt plays a significant role in the quality of Cheddar cheese by controlling the final pH of the cheese (Thomas and Pearce, 1981; Lawrence and Gilles, 1982), the growth of microorganisms and the overall texture and flavour of the cheese. The salt-in-moisture level controls the rate of proteolysis of the caseins by the different proteases. Proteolysis, and therefore the incidence of bitterness and other off-flavours, decreases with an increase in salt concentration (Thomas and Pearce, 1981). Insufficient salt was added during the manufacturing of the cheese and therefore results varied between 1.05% and 1.6% (Table 1) which were much lower compared with the values ranging from 1.7% to 2.38% reported by Laubscher and Viljoen (1999b). The error contributed to the development of a slightly bitter taste in the cheese. According to Laubscher and Viljoen (1999b), *D. hansenii* shows positive growth at salt concentrations as high as 5.0%. The fat content decreased slightly from 35.0% immediately after processing to 34.0% after 6 months of maturation (Results not shown). Laubscher and Viljoen (1999b) reported a slight increase in fat content during the first 12 days of ripening.

The lactose content initially present in the milk in the cheese vat before processing was 4.9% (Table 1; Fig. 4), being rapidly utilised by the starter cultures and depleted at the beginning of the cheddaring process. The lactose in the control cheese was only depleted after 10 days of ripening. Laubscher and Viljoen (1999a) reported a depletion in the lactose content after 2 days of ripening. Welthagen and Viljoen (1999) reported a decrease in the lactose content of Cheddar cheese to a value of 0.26% after 51 days of ripening. Since *D. hansenii* cannot utilise lactose, the main sugar in milk was metabolised by the lactic acid bacteria (Kreger-van Rij, 1984). The lactic acid bacteria, however, depend on several growth factors supplied by yeasts (Riviere, 1969) and this was the possible reason for the enhanced metabolisation of the lactose in the model cheese.

Lactose is a disaccharide that must be hydrolysed to glucose and galactose fractions before it can enter the catabolic pathway for hexoses (Schlegel, 1993). All the lactose present in the milk was hydrolysed to glucose and galactose. Glucose was immediately utilised by the microorganisms (Schlegel, 1993) leading to an accumulation of galactose (Table 1; Fig. 4). The galactose content of 0.36% during cutting increased to a value of 1.42% after 24 hrs of ripening. At this stage, the galactose content within the model cheese started to decrease, being depleted after 5 months, whereas the galactose content of the control cheese was only depleted after 6 months of ripening. This enhanced decrease of the galactose content in the model cheese might be attributed to the presence of *D. hansenii* (Kreger-van Rij, 1984).

The utilisation of lactose by the lactic acid bacteria resulted in a rapid increase in the lactic acid content from 0.36% during cutting to a value of 1.35% during cheddaring (Table 1; Fig 3). The monosaccharide end products based on the breakdown of lactose, glucose and galactose, were fermented and consequently lactic acid, ethanol and CO_2 were produced (Schlegel, 1993). The lactic acid content of the cheese remained constant for the first three months of the ripening period followed by an increase to a value of 2.01% after six months of ripening. In the control cheese, the lactic acid content was significantly lower, reaching a level of 1.52% after 6 months. These enhanced levels of lactic acid in the model cheese were due to increased lactic acid bacterial activity resulting from the utilisation of yeast excreted metabolites.

There was a decrease in the amount of citric acid in the model cheese. According to Kreger-van Rij (1984) some strains of *D. hansenii* have the ability to assimilate citric acid. The citric acid content of 0.22% at the beginning of processing decreased to a value of 0.05% after six months of ripening (Table 1; Fig. 3). A constant trend in the vicinity of 0.20% citric acid was observed in the control cheese.

CONCLUSION

Debaryomyces hansenii has the ability to grow and compete with other naturally occurring yeasts in the cheese. The species also grows in association with lactic acid bacteria without any inhibition of the starter culture. The yeast contributed to the aroma of the cheese, although a slightly bitter taste developed. This was partly due to the addition of insufficient salt. Continued hydrolysis of protein and fat could contribute to the bitter flavour. The cheese had a very good texture and body. Despite the ability of *D. hansenii* to grow and survive in matured Cheddar cheese and the contribution to flavour and texture, the development of bitter flavours is a major constraint. The inclusion of yeasts as starter cultures therefore remains uncertain. More yeasts need to be evaluated.

Table 1. Analytical data of matured Cheddar cheese during processing and ripening over a period of 6 months.

Sampling time	pH	pH (control)	Salt (%)	Organic acids		Organic acids (control)		Sugars		Sugars (control)	
				Lactic acid (%)	Citric acid (%)	Lactic acid (%)	Citric acid (%)	Lactose (%)	Galactose (%)	Lactose (%)	Galactose (%)
Cheese vat (start)	6.53	6.62	-	0.48	0.22	0.58	0.15	4.90	0.00	4.72	0.00
Cheese vat (end)	6.54	6.59	-	0.62	0.22	0.60	0.15	4.53	0.00	4.83	0.00
Rennting (start)	6.54	6.59	-	0.47	0.15	0.69	0.15	4.30	0.00	4.85	0.09
Renneting (end)	6.54	6.57	-	0.59	0.14	0.62	0.15	4.34	0.00	4.69	0.17
Cutting	6.04	6.53	-	0.36	0.19	1.60	0.21	4.09	0.36	1.03	0.57
Cheddaring	5.36	6.00	-	1.35	0.16	1.24	0.20	0.00	0.58	1.05	0.97
Cheddaring (end)	5.21	5.48	-	1.38	0.19	1.20	0.19	0.00	0.69	1.06	1.61
24 hours	5.20	5.28	1.30	1.20	0.24	1.11	0.16	0.00	1.42	0.91	1.13
48 hours	5.17	5.22	1.60	1.17	0.20	1.21	0.18	0.00	0.81	0.52	1.10
12 days	5.25	5.26	1.45	1.29	0.22	1.14	0.15	0.00	0.57	0.00	1.06
1 month	5.26	5.29	1.40	1.41	0.17	1.31	0.16	0.00	0.44	0.00	0.56
2 months	5.23	5.27	1.05	1.39	0.15	1.58	0.18	0.00	0.18	0.00	0.49
3 months	5.18	5.26	1.05	1.43	0.14	1.64	0.20	0.00	0.23	0.00	0.13
4 months	5.18	5.29	1.35	1.99	0.10	1.68	0.21	0.00	0.14	0.00	0.11
5 months	5.16	5.29	1.40	2.11	0.07	1.89	0.18	0.00	0.00	0.00	0.04
6 months	5.17	5.31	1.38	2.01	0.05	1.52	0.22	0.00	0.00	0.00	0.00

* Data are the means of 3 repetitions

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ADDENDUM

CHAPTER 3

THE ROLE OF *YARROWIA LIPOLYTICA* IN THE RIPENING OF MATURED CHEDDAR CHEESE

ABSTRACT

Yarrowia lipolytica is a yeast which occurs frequently in milk products and the species has the ability to predominate in real system over the naturally occurring yeasts. The yeast fulfils a number of criteria to be regarded as a co-starter for cheesemaking. The species is known for its outstanding proteolytic and lipolytic activity, its compatibility with starter cultures and stimulating effect when coinoculated with lactic acid bacteria. The potential of *Y. lipolytica* to contribute to the production and ripening of matured Cheddar cheese at a pilot plant level has been evaluated. The interaction between the yeast and the lactic acid bacteria, as well as the physical and chemical properties of the cheese, were determined. The yeast grew and competed with the lactic acid bacteria without any inhibition of the starter culture but died off after 5 months of maturation. The species contributed to the accelerated development of a strong Cheddar flavour, despite the development of a slightly fruity flavour. The body, flavour and texture of the cheese, however, granted it with a very good overall impression.

INTRODUCTION

High numbers of yeasts (>10^6 cfu/g) are frequently observed in cheeses and are believed to make a significant contribution to the process of maturation (Viljoen and Greyling. 1995; Welthagen and Viljoen, 1998; Welthagen and Viljoen, 1999). Their presence may be attributed to the yeasts ability to grow at low temperatures, the assimilation/fermentation of lactose, the assimilation of organic acids like succinic, lactic and citric acid, their proteolytic and lipolytic activities and resistance against high salt concentrations, cleaning compounds and sanitizers (Fleet, 1990; Fleet, 1992; Jakobsen and Narvhus, 1996; Laubscher and Viljoen, 1999a). Furthermore yeasts have the ability to tolerate low pH and moisture values (Wyder and Puhan, 1999a; 1999b).

The occurrence of yeasts in dairy products is significant. They can either cause spoilage or effect desirable biochemical changes (Seiler and Busse, 1990; Eliskases-Lechner, 1998). Yeasts are involved in the ripening process of cheese contributing to microbial interactions, texture changes and the biosynthesis of aromatic compounds like volatile acids and carbonyl compounds (Fleet and Mian, 1987; Roostita and Fleet, 1996; Rossi *et al*., 1998; Welthagen and Viljoen, 1999). Due to features such as high proteolytic and lipolytic activities, some yeast species play an important role in the formation of aroma precursors such as amino acids, fatty acids and esters (Lenoir, 1984). In addition, yeasts can inhibit undesired microorganisms (Kaminarides and Laskos, 1992) and excrete growth factors like B-vitamins, pantothenic acid, niacin, riboflavin and biotin (Purko *et al*, 1951; Lenoir, 1984; Fleet, 1990; Jakobsen and Narvhus, 1996). The main contribution of yeasts to the maturation process, however, is the utilisation of lactic acid, which as a result increases the pH, favouring bacterial growth and initiates the second stage of cheese ripening (Fleet, 1990; Rhom *et al*., 1992).

Yarrowia lipolytica occurs frequently in milk products and the species has the ability to predominate in real system on the naturally occurring yeasts (Guerzoni *et al*., 1998). Its compatibility with starter cultures and possible stimulating action when co-inoculated have been evidenced by Guerzoni *et al*. (1998) and Van den Tempel and Jakobsen (2000). The species is further known for its exceptional strong proteolytic

and lipolytic activity (Roostita and Fleet, 1996; Wyder and Puhan, 1999b; Guerzoni *et al*., 2001; Suzzi *et al*., 2001). In a study by Wyder and Puhan (1999a), *Y. lipolytica* was indicated as the yeast species with the strongest proteolytic activity whereas Choisy *et al*. (1987) considered it as the most predominant species contributing to lipolytic activity. Based on these features, it was possible to accelerate cheese ripening and to improve the quality of cheese by the addition of this yeast species (Lenoir *et al*., 1985; Devoyod, 1990).

Y. lipolytica is regarded as a good candidate as a ripening agent in cheese (Guerzoni *et al*., 1998), since it fulfils a number of specific criteria to be regarded as a co-starter in cheesemaking (Guerzoni *et al*., 2001; Suzzi *et al*., 2001). Despite the frequent references to interactions between yeasts and the lactic acid starter cultures in cheese, limited studies were performed during the actual cheese making process on industrial scale. Ripening studies at laboratory level with a model system, for example with cheese curd slurries, are usually not sufficient to reproduce the conditions in the cheese and can only be seen as a preliminary approach (Wyder *et al*., 1999). It is always necessary to perform cheese trials at a pilot plant level and therefore, in this study, we endeavoured to study the interaction between *Y. lipolytica* and the lactic acid bacteria by incorporating this yeast as part of the starter culture for the production of matured Cheddar cheese. The physical and chemical properties of the cheese were monitored in order to evaluate the contribution of the yeast to cheese production.

MATERIALS AND METHODS

Starter culture preparation

A *Yarrowia lipolytica* strain, previously isolated from Cheddar cheese and identified, was cultured in 400 ml YM-broth (Wickerham, 1951) under agitation at 30°C for 96 hrs. Cells were counted by means of a Haemacytometer and a total yeast count of 4 x 10^9 cells was prepared form the broth. The cells were collected by centrifugation of the broth for 5 min at 6000 rpm in sterile centrifuge tubes. The cells were resuspended in UHT milk, stored at 4-5°C and used within 24 hrs. In addition to the yeasts species, ten units (as proposed by the supplier) of the lactic acid bacteria starter culture (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* spp. *thermophilus*), RAO21 (Rhodia Foods, France) were used as starter culture for the production of matured Cheddar cheese. The RAO 21 lactic acid starter strains were also applied for the production of a traditional matured Cheddar cheese as control at the same time.

Matured Cheddar cheese manufacture

Pasteurised cows milk (400 l) was used for the manufacture of matured Cheddar cheese at a cheese plant in the Free State in South Africa on three occasions. The procedure for cheesemaking was carried out as described by Kosikowski (1970a).

Sampling description

Samples were taken at selected points during the processing of matured Cheddar cheese – from the cheese vat, during and after the renneting process, after cutting and during and after cheddaring. The cheese was kept under controlled conditions (8-12ºC) and sampled after 24 hrs, 48 hrs, 12 d, 30 d, followed by consecutive intervals of 30 d for 6 months. Similar samples were collected from the model cheese and the control cheese.

Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by opening the cheeses aseptically and cutting portions with a sterile knife. For each sample, 10 g cheese were aseptically weighed and added to 90 ml of sterile peptone water into Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.). Further decimal dilutions were carried out as required for microbial assays in 9 ml sterile peptone water and plated in duplicate by the spread plate technique onto selective media. DeMann, Rugosa and Sharpe (MRS) plates (Oxoid, Basingstoke, U.K.) which are selective for lactic acid bacteria were incubated under aerobic conditions for 48 hrs at 28ºC, whereas Chloramphenicol-Agar plates (Oxoid, Basingstoke, U.K.), selective for yeasts, were incubated under aerobic conditions for 96 hrs at 28ºC.

Sample analysis

All plates containing between 25 and 150 colony forming units (cfu) on the highest dilution (or the highest number if below 25) were counted and the mean values determined from duplicate plates. Results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions (2x2x3).

Physical and chemical analysis

During sampling, as described for the microbial samples, an additional 10 g of cheese were weighed into 10 ml of distilled water in Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.) for chemical analysis.

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Bouzas et al., 1991), whereas the organic acid contents were measured by means of a HPLC system equipped with a variable wavelength detector set at 220nm. 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 24ºC with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1970b).

Sensory analysis

The sensory quality of the model and control cheeses was judged by a panel of experts in the field of cheese evaluation, based on a standard protocol including openness, texture, taste and maturity at various intervals during the ripening period. The cheeses were evaluated after 2 months, 4 months, 6 months and 9 months and the results compared.

RESULTS AND DISCUSSION

Microbial populations

Yeasts in dairy products may interact with other microorganisms in three different ways: they may inhibit or eliminate undesired microorganisms causing quality defects or possessing potential pathogenic characteristics, they may inhibit the starter culture or contribute positively to the fermentation or maturation process by supporting the function of the starter culture (Jakobsen and Rossi, 1994). Studies on the interaction between yeasts and the starter cultures in Cheddar and Gouda cheeses by Welthagen and Viljoen (1998; 1999) and Laubscher and Viljoen (1999b) also indicated that yeasts play a significant role during the ripening of these cheeses by supporting the function of the starter cultures. The lactic acid bacterial counts in the matured Cheddar cheese during the manufacturing process reached a value of 7.50×10^7 cfu/g after the cheddaring process (Fig. 1). The numbers continued to increase during the ripening period reaching values of 8.99×10^8 cfu/g after 3 months of maturation. This was followed by a decrease in lactic acid bacterial numbers reaching a final value of 1.5×10^7 cfu/g after 6 months of maturation. In the traditional matured Cheddar cheese produced as a control, a more enhanced decrease in the lactic acid bacterial counts was observed than in the model cheese (Fig. 2). The improved survival of the lactic acid bacteria is remarkable and can be attributed to the supporting function of the yeasts, providing growth factors to the starter bacteria. *Yarrowia lipolytia* strains could only be found during the first four months of the ripening period. Due to their tolerance to low pH-values and high NaCl-concentrations, yeasts normally grow well

during the initial period of cheese ripening (Eliskases-Lechner and Ginzinger, 1995). Yeast numbers of 1.45 x 10^4 cfu/g was detected in the milk in the cheese vat whereafter the numbers of *Y. lipolytica* slowly decreased until the yeast died off after 5 months of maturation (Fig. 1). According to Bartchi *et al*. (1994) a decrease in yeast counts towards the end of the ripening period is generally observed in cheeses. Wyder and Puhan (1999b) were also only able to find *Y. lipolytica* during the first half of the ripening period of Limburger cheese. The decrease in yeast counts may be attributed to the depletion of the available sugars in the cheese as well as to competitive interaction between the lactic acid bacteria and the yeast, with the latter being overgrown. Although no yeasts could be detected in the final product after six months, they contributed to the cheese ripening process based on enzymes released after cell lysis (Wyder *et al*., 1999).

Physical and chemical analysis

The initial pH of the milk in the cheese vat was 6.64 followed by a decrease during processing to reach a value of 5.32 after the cheddaring process (Table 1; Fig. 3). The pH of the cheese continued to decrease during the ripening period and reached a final value of 5.05 after 6 months of maturation. This decrease in the pH-value is attributed to the growth of the lactic acid starter bacteria, producing lactic- and other organic acids. Yeasts, however, are generally known for their de-acidifying action in cheese by utilising lactic acid and the formation of alkaline metabolism products due to proteolysis (Eliskases-Lechner and Ginzinger, 1995; Wyder *et al*., 1999). Even though *Yarrowia lipolytica* is proteolytic and assimilate lactate (Kreger-van Rij, 1984; Barnett *et al*., 1990; Kurtzman and Fell, 1998), the model cheese remained at a lower pH than the control cheese during the ripening period (Table 1). This could be due to higher numbers of lactic acid bacteria in the model cheese (Fig. 2) producing organic acids and insufficient numbers of yeasts (Fig. 1) to assimilate these acids. In the control cheese, with no addition of yeasts, the pH declined to a value of 5.48 after the cheddaring process. The pH of the control cheese continued to decrease during the ripening period and reached a final value of 5.31 after 6 months of maturation.

The major sugar present in dairy products is lactose (Scott, 1981). Lactose is a disaccharide that must be hydrolysed to glucose and galactose before it can enter the

catabolic pathway for hexoses (Schlegel, 1993). All the lactose present in the milk was hydrolysed to glucose and galactose (Table 1; Fig. 4). Although yeasts are traditionally associated with fermentation, nearly half of the presently known yeast species lack the ability to ferment sugars (Van der Walt and Yarrow, 1984), and furthermore, lactose fermenting species are not typical representatives in the cheese environment (Eliskases-Lechner, 1998). *Y. lipolytica* lacks the ability to utilise or ferment lactose (Kreger-van Rij, 1984; Barnett *et al*., 1990; Kurtzman and Fell, 1998) and the depletion was therefore attributed to the lactic acid bacteria and other contaminating microorganisms. The lactose content initially present in the milk in the cheese vat was 3.48% (Table 1; Fig. 4) but was depleted during the cheddaring process, whereas the lactose content of the control cheese was only depleted after 10 days of ripening. Welthagen and Viljoen (1999) reported a decrease in the lactose content of Cheddar cheese to a value of 0.26% after 51 days of ripening, whereas Laubscher and Viljoen (1999b) reported depletion of the lactose content after 2 days of ripening. The stimulation of lactic acid bacteria due to the addition of *Y. lipolytica* as part of the starter culture therefore indirectly also stimulated lactose utilisation. No member of the lactic acid bacteria group can grow without growth factors such as vitamins (lactoflavin, thiamine, pantothenic acid, nicotinic acid, folic acid, biotin) and amino acids (Schlegel, 1993). These growth factors are obtained from the milk and partially supplied by the yeasts (Riviere, 1969; Lenoir, 1984) which as a result encouraged the enhanced growth of the starter cultures.

The lactose present in the milk was hydrolysed to glucose and galactose (Schlegel, 1993). *Y. lipolytica* has the ability to utilise glucose and galactose as carbohydrate sources (Kurtzman and Fell, 1998). The glucose, however, was rapidly utilised by the microorganisms and therefore only traces were detected. This resulted in the accumulation of galactose to a value of 0.85% after 24 hours of ripening (Table 1; Fig. 4), followed by utilisation and the consequent depletion during the third month of ripening. The galactose content of the control cheese was only depleted after 6 months of ripening. Galactose is phosphorylised by a specific galactokinase and isomerised to glucose-1-phosphate, before being utilised by the microorganisms (Schlegel, 1993). The accelerated depletion of the sugar in the model cheese might be attributed to the presence of *Y. lipolytica* (Kreger-van Rij, 1984; Barnett *et al*., 1990;

Kurtzman and Fell, 1998) and as a consequence the higher number of lactic acid bacteria.

The monosaccharide end products derived from the breakdown of lactose namely glucose and galactose, were fermented and consequently lactic acid was produced (Schlegel, 1993). The lactic acid content of 0.62% at the beginning of the manufacturing process increased to a value of 1.46% after the cheddaring process (Table 1; Fig. 3). The lactic acid content of the cheese continued to increase until a value of 2.09% was reached after six months of maturation. This was significantly higher than the value of 1.52% lactic acid obtained in the control cheese after 6 months of ripening. The higher lactic acid content in the model cheese was due to increased lactic acid bacterial activity. This corresponds with the results of Wyder *et al*. (1999) who attributed the increase in lactic acid concentration to the stimulation of lactic acid bacteria, rather than to a direct result of yeast metabolism. The growth and frequent occurrence of the majority of yeasts in dairy products partially rely on the assimilation of lactic acid, citric acid and other organic acids (Fleet, 1990; Deàk and Beuchat, 1996) and therefore the assimilation of lactic acid is a common feature for yeast isolates in fermented dairy products (Loretan *et al*., 1998). Yeasts thereby contribute to the neutralisation of cheese (Lenoir, 1984; Valdés-Stauber *et al*., 1997; Viljoen, 1998). The majority of yeasts in dairy products, including *Y. lipolytica*, assimilate citric acid (Barnett *et al*., 1990; Fleet, 1990). The citric acid content of 0.18%, however, remained constant during the ripening period (Table 1; Fig. 3). A similar constant trend at 0.20% in the contents of the citric acid was noticed in the control cheese. *Y. lipolytica* does not have the ability to produce or utilise acetic acid (Barnett *et al*., 1990) and therefore the occurrence of this organic acid (results not shown) must be attributed to the growth of the lactic acid starter bacteria.

Sensory analysis

The biochemical activities of *Yarrowia lipolytica* strains play an important role in the organoleptic features of cheeses due to the production of aromatic compounds and/or their precursors such as methyl ketones, alcohols, lactones and esters (Suzzi *et al*., 2001). Martin *et al*. (1999) stated that the synthesis of aroma compounds by bacteria might be enhanced when these microorganisms are associated with *Y. lipolytica.* The

yeast is recognised for its particularly good lipolytic activity (Guerzoni *et al*., 1998; Van den Tempel and Jakobsen, 2000; Guerzoni *et al*., 2001), releasing fatty acids which can be further transformed into desirable or undesirable volatile or non-volatile compounds with characteristic aroma (Guerzoni *et al*., 2001). The proteolytic activity of *Y. lipolytica* strains, converting casein to peptides of different molecular weight and free amino acids, has a direct influence on cheese flavour or serve as a precursor of flavour components (Parra *et al*., 1996; Suzzi *et al*., 2001). After 4 months of ripening, the matured Cheddar cheese with *Y. lipolytica* as a co-inoculum was characterised by the development of a stronger Cheddar flavour than the control cheese despite a slightly fruity flavour. During the final 2 months of ripening, the Cheddar flavour intensified while the slightly fruity aroma could still be detected. The off-flavour intensified after 9 months of ripening. The accelerated formation of the desired flavour can be attributed to the enhanced breakdown of proteins by *Y. lipolytica*. The development of a fruity flavour agrees with previous studies by Hansen *et al*. (1984) and Lee and Richard (1984) who reported the ability of yeasts to produce aroma compounds responsible for fruity flavours such as alcohols, aldehydes, esters and monoterpenes. The body, flavour and texture of the matured cheese, however, granted it with a very good overall impression.

CONCLUSION

Yarrowia lipolytica had the ability to remain viable in the cheese for 4 months, growing in association with the lactic acid bacteria without any inhibition of the starter culture. The yeast contributed to the accelerated formation of the desired Cheddar flavour. Although a slightly fruity flavour was detected, the body, flavour and texture of the matured cheese granted it with a very good overall impression. *Y. lipolytica* may be regarded as a co-starter for cheesemaking.

Table. 1. Analytical data of matured Cheddar cheese during ripening over a period of 6 months.

Sampling time	pH	pH (control)	Organic acids		Organic acids (control)		Sugars		Sugars (control)	
			Lactic acid (%)	Citric acid (%)	Lactic acid (%)	Citric acid (%)	Lactose (%)	Galactose (%)	Lactose (%)	Galactose (%)
Cheese vat (start)	6.64	6.62	0.62	0.18	0.58	0.15	3.48	0.01	4.72	0.00
Cheese vat (end)	6.58	6.59	0.75	0.21	0.60	0.15	4.14	0.02	4.83	0.00
Renneting (start)	6.52	6.59	0.71	0.17	0.69	0.15	3.10	0.02	4.85	0.09
Renneting (end)	6.51	6.57	0.54	0.25	0.62	0.15	4.18	0.02	4.69	0.17
Cutting	6.51	6.53	1.79	0.19	1.60	0.21	0.92	0.41	1.03	0.57
Cheddaring	5.92	6.00	1.68	0.19	1.24	0.20	0.26	0.29	1.05	0.97
Cheddaring (end)	5.32	5.48	1.46	0.25	1.20	0.19	0.00	0.54	1.06	1.61
24 hours	5.30	5.28	1.46	0.24	1.11	0.16	0.00	0.85	0.91	1.13
48 hours	5.26	5.22	1.34	0.21	1.21	0.18	0.00	0.67	0.52	1.10
12 days	5.19	5.26	1.34	0.18	1.14	0.15	0.00	0.31	0.00	1.06
1 month	5.16	5.29	1.73	0.18	1.31	0.16	0.00	0.30	0.00	0.56
2 months	5.05	5.27	1.99	0.17	1.58	0.18	0.00	0.07	0.00	0.49
3 months	5.07	5.26	2.11	0.16	1.64	0.20	0.00	0.00	0.00	0.13
4 months	5.05	5.29	1.84	0.18	1.68	0.21	0.00	0.00	0.00	0.11
5 months	5.06	5.29	2.07	0.22	1.89	0.18	0.00	0.00	0.00	0.04
6 months	5.05	5.31	2.09	0.21	1.52	0.22	0.00	0.00	0.00	0.00

* Data are the means of 3 repetitions

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ADDENDUM

CHAPTER 4

CO-INOCULATION OF *DEBARYOMYCES HANSENII* AND *YARROWIA LIPOLYTICA* AS POTENTIAL STARTER CULTURES IN THE MAKING OF MATURED CHEDDAR CHEESE

ABSTRACT

A worldwide market increase in the demand for, and consequent production of cheese and cheese products evolved during the last century and therefore numerous studies have been conducted to accelerate cheese ripening. Recent studies indicated that yeasts could be included as part of starter cultures for the manufacturing of cheese, enhancing flavour development. *Debaryomyces hansenii* and *Yarrowia lipolytica* possess some of the essential attributes for use as cheese starter cultures. The potential of these species as agents for accelerated ripening of matured Cheddar cheese, has been evaluated. The microbial profiles of the two yeasts and the lactic acid bacteria, as well as the physical and chemical properties of the cheese were determined. The yeasts had the ability to survive and proliferate in the presence of each other, other natural occurring yeasts in the cheese, and with the lactic acid bacteria without inhibition of the bacterial starter culture. After a reduced period of ripening, the cheese reflected a good strong flavour, texture and body. The co-inoculation of *D. hansenii* and *Y. lipolytica* as adjunct starter cultures for the production of matured Cheddar cheese is evaluated.

INTRODUCTION

Numerous studies have been conducted on the acceleration of cheese ripening, urged by the economic advantages of a rapid development of stronger cheese flavour in a shorter time (Law, 1984). Almost all of these attempts fall into one of four categories which include the use of elevated temperatures, the addition of enzymes for speeding up flavour-producing reactions, the use of modified starter cultures and liquid slurry methods (Law, 1984). Lactic acid bacteria as starter cultures contain the desired enzymes and do not produce flavours that alter the specific attributes of the various cheeses (Guerzoni *et al*., 1998). Yeasts, however, were always considered as contaminants in dairy products causing spoilage during the fermentation process and were recognised as a potential problem in cheese (Fleet, 1990; 1992). Recent studies, however, indicated that yeasts could be applied as starters for the manufacturing of cheeses, adding to the flavour, instead of trying to reproduce the flavour profiles of traditional cheeses (Guerzoni *et al*., 1998). Consequently, the inclusion of yeasts as part of the starter culture may accelerate the ripening of cheese and result in new cheese flavours due to the interaction between the yeasts and the lactic acid bacteria with the starter cultures being supported by the yeasts (Viljoen, 2001).

The possibility of using *Debaryomyces hansenii* and *Yarrowia lipolytica* as adjunct starter cultures for cheese production were proposed, due to their positive attributes to cheese ripening (Devoyod, 1990; Fleet, 1990; Suzzi *et al*., 2001). *D. hansenii*, the perfect form of *Candida famata*, predominated in most studies of yeasts associated with dairy products (Walker and Ayres, 1970; Seiler and Busse, 1990; Eliskases-Lechner, 1998; Welthagen and Viljoen, 1998; Wyder and Puhan, 1999a). The reasons for the high numbers of *D. hansenii* in cheeses, are due to the species ability to grow at low temperatures (Davenport, 1980), high salt concentrations (Mrak and Bonar, 1939), low a_w values (Tilbury, 1980) and their lipolytic and proteolytic activity (Fleet and Mian, 1987; Wyder and Puhan, 1999a). *D. hansenii* also inhibits the germination of undesired microorganisms like *Clostridium butyricum* and *C. tyrobutyricum* in cheese brines (Fatichenti *et al*., 1983). Furthermore, a synergistic effect between lactic acid bacteria and *D. hansenii* with a resulting prolonged survival of the lactic acid bacteria has been reported by Yamauchi *et al*. (1975).

Y. lipolytica also possesses some of the essential attributes for use as a cheese starter culture, like the ability to grow and compete with other naturally occurring yeasts, compatibility with lactic acid bacteria and possible stimulating action when co-inoculated and a remarkable proteolytic activity (Guerzoni *et al*., 1998; Suzzi *et al*., 2001). The species is known for its strong proteolytic and lipolytic activity (Roostita and Fleet, 1996; Wyder and Puhan, 1999b) and plays a significant role during the ripening of cheeses by metabolising the lactic acid present, which as a result raises the pH (Yamauchi *et al*., 1976; Wyder and Puhan, 1999a). The yeast is considered as the most predominant species contributing to lipolytic activity (Choisy *et al*., 1987). In a study by Wyder and Puhan (1999a) the species was indicated as an exceptional inducer of proteolytic activity. It was possible to accelerate cheese ripening and to improve the quality of the product by the addition of this yeast species (Lenoir *et al*., 1985; Devoyod, 1990).

The technological properties of yeasts, such as aroma formation, lipolytic and proteolytic activities, osmotolerance, possible microbial interactions and inhibitory activity against spoilage organisms, all make them potentially viable organisms for use as starter cultures in the dairy industry (Loretan *et al*., 1998; Viljoen, 2001). According to Wyder *et al*. (1999) and Welthagen and Viljoen (1998, 1999) the combination of *D. hansenii* and *Y. lipolytica* might have a positive effect on flavour development in cheese. Consequently in this study, we endeavoured to apply both species simultaneously as adjunct starter cultures for the production of matured Cheddar cheese on industrial scale. The growth profiles of the two yeasts and the lactic acid bacteria were monitored as well as the physical and chemical properties of the cheese in order to evaluate the contribution of the yeasts to cheese production.

MATERIALS AND METHODS

Starter culture preparation

The LAF 3 starter strains of *Debaryomyces hansenii* were obtained from Chr. Hansen (Hørsholm, Denmark). Two units (each comprising of 2 x 10^9 cells) of this species were incorporated as part of the starter culture for the production of matured Cheddar cheese as indicated by the manufacturer. A *Yarrowia lipolytica* strain, previously isolated from Cheddar cheese and identified (Welthagen and Viljoen, 1999) was cultured in 400 ml YM-broth (Wickerham, 1951) under agitation at 30ºC for 96 hrs. Cells were counted by means of a Haemacytometer and a total yeast count of 4 x 10^9 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 resuspended in UHT milk, stored at 4-5ºC, and used within 24 hrs. In addition to the two yeast species, ten units (as proposed by the supplier) of the lactic acid bacteria starter strains (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* spp. *thermophilus*), RAO 21 (Rhodia Foods, France) were used as starter culture for the production of matured Cheddar cheese and simultaneously added with the yeasts. The RAO 21 lactic acid starter strains were also applied for the production of a traditional matured Cheddar cheese as control.

Matured Cheddar cheese manufacture

Pasteurised cows milk (400 l) was used for the manufacture of matured Cheddar cheese at a cheese plant in the Free State in South Africa on three occasions. The procedure for cheese making was carried out as described by Kosikowski (1970a).

Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by opening the cheeses, representatives of each batch, aseptically and cutting portions with a sterile knife. For each sample, 10 g cheese were aseptically weighed and added to 90 ml of sterile peptone water into Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.). Further

decimal dilutions were carried out as required for microbial assays in 9 ml sterile peptone water and plated in duplicate by the spread plate technique onto selective media. DeMann, Rugosa and Sharpe (MRS) plates (Oxoid, Basingstoke, UK) which are selective for lactic acid bacteria were incubated under aerobic conditions for 48 hrs at 28ºC, whereas Chloramphenicol-Agar plates (Oxoid, Basingstoke, UK), selective for yeasts, were incubated under aerobic conditions for 96 hrs at 28ºC.

Sample analysis

All plates containing between 25 and 150 colony forming units (cfu) on the highest dilution (or the highest number if below 25) were counted and the mean values determined from duplicate plates. The number of *Debaryomyces hansenii* and *Yarrowia lipolytica* strains were individually counted based on visually distinguished appearances. Results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions (2x2x3).

Physical and chemical analysis

During sampling, as described for the microbial samples, an additional 10 g of cheese were weighed into 10 ml of distilled water in Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.) for chemical analysis.

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Bouzas *et al*., 1991), whereas the organic acid contents were measured by means of a HPLC system equipped with a variable wavelength detector set at 220nm. 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 24ºC with a HI 9321 Microprocessor pH meter (HANNA Instruments, Germany) according to the method described by Kosikowski (1970b). The moisture content was determined throughout the ripening period according to the method described by Marshall (1993). The fat content of the cheese samples was

determined based on the Schmid-Bondzynski-Ratzlaff method (IDF 5B, 1986) and the salt determined as described by Marshall (1993).

Sensory analysis

The sensory quality of the model and control cheeses was judged by a panel of experts in the field of cheese evaluation, based on a standard protocol including openness, texture, taste and maturity at various intervals during the ripening period. Both cheeses were evaluated after 2 months, 4 months, 6 months and 9 months and the results compared.

RESULTS AND DISCUSSION

Microbial populations

The lactic acid bacterial counts of 1.97 x 10^8 cfu/g after 40 min in the cheese vat, remained constant during the processing of the cheese and increased to a value of 7.45 x 10^8 cfu/g after the first 24 hrs of ripening (Fig. 1). The number of lactic acid bacteria continued increasing, until a value of 7.6 x 10^8 cfu/g was reached after 4 months of maturation. This was followed by a gradual decrease to a value of 6 x 10^7 cfu/g after 6 months of maturation. The survival and actual increase of the lactic acid bacterial counts throughout the ripening period are remarkable and can be attributed to the supporting role of yeasts, providing growth factors to the starter bacteria. In the traditional matured Cheddar cheese produced as a control, a definite decrease in the lactic acid bacterial counts was observed during ripening compared to the cheese inoculated with yeasts (Fig. 1). The total yeast counts decreased during the manufacturing process, from 5.4 x 10^4 cfu/g after 40 min in the cheese vat to 3.6 x 10^3 cfu/g after cheddaring (Fig. 1). This was followed by an increase to a value of 9.7 x 10^6 cfu/g after 48 hrs of maturation. The total yeast counts thereafter gradually decreased for the first 2 months of maturation followed by a rapid decrease until a value of 2.0 x 10^2 cfu/g was reached after 6 months of maturation. This decrease of viable yeast cells correlated with the depletion of the carbohydrates present in the cheese (Table 1). Due to their tolerance of low pH-values and high NaCl-concentrations, the yeasts grew particularly well during the initial period of ripening,

which corresponds with the results of Eliskases-Lechner and Ginzinger (1995). The initial high yeast numbers contributed to the ripening process by the utilisation of lactic acid produced by the lactic acid bacteria (Lenoir, 1984; Wyder *et al*., 1999) which consequently led to an increase in pH that usually encourages bacterial growth and initiates the second stage of maturation (Siewert, 1986; Jakobsen and Narvhus, 1996). In addition, the production of growth factors such as vitamins (Purko *et al*., 1951) and amino acids (Wyder *et al*., 1999) by the yeasts, promoted the growth of a bacterial microflora which remained viable at high numbers throughout the ripening period.

Debaryomyces hansenii was the predominant yeast species throughout the ripening period of the cheese. The counts decreased from 2.04 x 10^4 cfu/g after 40 min in the cheese vat to 3.25 x 10^3 cfu/g after the cheddaring process (Fig. 2). The initial decrease may be attributed to the addition of salt and competitive interaction with the lactic acid bacteria for nutrients and especially the lack of other carbohydrates before the breakdown of lactose occurred. The decrease was followed by an increase to 1.18 x 10^7 cfu/g after 24 hrs of ripening, whereafter the counts decreased gradually until a value of 2.0 x 10^2 cfu/g was reached after 6 months of maturation. The viability of the species throughout the ripening period after depletion of the carbohydrates might be attributed to the utilisation of available organic acids derived from the breakdown of lactose and produced by the lactic acid bacteria. The *Yarrowia lipolytica* counts increased and decreased proportionally to those of *D. hansenii*, but in lower numbers. The counts decreased from 3.36 x 10^4 cfu/g after 40 min in the cheese vat to a value of 3.5 x 10^2 cfu/g after cheddaring (Fig. 2). The decrease was followed by an increase to a value of 1.35 x 10^4 cfu/g after 48 hrs of ripening after which the counts decreased gradually until the yeast died off after 3 months of maturation. In a study by Wyder and Puhan (1999b), who monitored Limburger cheese for 35 d, *D. hansenii* was also the dominating species during the ripening period while *Y. lipolytica* could be found solely in the first half of the ripening period. According to Bartschi et al. (1994), a decrease in yeast counts towards the end of the ripening period are generally observed in cheeses. The reason for *Y. lipolytica* being overgrown by *D. hansenii* may be due to the latter yeast's remarkable ability to grow at low temperatures (Davenport, 1980), high salt concentrations (Mrak and Bonar, 1939), and low a_w values (Tilbury, 1980),

which are the conditions prevailing during the manufacturing of matured Cheddar cheese.

Physical and chemical analysis

During the processing of matured Cheddar cheese inoculated with yeasts, the pH declined from 6.69 in the raw milk to a value of 5.30 at the end of cheddaring (Table 1; Fig. 3). This decrease in the pH value is attributed to lactic acid production by the mesophilic lactococci applied as starter cultures. The decrease in pH is also aggravated by the enzymatic release of free fatty acids from tryglicerydes by the yeasts (Guerzoni *et al*., 2001). After this rapid decline, the pH remained constant for the first two months of ripening followed by a decrease to a value of 5.24 after 4 months (Table 1; Fig. 3). The pH decreased further to a value of 5.22 after 6 months of ripening.

In the control cheese, where no yeasts were added as part of the starter culture for matured Cheddar cheese production, the pH declined to a value of 5.48 at the end of cheddaring (Table 1). This was followed by a decrease to a value of 5.22 after 48 hours of ripening and an increase to a value of 5.31 after 6 months of ripening. During the last 4 months of the ripening period, the model cheese remained at a lower pH than the control cheese. This was due to higher numbers of lactic acid bacteria in the model cheese (Fig. 1) producing lactic- and citric acid. The yeasts, however, still contributed to the neutralisation of the cheese (Lenoir, 1984; Valdés-Stauber *et al*., 1997; Viljoen, 1998). Yeasts are generally known for their de-acidifying action in cheese by utilising lactic acid with a subsequent increase in pH (Eliskases-Lechner and Ginzinger, 1995; Wyder *et al*., 1999). Changes in pH are, however, not necessarily the result of lactic acid degradation only, but also of alkaline metabolism products due to proteolysis (Eliskases-Lechner and Ginzinger, 1995; Wyder *et al*., 1999). *Debaryomyces hansenii* and *Yarrowia lipolytica* exhibit typical proteolytic activity (Choisy *et al*., 1987; Suzzi *et al*., 2001) and therefore may increase the pH (Wyder and Puhan, 1999a). Starter bacteria die off due to lower pH values (McSweeney *et al*., 1994), but the neutralisation of the cheese encourages the growth of bacteria essential for cheese ripening (Fleet, 1990). According to Guerzoni *et al*. (2001) the use of *Y. lipolytica* as co-inoculum stimulated both the lactococci and

lactobacilli starter cultures. This corresponds with the results obtained in our study where the lactic acid bacteria remained viable throughout the ripening period at higher numbers when compared with the control cheese. A synergistic effect therefore may exist between *Y. lipolytica*, *D. hansenii* and the lactic acid bacteria (Viljoen, 2001, Wyder and Puhan 1999a).

The moisture content of the cheeses remained constant during the ripening period with values varying between 36.04% and 39.09% (Results not shown). The retaining of the moisture values is attributed to the wrapping of the cheeses in impermeable plastic film imperative for good texture. The salting of cheese improves its flavour, texture and appearance, suppresses the growth of spoilage organisms and reduces the moisture, therefore serving to control the undesired microorganisms in the final cheese (Kosikowski, 1970b). The salt content varied between 3.67% and 5.50% (Table 1) during the ripening period. Proteolysis, and therefore the incidence of bitterness and off-flavours, decreases with an increase in salt concentration (Thomas and Pearce, 1981). Fat plays an important role as a flavour precursor in Cheddar cheese (Lawrence *et al*., 1993). The fat content for both cheeses varied between 31.50% and 35.0% during the ripening period (Results not shown). *Y. lipolytica* is recognised as the species having the highest lipolytic activity (Choisy *et al*., 1987; Suzzi *et al*., 2001). Free fatty acids are released from fat and can be further transformed in desirable or undesirable volatile or non-volatile compounds characterising the aroma (Guerzoni *et al*., 2001). The moisture, salt and fat contents obtained within the yeasts inoculated cheese were similar to the control cheese (Results not shown).

Lactose is the major sugar present in dairy products (Scott, 1981), being a disaccharide that must be hydrolysed to glucose and galactose before entering the catabolic pathway for hexoses (Schlegel, 1993). All the lactose present in the milk was hydrolysed to glucose and galactose (Table 1; Fig. 4). Neither *D. hansenii*, nor *Y. lipolytica*, has the ability to ferment or utilise lactose (Kreger-van Rij, 1984; Barnett *et al*., 1990; Kurtzman and Fell, 1998) and the depletion was therefore attributed to the lactic acid bacteria and other contaminating microorganisms. The lactose content initially present in the milk in the cheese vat was 3.42% (Table 1), being depleted during the cheddaring process, whereas the lactose in the control cheese was only

depleted after 10 days. Welthagen and Viljoen (1999) reported a decrease in the lactose content of Cheddar cheese to a value of 0.26% after 51 days of ripening, whereas Laubscher and Viljoen (1999) reported a depletion in the lactose content after 2 days of ripening. The addition of yeasts as part of the starter culture therefore might have increased the utilisation of lactose, mainly due to enhanced lactic acid bacterial growth. The lactic acid bacteria depend on several growth factors supplied by the yeasts (Riviere, 1969), which was the possible reason for the enhanced utilisation of the lactose.

The breakdown of lactose resulted in the formation of galactose and trace amounts of glucose (Schlegel, 1993). The latter was rapidly utilised, whereas the galactose content initially accumulated (Table 1; Fig. 4). Both yeast species added as starters have the ability to utilise glucose and galactose as carbohydrate source (Kurtzman and Fell, 1998), whereas only *D. hansenii* has variable capabilities of fermenting the carbohydrates. In a study by Eliskases-Lechner (1998) 45% of the screened isolates of *D. hansenii* fermented glucose. The galactose content of 0.68% during cutting (Table 1; Fig. 4) increased to a value of 0.86% after cheddaring. At this stage, the utilisation of galactose was initiated, being depleted after 12 days of ripening. In contrast, the galactose content of the control cheese was only depleted after 6 months of ripening. The enhanced utilisation of galactose might be attributed to the presence of both *D. hansenii* and *Y. lipolytica* (Kreger-van Rij, 1984; Barnett *et al*., 1990, Kurtzman and Fell, 1998), other contaminating microorganisms and the lactic acid bacteria.

The utilisation of lactose by the lactic acid bacteria also resulted in an increase in the lactic acid content from 0.33% after renneting to a value of 1.27% after the cheddaring process (Table 1; Fig. 3). The monosaccharide end products derived from the breakdown of lactose, namely glucose and galactose, were fermented and consequently lactic acid was produced. According to Schlegel (1993) the breakdown products of lactose, glucose and galactose, are fermented to lactic acid, ethanol and CO_2. The lactic acid content of the cheese remained relatively constant for the first period of ripening (Table 1; Fig. 3), followed by a gradual increase, until a value of 1.93% was reached after 6 months of maturation (Table 1; Fig. 3). The lactic acid content in the control cheese was lower and reached a value of only 1.52% after 6

months. The enhanced lactic acid content in the yeast-inoculated cheese was due to increased lactic acid bacterial activity. This corresponds with the work of Wyder *et al*. (1999) who attributed the increased lactic acid concentration to the stimulation of lactic acid bacteria, rather than to a direct result of yeast metabolism. The yeasts, however, assimilated the lactic acid and thereby contributed to the neutralisation of the cheese (Lenoir, 1984; Valdés-Stauber *et al*., 1997; Viljoen, 1998). The growth and the occurrence of the majority of yeasts in dairy products also rely on the assimilation of citric acid (Fleet, 1990). According to Kurtzman and Fell (1998) some strains of *D. hansenii* and *Y. lipolytica* have the ability to assimilate citric acid. The citric acid content of 0.11% after renneting, increased to a value of 0.21% after 12 days of ripening (Table 1; Fig. 3). This was followed by a decrease to a value of 0.05% after 6 months of maturation. A constant trend in the vicinity of 0.20% citric acid was observed in the control cheese.

Sensory analysis

With the addition of the two yeast species as adjunct starter cultures, the cheese developed a good, slightly sweet Cheddar taste after 2 months of ripening. After 4 months, a stronger Cheddar taste development was detected compared to the control cheese. At the end of the ripening period, the development of a mature taste was more enhanced in the model cheese than in the control cheese. The model cheese had a clean, slightly sweet, pleasant taste and still retained its good, strong flavour after 9 months, while the control cheese developed a bitter and slightly impure taste. The reason for this prolonged expiry date of the model cheese may be due to continued interaction between the yeasts and the lactic acid bacterial starter cultures, with the yeasts maintaining the balance between the different starter strains. This resulted in the starter strains growing at a constant rate with no overproduction of any of the flavour compounds and consequently no off-flavours.

CONCLUSION

Debaryomyces hansenii and *Yarrowia lipolytica* had the ability to remain viable in the cheese for a prolonged period of time, growing in association with the other natural occurring contaminants in the cheese and with the lactic acid bacteria without inhibition of the starter culture. The cheese already had a good strong flavour after 4 months of ripening, with a very good texture and body as judged by a taste panel. It is proposed that the combination of *D. hansenii* and *Y. lipolytica* can be applied as adjunct starter cultures for the production of matured Cheddar cheese with resulting accelerated ripening.

Table 1. Analytical data of matured Cheddar cheese during processing and ripening over a period of 6 months.

Sampling time	pH	pH (control)	Salt (%)	Organic acids		Organic acids (control)		Sugars		Sugars (control)	
				Lactic acid (%)	Citric acid (%)	Lactic acid (%)	Citric acid (%)	Lactose (%)	Galactose (%)	Lactose (%)	Galactose (%)
Cheese vat (start)	6.69	6.62	-	0.00	0.00	0.58	0.15	3.42	0.00	4.72	0.00
Cheese vat (end)	6.62	6.59	-	0.00	0.00	0.60	0.15	3.96	0.00	4.83	0.00
Renneting (start)	6.62	6.59	-	0.00	0.00	0.69	0.15	3.54	0.00	4.85	0.09
Renneting (end)	6.57	6.57	-	0.33	0.11	0.62	0.15	3.80	0.00	4.69	0.17
Cutting	6.20	6.53	-	1.10	0.26	1.60	0.21	3.80	0.68	1.03	0.57
Cheddaring	5.50	6.00	-	0.98	0.16	1.24	0.20	0.00	0.62	1.05	0.97
Cheddaring (end)	5.30	5.48	-	1.27	0.19	1.20	0.19	0.00	0.86	1.06	1.61
24 hours	5.33	5.28	3.67	1.43	0.25	1.11	0.16	0.00	0.51	0.91	1.13
48 hours	5.29	5.22	3.88	1.34	0.22	1.21	0.18	0.00	0.51	0.52	1.10
12 days	5.30	5.26	3.94	1.41	0.21	1.14	0.15	0.00	0.22	0.00	1.06
1 month	5.31	5.29	3.99	1.17	0.06	1.31	0.16	0.00	0.00	0.00	0.56
2 months	5.29	5.27	3.86	1.69	0.07	1.58	0.18	0.00	0.00	0.00	0.49
3 months	5.25	5.26	4.00	1.88	0.05	1.64	0.20	0.00	0.00	0.00	0.13
4 months	5.24	5.29	4.31	1.71	0.05	1.68	0.21	0.00	0.00	0.00	0.11
5 months	5.23	5.29	5.50	1.91	0.06	1.89	0.18	0.00	0.00	0.00	0.04
6 months	5.22	5.31	5.26	1.93	0.05	1.52	0.22	0.00	0.00	0.00	0.00

- Data are the means of 3 repetitions

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ADDENDUM

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CHAPTER 5

YEASTS AS ADJUNCT STARTERS IN MATURED CHEDDAR CHEESE

ABSTRACT

Debaryomyces hansenii and *Yarrowia lipolytica* are typical foodborne yeast species frequently associated with dairy products and capable of predominating the yeast composition in such systems. The two species fulfil a number of criteria to be regarded as co-starters for cheesemaking. They are known for their proteolytic and lipolytic activity as well as their compatibility and stimulating action with the lactic acid starter cultures when co-inoculated. Recent studies indicated that yeasts could be included as part of starter cultures for the manufacturing of cheese, enhancing flavour development during the maturation. The potential of *D. hansenii* and *Y. lipolytica* as agents for accelerated ripening of matured Cheddar cheese has been evaluated during four cheese treatments. The interaction between the two yeast species and the lactic acid bacteria was surveyed incorporating (i) *D. hansenii*, (ii) *Y. lipolytica* and (iii) both species as adjuncts to the starter culture, (iv) and a control cheese without any additions for the production of matured Cheddar cheese. The physical and chemical properties of the cheeses were monitored in order to evaluate the contribution of the yeasts to cheese maturation. The yeasts grew in association with the lactic acid bacteria without any inhibition. The yeasts species when individually added contributed to the development of bitter flavours despite accelerated development of strong Cheddar flavours. When both species were incorporated as part of the starter culture, the cheese however had a good strong flavour after a reduced ripening period. The cheese retained this good flavour and aroma 9 months after production. The simultaneous application of *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese is proposed.

INTRODUCTION

Reports on the occurrence of yeasts in cheeses date back to the early part of this century, but it is still not widely appreciated that yeasts can be an important component of many, if not all, cheese varieties (Fleet and Mian, 1987; Walker, 1988; Devoyod, 1990; Fleet, 1990). High numbers of yeasts are frequently observed in cheeses and are believed to make a significant contribution to the maturation process (Viljoen and Greyling, 1995; Welthagen and Viljoen, 1998; 1999). Their occurrence may be attributed to the yeasts ability to grow at low temperatures, the assimilation/fermentation of lactose, the assimilation of organic acids like succinic, lactic and citric acid, their proteolytic and lipolytic activities, resistance against high salt concentrations and resistance to cleaning compounds and sanitizers (Fleet, 1990; Fleet, 1992; Jakobsen and Narvhus, 1996; Laubscher and Viljoen, 1999a). Furthermore yeasts have the ability to tolerate low pH and water activity values (Wyder and Puhan, 1999a, b).

Yeasts frequently occur in dairy products. They can either cause spoilage or effect desirable biochemical changes (Seiler and Busse, 1990; Eliskases-Lechner, 1998). Yeasts are involved in the ripening process of cheese and partake in microbial interactions, and contribute to texture changes and the biosynthesis of aromatic compounds like volatile acids and carbonyl compounds (Fleet and Mian, 1987; Roostita and Fleet, 1996; Rossi *et al*., 1998; Welthagen and Viljoen, 1999). Due to features such as high proteolytic and lipolytic activities, some yeast species play an important role in the formation of aroma precursors such as amino acids, fatty acids and esters (Lenoir, 1984). They can inhibit undesired microorganisms (Kaminarides and Laskos, 1992) and excrete growth factors like B-vitamins, pantothenic acid, niacin, riboflavin and biotin (Purko *et al.*, 1951; Lenoir, 1984; Fleet, 1990; Jakobsen and Narvhus, 1996). The main contribution of yeasts to the cheese maturation process is the utilisation of lactic acid which in turn increases the pH and therefore favouring bacterial growth and initiates the second stage of cheese ripening (Fleet, 1990; Rhom *et al*., 1992).

The possibility of using *Debaryomyces hansenii* and *Yarrowia lipolytica* as starter cultures for cheese production was proposed due to their positive attributes to cheese

ripening (Devoyod, 1990; Fleet, 1990; Suzzi *et al*., 2001). *D. hansenii*, the perfect form of *Candida famata*, predominated in most studies of yeasts associated with dairy products (Walker and Ayres, 1970; Seiler and Busse, 1990; Eliskases-Lechner, 1998; Welthagen and Viljoen, 1998; Wyder and Puhan, 1999a). The reasons for the high numbers of *D. hansenii* in cheeses are due to the species' ability to grow at low temperatures (Davenport, 1980), high salt concentrations (Mrak and Bonar, 1939), low a_w values (Tilbury, 1980) and also to their lipolytic and proteolytic activity (Fleet and Mian, 1987; Wyder and Puhan, 1999a). *D. hansenii* also inhibits the germination of undesired microorganisms, like *Clostridium butyricum* and *C. tyrobutyricum* in cheese brines (Fatichenti *et al*., 1983). Furthermore, a synergistic effect between lactic acid bacteria and *D. hansenii*, with a resulting prolonged survival in the cheese of the lactic acid bacteria, has been reported by Yamauchi *et al*. (1975). Welthagen and Viljoen (1998) and Laubscher and Viljoen (1999b) suggested further research on the possibility of including *D. hansenii* as part of cheese starter cultures due to its great resistance against high salt concentrations, low temperatures and ability to multiply in cheese systems.

Y. lipolytica occurs frequently in milk products and the species has the ability to predominate over the naturally occurring yeasts (Guerzoni *et al*., 1998). Its compatibility with starter cultures and possible stimulating action when co-inoculated have been indicated by Guerzoni *et al*. (1998) and Van den Tempel and Jakobsen (2000). The species is known for its strong proteolytic and lipolytic activity (Roostita and Fleet, 1996; Wyder and Puhan, 1999b; Guerzoni *et al*., 2001; Suzzi *et al*., 2001). In a study by Wyder and Puhan (1999a), *Y. lipolytica* was indicated as the species with the strongest proteolytic activity. This yeast is considered as the most predominant species contributing to lipolytic activity (Choisy *et al*., 1987). It was possible to accelerate cheese ripening and to improve the quality of cheese by the addition or this yeast species (Lenoir *et al*., 1985; Devoyod, 1990).

D. hansenii and *Y. lipolytica* have been regarded as good candidates for ripening agents in cheese (Guerzoni *et al*., 1998) fulfilling specific criteria to be regarded as co-starters for cheesemaking (Guerzoni *et al*., 2001; Suzzi *et al*., 2001). However, despite the frequent references to the presence of yeasts and the lactic acid starter cultures in cheese, few studies refer to these interactions. Ripening studies at

laboratory level with a model system, for example with cheese curd slurries, are usually not sufficient to reproduce the conditions in the cheese and can only be seen as a preliminary approach (Wyder *et al*., 1999). It is always necessary to perform cheese treatments at a pilot plant level and therefore, in this study, we endeavoured to study the interaction between the two yeast species and the lactic acid bacteria by incorporating (i) *D. hansenii*, (ii) *Y. lipolytica* and (iii) both these species together as part of the starter culture for the production of matured Cheddar cheese. The physical and chemical properties of the cheeses were monitored in order to evaluate the contribution of the yeasts to cheese ripening.

MATERIALS AND METHODS

Starter culture preparation

The *Debaryomyces hansenii* culture (Product LAF3) was obtained from Chr. Hansen (Hørsholm, Denmark). Two units (each comprising of 2 x 10^9 cells) of this culture were incorporated together with the starter culture for the production of matured Cheddar cheese as recommended by the manufacturer. A strain of *Yarrowia lipolytica*, previously isolated from Cheddar cheese (Welthagen and Viljoen, 1999) was cultured in 400 ml YM-broth (Wickerham, 1951) under agitation at 30ºC for 96 hrs. Cells were counted by means of a Haemocytometer and a yeast suspension containing 4 x 10^9 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 resuspended in UHT milk, stored at 4-5ºC, and used within 24 hrs. In addition to the two yeast species, 10 units (as recommended by the supplier) of the lactic acid bacteria starter culture (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* spp. *thermophilus*), RAO 21 (Rhodia Foods, France) were used as starter culture for the production of matured Cheddar cheese and simultaneously added with the yeasts. The RAO 21 lactic acid starter culture was also applied for the production of a traditional matured Cheddar cheese with no additions as control under similar conditions.

Matured Cheddar cheese manufacture

Pasteurised cows milk (400 l) was used on each occasion for the manufacture of matured Cheddar cheese for four (including the control) cheese treatments. Each treatment was repeated three times. For the first treatment, *Debaryomyces hansenii* was added (2 units) as part of the starter culture while *Yarrowia lipolytica* was added (2 x suspended cells in UHT milk) as part of the starter culture treatment 2. For the third treatment both these yeast species were added with the starter culture. The fourth treatment was the control with only the starter culture. Cheesemaking was done at a cheese plant in the Free State in South Africa using a standard procedure described by Kosikowski (1970a).

Sampling description

Samples were taken at selected points during the processing of matured Cheddar cheese – from the cheese vat, during and after the renneting process, after cutting and during and after cheddaring. The cheese was kept under controlled conditions (8-12ºC) and sampled after 24 and 48 hrs, and 12 and 30 d, followed by consecutive intervals of 30 d for 6 months. Similar samples were collected from both the experimental cheeses and the control cheese.

Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by aseptically opening the cheeses, representatives of each batch, and cutting portions out with a sterile knife. For each sample, 10 g cheese were aseptically weighed and added to 90 ml of sterile peptone water into Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.). Further decimal dilutions were carried out as required for microbial assays in 9 ml sterile peptone water and plated in duplicate by the spread plate technique onto selective media. De Mann, Rogosa and Sharpe (MRS) plates (Oxoid, Basingstoke, UK) which are elective for lactic acid bacteria were incubated aerobically for 48 hrs at 28ºC whereas Chloramphenicol-Agar plates (Oxoid, Basingstoke, UK), selective for yeasts, were incubated aerobically for 96 hrs at 28ºC.

Sample analysis

All plates containing between 25 and 150 colony forming units (cfu) on the highest dilution (or the highest number if below 25) were counted and the mean values were determined from duplicate plates. For treatment 3, the number of *Debaryomyces hansenii* and *Yarrowia lipolytica* colonies were individually counted based on their visually distinguishable appearances. Results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions (2x2x3).

Physical and chemical analysis

During sampling, as described for the microbial samples, an additional 10 g of cheese were weighed into 10 ml of distilled water in Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.) for chemical analysis.

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Bouzas *et al*., 1991) whereas the organic acids were quantified by means of a HPLC system equipped with a variable wavelength detector set at 220nm. 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 24ºC with a HI 9321 Microprocessor pH meter (HANNA Instruments, Germany) according to the method described by Kosikowski (1970b).

Sensory analysis

The sensory quality of the experimental and control cheeses was judged by a panel of experts in cheese evaluation based on a standard protocol including openness, texture, taste and maturity. The cheeses were evaluated after 2, 4, 6 and 9 months.

RESULTS AND DISCUSSION

Microbial populations

Yeasts and other microorganisms present in dairy products may interact in different ways. The yeasts may inhibit or eliminate undesired microorganisms that cause quality defects or potential pathogenic characteristics. They may inhibit the starter culture or contribute positively to the maturation process by supporting the function of the starter culture (Jakobsen and Narvhus, 1996). Studies on the yeast diversity associated with Cheddar and Gouda cheeses by Welthagen and Viljoen (1998; 1999) and Laubscher and Viljoen (1999c) indicated that yeast contaminants from the

environment, play a significant role during the ripening of these cheeses by supporting the function of the starter cultures.

Enumeration of the yeasts and lactic acid bacteria present during the processing and maturation stages of the three treatments indicated an enhanced survival of the lactic acid bacteria compared to the traditional matured Cheddar cheese, produced as a control under similar conditions (Fig. 1). In all three cheese treatments in the present study the lactic acid bacterial numbers remained high during the maturation period and never declined to values below 10^7 cfu/g cheese (Fig. 1). In contrast, in the control, traditional matured Cheddar cheese, a decrease (more than 1 log unit) in the lactic acid bacterial counts was observed, initiated within 2 days after processing (Fig. 1). Declining lactic acid bacterial counts in the three treatments were only detected after 3 to 4 months.

When *Debaryomyces 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 6 months of maturation (Fig. 1a). When *Yarrowia lipolytica* was used as the sole co-inoculum, the number of yeasts also gradually decreased and ceased to survive after 4 months of the ripening period (Fig. 1b). When both yeast species were incorporated simultaneously as part of the starter culture for the production of matured Cheddar cheese (Fig.1c) the combined yeast count initially decreased during the manufacturing process, followed by a substantial increase (> 3 log units) during the first 48 hrs of ripening (Fig. 1c). The number of yeasts in the cheese with *Debaryomyces* and *Yarrowia* cultures remained significantly higher (> 2 - 3 log units) compared to the cheeses with individual inoculated yeasts during the initial 2 months of maturation. Thereafter, the yeast count decreased until a value of 2 x 10^2 cfu/g was obtained after 6 months of maturation. *D. hansenii* predominated *Y. lipolytica* species during the maturation stage in the cheese exhibiting counts of 3 log units higher. During the final 3 months of ripening only *D. hansenii* was found (Fig. 2).

Survival of yeasts in the cheese might be attributed to the utilisation of organic acids produced by the lactic acid bacteria and their proteolytic and lipolytic abilities (Fleet, 1990; Welthagen and Viljoen, 1998). The reason for *Y. lipolytica* being overgrown by *D. hansenii* may be due to the latter's greater ability to grow at low temperatures

(Davenport, 1980), high salt concentrations (Mrak and Bonar, 1939) and low a_w values (Tilbury, 1980) which are the conditions prevailing during the manufacturing of matured Cheddar cheese. Due to their tolerance of low pH-values and high NaCl-concentrations, yeasts grow particularly well during the initial period of ripening (Eliskases-Lechner and Ginzinger, 1995). According to Bartchi *et al*. (1994) a decrease in yeast counts towards the end of the ripening period is observed in cheeses. Wyder and Puhan (1999b) also found *Y. lipolytica* only during the first half of the ripening period of Limburger cheese. Generally, the utilisation of lactic acid by the yeasts leads to an increase in pH that encourages bacterial growth and contributes to the ripening process of the cheese (Fleet, 1990). A lower final lactic acid content was evident in the experimental cheeses compared to the control cheese (results not shown). Yeasts liberate growth factors such as vitamins (Purko *et al*., 1951) and amino acids (Wyder *et al*., 1999) through autolysis or excretion, thus promoting growth of the bacterial population that remained viable at high numbers throughout the ripening periods of all three cheese treatments. The high number of lactic acid bacteria is expected in a mature Cheddar cheese, although this is not necessarily a reflection of only representatives of the starter culture. The high count may also be exaggerated due to nonstarter lactic acid bacteria (NSLAB). Similarly, a yeast count of almost 10^3 cfu/g, originated as contaminants, was also observed within the control cheese (results not shown).

Physical and chemical analysis

The major sugar present in dairy products is lactose (Scott, 1981). Lactose is a disaccharide that must be hydrolysed to glucose and galactose before it can enter the catabolic pathway for hexoses (Schlegel, 1993). All the lactose present in the milk was hydrolysed during maturation (Fig. 3). Although yeasts are traditionally associated with fermentation, nearly half of the presently known yeast species lack the ability to ferment sugars (Van der Walt and Yarrow, 1984) and furthermore, lactose fermenting species are not typical representatives in the cheese environment (Eliskases-Lechner, 1998). Since both *Debaryomyces hansenii* and *Yarrowia lipolytica* are non-fermenting yeasts incapable of utilising lactose (Kreger-van Rij, 1984; Barnett *et al*., 1990; Kurtzman and Fell, 1998) its depletion can be attributed to the lactic acid bacteria and other contaminating microorganisms. In all three cheese

treatments with the addition of yeasts as adjunct starters, the lactose was depleted during the manufacturing process (Fig. 3) whereas the lactose in the control was only depleted after 10 days (results not shown). Welthagen and Viljoen (1999) reported a decrease in the lactose content of Cheddar cheese to a value of 0.26% after 51 days of ripening, whereas Laubscher and Viljoen (1999c) reported depletion of the lactose content after 2 days of ripening. The addition of *Y. lipolytica* and *D. hansenii* as part of the starter cultures therefore may have enhanced the utilisation of lactose by the lactic acid bacteria probably due to increased lactic acid bacterial numbers at this stage (Fig. 1). No lactic acid bacteria can grow without growth factors such as vitamins (lactoflavin, thiamine, pantothenic acid, nicotinic acid, folic acid, biotin) and amino acids (Schlegel, 1993). These growth factors are supplied by yeasts (Riviere, 1969; Lenoir, 1984) and were the possible reason for the increased metabolisation of the lactose.

The lactose present in the milk was hydrolysed to glucose and galactose (Schlegel, 1993) resulting in the accumulation of galactose (Fig. 3). Normally the glucose moiety will be utilised directly, intracellularly, by the starter cultures and not excreted into the cheese. The unusual high concentration of accumulated galactose in the cheeses was probably due to the application of a thermophilic starter strain. Both *D. hansenii* and *Y. lipolytica* have the ability to utilise glucose and galactose as carbohydrate sources (Kurtzman and Fell, 1998). In all four cheese treatments the glucose was rapidly utilised by the microorganisms and therefore only traces of this sugar were detected. A maximum galactose content of almost 1.5% was obtained after 24hr of maturation when *D. hansenii* was added, whereas a maximum of 0.86% was detected with the inclusion of *Y. lipolytica*, and a maximum of 0.89% at the end of cheddaring when both yeast species were added as adjunct starters (Fig. 3a-c). After reaching the maximum contents, a decrease in contents started and galactose was depleted after 5, 3 and 1 months during the first, second and third cheese treatments, respectively. In contrast, a galactose content of 0.11% was still present in the control cheese after 4 months of maturation (results not shown). The depletion of the galactose content might be attributed to the growth of *D. hansenii* and *Y. lipolytica* (Kreger-van Rij, 1984; Barnett *et al*., 1990; Kurtzman and Fell, 1998), other contaminating microorganisms and non starter lactic acid bacteria.

During each of the four cheese treatments the lactic acid content of the cheeses rapidly increased (Fig. 4). The lactic acid content of the cheeses continued to increase reaching maximum values in the vicinity of 2% after 6 months of maturation. It is interesting to note, however, that the lactic acid content in the control cheese was lower at all sampling intervals maximising to a content of 1.5% after 6 months maturation (results not shown). The enhanced lactic acid content in the yeast inoculated cheeses may be due to increased lactic acid bacterial activity, resulting from their utilisation of yeast metabolites. This corresponds with the work of Wyder *et al*. (1999) who attributed the greater lactic acid concentration to the stimulation of lactic acid bacteria, rather than to a direct result of yeast metabolism. The growth and frequent occurrence of most yeasts in dairy products relies on the assimilation of lactic acid, citric acid and other organic acids (Fleet, 1990; Deàk and Beuchat, 1996) and therefore the assimilation of lactic acid is a common feature for yeast isolates from fermented milks (Loretan *et al*., 1998). Generally, yeasts thereby contribute to the deacidification of cheese which was however not supported by the present results (Lenoir, 1984; Valdés-Stauber *et al*., 1997; Viljoen, 1998) probably due to the greater quantities of lactic acid (Fig. 4) than normally produced. The majority of yeasts in dairy products, including *D. hansenii* are capable to utilise citric acid (Barnett *et al*., 1990; Fleet, 1990). The citric acid content in the cheeses decreased with the addition of *D hansenii* to a minimum of 0.048% after 6 months (Fig. 4a) and 0.049% with the co-inoculation of both species (Fig. 4c) but remained constant in the vicinity of 0.2% with the addition of *Y. lipolytica* (Fig. 4b). A similar constant trend at 0.2% in the contents of the citric acid was noticed in the control cheese (results not shown).

The growth of the lactic acid starter bacteria resulted in the production of lactic- and other organic acids with a consequent decrease in the pH of the cheese. In all four cheese treatments, the pH of the cheeses decreased rapidly during the manufacturing process, followed by a more gradual decrease during the ripening period (Fig. 4). The decrease in the pH value may also be aggravated, although limited, by the enzymatic release of free fatty acids from tryglicerydes by the yeasts (Guerzoni *et al*., 2001). During the final 3 months of ripening the experimental cheeses remained at a slightly lower pH than the control cheese especially during the second cheese treatment (significantly lower) where *Y. lipolytica* was used as sole co-inoculum (Fig. 4b). The lower pH values may be attributed to the higher numbers of lactic acid bacteria in the

experimental cheeses (Fig. 1) and therefore producing mainly lactic acid at higher concentrations (Fig. 3). No significant difference in the pH value was however observed between the control cheese and the experimental cheese inoculated with both yeasts (Fig. 3c). Yeasts, however, are generally known for their de-acidifying action in cheese by utilising lactic acid and the formation of alkaline metabolism products due to proteolysis (Eliskases-Lechner and Ginzinger, 1995; Wyder *et al*., 1999). Due to the enhanced lactic acid contents produced in the experimental cheeses, de-acidification was not detected.

Sensory analysis

Debaryomyces hansenii is a yeast species with high proteolytic activity (El Soda, 1986; Choisy *et al*., 1987). Peptidases like aminopeptidases and carboxypeptidases present in yeasts play an important role in the proteolysis of milk proteins (Lenoir *et al*., 1985; Choisy *et al*., 1987). Furthermore, synergistic effects have been observed between *D. hansenii* and lactic acid bacteria resulting in stronger proteolysis (Deiana *et al*., 1984). *D. hansenii* also produces lipases that hydrolyse milk fat (Fleet and Mian, 1987). The biochemical activities of *Yarrowia lipolytica* strains can play an important role in the organoleptic features of cheeses due to the production of aromatic compounds and their precursors such as methyl ketones, alcohols, lactones and esters (Suzzi *et al*., 2001). Martin *et al*. (1999) assumed that the synthesis of aroma compounds by bacteria might be enhanced when these microorganisms are associated with *Y. lipolytica.* The yeast was recognised for its particularly good lipolytic activity releasing fatty acids which can be further transformed into desirable or undesirable volatile or non-volatile compounds with characteristic aroma (Guerzoni *et al*., 1998, 2001; Van den Tempel and Jakobsen, 2000). The proteolytic activity of *Y. lipolytica* strains, converting casein to peptides of different molecular weight and free amino acids, which may have a direct influence on cheese flavour or be precursors of flavour components (Parra *et al*., 1996; Suzzi *et al*., 2001).

Cheese of treatment 1 developed a fruity flavour after 2 months of ripening in addition to an enhanced development of the desired Cheddar flavour compared to the control cheese (results not shown). This fruity flavour was detected throughout the ripening period. After 6 months the cheese developed a bitter taste. A similar

enhanced Cheddar flavour development was also obtained with cheese-added *Y. lipolytica*, where a fruity aroma developed after 2 months of ripening with a stronger development of the desired Cheddar flavour in the experimental cheese after 4 months (results not shown). After 6 months the cheese retained its strong Cheddar flavour while the fruity taste could still be detected. In both cheese treatments the off-flavours were intensified after 9 months of ripening. The accelerated formation of the desired Cheddar flavour may be attributed to the enhanced breakdown of proteins by the yeasts. The development of a fruity flavour corresponds with previous studies (Hansen *et al*., 1984; Lee and Richard, 1984) which reported the ability of yeasts to produce aroma compounds responsible for fruity flavours such as alcohols, aldehydes, esters and monoterpenes. Fruity flavours can also be the result of the formation of esters from formic or acetic acids (Wyder and Puhan, 1999a) while continued hydrolysis of protein and fat could contribute to bitter and rancid flavours (Fleet and Mian, 1987).

With the addition of both yeast species to the starter culture (treatment 3), 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 compared to the control cheese (results not shown). At the end of the ripening period, the development of a mature taste was more significant in experimental cheese 3 compared to the control cheese. The experimental cheese had a clean, slightly sweet, pleasant taste and retained its good, strong flavour after 9 months, while the control cheese developed a bitter and slightly impure taste at that time (results not shown). Wyder *et al*. (1999) reported that *Y. lipolytica* was able to diminish the negative effect of *D. hansenii* on sensory quality. The reason for the extended expiry date of experimental cheese 3 based on its sensory analysis, may also be due to interaction between the yeasts and the lactic acid bacterial starter cultures, with the yeasts maintaining the balance between the different starter strains.

CONCLUSION

Debaryomyces hansenii and *Yarrowia lipolytica* grew and competed with other naturally occurring yeasts in the cheese and with the starter bacteria without any inhibition of the starter culture. The species contributed to the accelerated development of a strong Cheddar flavour, although bitter and fruity flavours were detected when the yeasts were inoculated individually. When both species were incorporated as part of the starter culture, the cheese had a good strong flavour after a ripening period of 4 months. The cheese had a clean, slightly sweet, pleasant taste and still retained its good, strong flavour after 9 months, while the control cheese developed a bitter and slightly impure taste. The application of both *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese is proposed.

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CHAPTER 6

CHANGES IN FATTY ACID CONTENTS PRESENT IN MATURED CHEDDAR CHEESE WITH THE ADDITION OF *DEBARYOMYCES HANSENII* AND *YARROWIA LIPOLYTICA* AS ADJUNCT STARTERS

ABSTRACT

When *Debaryomyces hansenii* and *Yarrowia lipolytica* were incorporated as part of the starter culture for the production of matured Cheddar cheese, the cheese had a good, strong flavour after a reduced period of ripening. The presence and amounts of free fatty acids (FFA) determine the flavour of many dairy foods and therefore the total lipid profiles as well as the FFA present in the cheese were determined and compared to those of a control cheese. Lower levels of FFA in the model cheese compared with the control, indicated the significance of lipolysis and FFA breakdown by the yeasts. Furthermore, the absence of some of the unsaturated fatty acids as well as the presence of γC18:3 only in the model cheese, designates the enzymatic activities of the yeast species. The lipolytic activities of the yeasts therefore contributed to the enhanced development of a matured taste in the model cheese.

INTRODUCTION

The numerous compounds involved in cheese aroma are mainly derived from three major metabolic pathways: lactose catabolism, protein catabolism and lipid catabolism (Molimard and Spinnler, 1995). Lipolysis is defined as the enzyme-catalysed hydrolytic cleavage of triglycerides resulting in the release of free fatty acids (FFA) (Arnold *et al*., 1975). Cheese FFA are derived from the hydrolysis of glycerides by natural lipases in the milk, while lipolysis may also be due to the action of microbial lipases (Choisy *et al*., 1986). Milk fat contains 98% of glyceride neutral lipids that are composed of a wide variety of fatty acids (FA) (Choisy *et al*., 1984). Lipases hydrolyse triglicerides to form diglicerides, monoglycerides and FFA. The latter are important components in the flavour of many cheese types and are also precursors of aroma compounds such as methyl ketones, secondary alcohols, lactones and esters (Choisy *et al*., 1986; Schrödter, 1990; Ha and Lindsay, 1991; Molimard and Spinnler, 1995).

The presence and amounts of FA, which are largely dependent on the milk fat substrate and the lipase selectivity and activity, determine the flavour of many dairy foods (Ha and Lindsay, 1993). Excessive quantities of FFA are associated with hydrolytic rancidity flavour defects in cheese (McDaniel *et al*., 1969; Arnold *et al*., 1975; Bynum *et al*., 1984). These changes in compounds are mainly attributed to the actions of various microorganisms, including the lactic acid bacteria, natural bacteria present in the milk and sometimes also yeasts (Stadhouders and Veringa, 1973; Fleet, 1990).

Debaryomyces hansenii and *Yarrowia lipolytica* have been regarded as good candidates for ripening agents in cheese (Guerzoni *et al*., 1998) by fulfilling specific criteria to be regarded as adjunct starters for cheesemaking (Guerzoni *et al*., 2001; Suzzi *et al*., 2001). *D. hansenii* predominated in most studies of yeasts associated with dairy products (Walker and Ayres, 1970; Seiler and Busse, 1990, Eliskases-Lechner, 1998; Welthagen and Viljoen, 1998; Wyder and Puhan, 1999) and the synergistic effect that exists between the species and the lactic acid bacteria was reported by Yamauchi *et al.* (1975) resulting in the prolonged survival of the lactic acid bacteria in matured Cheddar cheese (Ferreira and Viljoen, 2003). *Y. lipolytica*

has the ability to predominate in real system on the naturally occurring yeasts (Guerzoni et al., 1998) and is known for its exceptionally strong proteolytic and lipolytic activity (Choisy *et al*., 1987; Wyder and Puhan, 1999). Both strains have been co-inoculated as adjunct starter cultures for the production of matured Cheddar cheese (Ferreira and Viljoen, 2003) resulting in enhanced lactic acid bacterial growth and improved taste development.

Consequently, the aim of this study was to determine the total lipid profiles as well as the FFA present in a matured Cheddar cheese co-inoculated with both *D. hansenii* and *Y. lipolytica* as adjunct starters. These data were statistically compared with the control cheese produced by the standard procedure under similar conditions to establish the effect of the application of yeasts on the lipid profiles of the cheese.

MATERIALS AND METHODS

Starter culture preparation

The LAF 3 starter strains of *Debaryomyces hansenii* were obtained from Chr. Hansen (Hørsholm, Denmark). Two units (each comprising of 2 x 10^9 cells) of this species were incorporated as part of the starter culture for the production of matured Cheddar cheese as indicated by the manufacturer. A *Yarrowia lipolytica* strain, previously isolated from Cheddar cheese and identified (Welthagen and Viljoen, 1999) was cultured in 400 ml YM-broth (Wickerham, 1951) under agitation at 30ºC for 96 hrs. Cells were counted by means of a Haemacytometer and a total yeast count of 4 x 10^9 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 resuspended in UHT milk, stored at 4-5ºC, and used within 24 hrs. In addition to the two yeast species, ten units (as proposed by the supplier) of the lactic acid bacteria starter strains (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* spp. *thermophilus*), RAO 21 (Rhodia Foods, France) were used as starter culture for the production of matured Cheddar cheese and simultaneously added with the yeasts. The RAO 21 lactic acid starter strains were also applied for the production of a traditional matured Cheddar cheese as control.

Matured Cheddar cheese manufacture

Pasteurised cows milk (400 l) was used for the manufacture of matured Cheddar cheese at a cheese plant in the Free State in South Africa on three occasions. The procedure for cheese making was carried out as described by Kosikowski (1970).

Sampling description

The cheese was kept under controlled conditions (8-12ºC) and sampled after 1 d, 15 d and 30 d, followed by consecutive intervals of 30 d for 6 months. Similar samples were collected from the model cheese and the control cheese.

Lipid extraction and fatty acid analysis

Lipids were extracted from the cheese using chloroform:methanol (2:1 v/v) (Kendrick and Ratledge, 1992) and washed twice with distilled water (Folch *et al*., 1957). The organic solvents were evaporated under vacuum. The lipids were dissolved in a minimal volume of diethyl ether and transferred to vials after which they were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. The samples were dissolved in chloroform and methylated with trimethyl sulphonium hydroxide (TMSOH) (Butte, 1983). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph equipped with a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 $ml.min^{-1}$) as carrier gas (Kock, 1988). Lauric acid (C:12) (3 mg) were included as a standard while peaks were identified by reference to authentic standards.

Free fatty acid extraction and analysis

Free fatty acids were extracted from the cheese using ethyl acetate (Nigam, 1987), which was subsequently evaporated under vacuum. The lipids were dissolved in a minimal volume of diethyl ether and transferred to vials after which they were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. The samples were dissolved in methanol and methylated with diazomethane. The fatty acid methyl esters were analysed as described above.

Statistical analysis

Statistical differences between the model and control cheeses were determined by comparing treatments using an ANOVA procedure and a Tukey-Kramer multiple comparison procedure (NCSS, 2004). The level of significance was $P<0.05$, $P<0.01$ and $P<0.001$.

RESULTS AND DISCUSSION

Despite the frequent occurrences of yeasts in dairy products (Lenoir, 1984; Fleet and Mian, 1987; Fleet, 1990; Seiler and Busse, 1990; Welthagen and Viljoen, 1998, 1999; Viljoen *et al*., 2003), it is not generally accepted that these yeasts contribute significantly to the quality of the final product. Milk is fermented by bacterial starter cultures and therefore these bacteria are considered to be of major importance contributing to the final product adding to the aroma and taste (Cousin, 1982). Yeasts, however, play an essential role in the ripening of some fermented dairy products and contribute substantially to the final product (Lenoir, 1984; Choisy *et al*., 1987; Fleet and Mian, 1987; Devoyod, 1990; Fleet, 1990; Molimard *et al*., 1997; Spinnler *et al*., 2001; Klein *et al*., 2002; Bintsis *et al*., 2003). These contributions are attributed to various interactions between the yeasts and the lactic acid starter bacteria (Welthagen and Viljoen, 1998, 1999). With the addition of two yeast species as adjunct starters for the production of matured Cheddar cheese, the cheese developed a stronger Cheddar flavour in a reduced time compared to the control (Ferreira and Viljoen, 2003). The cheese retained this good strong flavour for more than nine months, while the control cheese developed off-flavours at that time.

Early reviews on cheese flavour by Harper (1959) and Day (1967) already referred to the importance of lipolytic release of free fatty acids in the development of flavour in aged cheeses. Long chain free fatty acids make up the major portion of FFA in Cheddar cheese, even though the short chain FFA may have a more significant impact on flavour (Woo and Lindsay, 1982; Bynum *et al*., 1984). The total FA of the model and the control cheeses are shown in Fig. 1 and Table 2, while the levels of FFA in these two cheeses are shown in Fig. 2 and Table 3.

The levels of total FA were generally higher in the control cheese than the model cheese. The predominant FA in the model cheese were C18:1 (oleic acid), C16:0 (palmitic acid), C18:0 (stearic acid) and C14:0 (myristic acid) (Fig. 3) which represented 28.9%, 28.7%, 16.3% and 10% of the total FA respectively. In the control cheese, the same FA predominated, but in a different order, with C16:0 representing 30.1% of the total FA, C18:1 –29.5%, C18:0 –15.8% and C14:0 -10% (Fig. 3). The levels of C14:1, C16:0, C16:1 and C18:1 in the model cheese differed

significantly from those in the control cheese (P-values ranged between $P<0.05$ and $P<0.001$). Furthermore, γC18:3 was part of the total FA profile in the model cheese, but was not detected in the control cheese. This designates the enhanced enzymatic activities contributed to the presence of the two yeast species in the model cheese, especially those of *Yarrowia lipolytica* (Guerzoni *et al*., 1998; Van den Tempel and Jakobsen, 2000).

Cheese flavour is obtained through a series of chemical changes that occur during ripening. Lipid hydrolysis leads to the formation of FFA which serve as substrates for further reactions (Molimard and Spinnler, 1995). This metabolism of FFA is important for the aromatic characterisation of cheese (Suzzi *et al*., 2001). Perception threshold is defined as the lowest concentration of a compound that allows more than half the judges present to distinguish between the control sample containing no compound and the sample containing the compound (Urbach *et al*., 1972). Long chain FFA (> C12:0) have a high perception threshold and play a minor role in flavour, while short and moderate-chain, even-numbered FFA (C4:0 to C12:0) have much lower perception thresholds and each has a characteristic flavour note (Table 1) (Molimard and Spinnler, 1995). FFA are present in the model cheese at significant levels less than in the control cheese (P-values ranged between $P<0.01$ and $P<0.001$). The low levels of FFA in the model cheese are further explained by the ability of yeasts to oxidise these fatty acids by the process of β-oxidation to yield FA having two carbon atoms less than the parent (Ercoli *et al*., 1992). Repeated cycles of β-oxidation diminished the total FFA in the model cheese and these molecules were transformed into flavour compounds like alcohols, ketones and lactones. Furthermore Wong *et al*., (1975) and Maga (1976) reported that cheese-related microorganisms are able to produce short chain hydroxy acids from long chain FA. These are then transformed to their corresponding lactones. The lower levels of FFA as well as the more intense Cheddar flavour of the model cheese compared to those of the control cheese therefore indicated the significance of FFA breakdown by the yeasts. Furthermore, results presented in Fig. 2 and Table 3 are evident of the absence of the unsaturated FFA C14:1 and C16:1 in the model cheese which are explained by Maga (1976), who reported that free unsaturated fatty acids can be transformed by the microbial enzymes (lipoxygenase, epoxidase and hydratase) into the relative hydroxy-

acids. These can be transformed into shorter chain molecules, including lactones. Armstrong (1989) and Ercoli *et al*. (1992) reported some strains of *Y. lipolytica* to be particularly effective in converting unsaturated FA into C10 and C12 gamma lactones. Polyunsaturated FA therefore had a relevant role in the aroma of the model cheese.

The predominant FFA in the model cheese were C18:1, C16:0, C18:0 and C14:0 (Fig. 4) which represented 32.1%, 30.8%, 13.9% and 9.6% of the total FFA respectively. These are among the predominant FFA reported by Roostita and Fleet (1996) in UHT-treated milk cheese and Kilcawley *et al*. (2001) in Cheddar cheese. In the control cheese where higher levels of FFA were observed, the same FFA predominated with C18:1 representing 30.1% of the total FFA, C16:0 –29.1%, C18:0 –12% and C14:0 – 10.8% (Fig. 4). The reason for the apparent absence of short chain FFA (<C10:0) is due to the fact that they are volatile and partitioned mainly in the aqueous phase with the remaining FFA associated with the fat phase and may therefore not be extracted and measured completely (Woo and Lindsay, 1982; IDF, 1991). Furthermore, the fats were hydrolysed with the liberation of even-numbered carbon FFA, while no odd-numbered FFA were detected. Odd-numbered FFA are present in milk in traces (Mietton *et al*., 1994).

CONCLUSION

Significant lower levels of FFA in the model cheese compared with the control cheese indicated the significance of lipolysis and FFA breakdown by the yeasts. Furthermore, all of the C14:1 and C16:1 in the model cheese were transformed into their relative aroma compounds, while the presence of γC18:3 only in the model cheese, designates the enzymatic activities of the two yeast species. This lipolytic activity of the yeasts probably contributed to the enhanced development of a matured taste in the model cheese when compared to the control cheese.

Table 1. Flavour notes of some short and moderate-chain fatty acids (Brennand *et al.*, 1989; Patton, 1964; Siek *et al*., 1969; Urbach *et al*., 1972; McDaniel *et al*., 1969; Woo and Lindsay, 1983).

Compound	Flavour note
Butyric acid	Vinegar, pungent, rancid, cheesy
Isobutyric acid	Sweat, mild, rotten apple
Isovaleric acid	Rotten fruit, mild, fruity, sweat
Hexanoic acid	Pungent, blue cheese
Octanoic acid	Wax, soapy, goaty, musty, rancid, fruity
Decanoic acid	Rancid, soapy
Dodecanoic acid	Rancid, soapy

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ADDENDUM

Table 2. Statistical analysis of the total fatty acids of matured Cheddar cheese with *Debaryomyces hansenii* and *Yarrowia lipolytica* as adjunct starters compared to the total fatty acids of the control cheese during ripening (mg/g cheese).

	C10:0			C12:0			C14:0		
	Model	**Control**	**Sign.**	**Model**	**Control**	**Sign.**	**Model**	**Control**	**Sign.**
Day 1	6.1325 ± 0.2565	6.8186 ± 0.9949	NS	8.6004 ± 1.4647	9.9793 ± 0.4423	NS	23.7043 ± 0.5309	27.8810 ± 4.4006	NS
Day 15	6.3110 ± 0.3230	7.3473 ± 0.3103	*	8.0945 ± 0.0836	10.6394 ± 1.2100	*	24.8510 ± 0.9960	30.2404 ± 1.2875	**
Day 30	6.4015 ± 0.2619	7.5676 ± 0.3958	*	8.8541 ± 0.9131	10.1464 ± 0.3357	NS	24.4448 ± 1.2185	31.1750 ± 1.7763	**
Day 60	7.2560 ± 0.4859	7.7368 ± 0.0495	NS	9.8820 ± 1.6688	10.8408 ± 1.2859	NS	28.4605 ± 1.9499	31.8987 ± 0.0468	*
Day 90	7.3768 ± 0.0255	7.6585 ± 0.3981	NS	10.2729 ± 1.2969	10.3673 ± 0.5010	NS	28.9506 ± 0.1571	31.2795 ± 1.6907	NS
Day 120	7.2886 ± 0.0754	7.3976 ± 0.5139	NS	9.9523 ± 1.8289	10.1124 ± 0.3841	NS	28.7335 ± 0.1797	30.5015 ± 2.0166	NS
Day 150	6.6286 ± 0.0295	7.1739 ± 0.6156	NS	9.4165 ± 1.3457	9.8150 ± 0.3259	NS	25.8994 ± 0.3420	29.5658 ± 2.4594	NS
Day 180	6.8989 ± 0.0739	7.2761 ± 0.5376	NS	9.7200 ± 1.4041	10.1060± 0.4822	NS	27.5961 ± 0.7270	29.9160 ± 2.2484	NS

	C14:1			C16:0			C16:1		
	Model	**Control**	**Sign.**	**Model**	**Control**	**Sign.**	**Model**	**Control**	**Sign.**
Day 1	1.7494 ± 0.0191	2.1122 ± 0.2798	NS	67.9331 ± 0.9661	83.6246 ± 13.1801	NS	3.7426 ± 0.0732	4.6303 ± 0.7103	NS
Day 15	1.7774 ± 0.0793	2.3002 ± 0.0008	***	71.6848 ± 1.9636	90.9979 ± 3.7989	**	3.8799 ± 0.2018	4.9662 ± 0.2411	**
Day 30	1.7186 ± 0.0927	2.3870 ± 0.0447	***	69.4921 ± 3.1695	93.9261 ± 4.8884	**	3.7711 ± 0.2825	5.1305 ± 0.2571	**
Day 60	2.2112 ± 0.3165	2.4509 ± 0.0941	NS	81.3524 ± 6.0482	96.1276 ± 0.3298	*	4.3703 ± 0.2709	5.2543 ± 0.0056	**
Day 90	2.1716 ± 0.0455	2.4087 ± 0.0329	**	83.5552 ± 1.4544	94.1816 ± 4.4209	*	4.3833 ± 0.0617	5.1876 ± 0.2810	**
Day 120	2.0582 ± 0.0550	2.3462 ± 0.0372	**	82.0526 ± 0.0796	92.3351 ± 5.2637	*	4.3300 ± 0.0012	5.0793 ± 0.3739	*
Day 150	1.8806 ± 0.0256	2.2623 ± 0.1014	**	73.7099 ± 0.8901	89.2529 ± 6.7161	*	3.9612 ± 0.1327	4.8403 ± 0.3884	*
Day 180	2.1886 ± 0.2803	2.3028 ± 0.0762	NS	78.2858 ± 1.8389	90.2522 ± 5.9668	*	4.5204 ± 0.3894	5.0003 ± 0.4040	NS

	C18:0			C18:1			C18:2		
	Model	**Control**	**Sign.**	**Model**	**Control**	**Sign.**	**Model**	**Control**	**Sign.**
Day 1	38.3992 ± 0.2507	43.5398 ± 6.9345	NS	68.4674 ± 0.4502	81.8999 ± 13.1715	NS	14.6496 ± 0.5739	14.6329 ± 2.0719	NS
Day 15	41.3674 ± 1.1643	47.6076 ± 1.6664	**	72.8348 ± 2.1766	88.9401 ± 3.5296	**	15.2896 ± 0.1056	15.9668 ± 0.2379	*
Day 30	39.4814 ± 2.0042	49.1276 ± 2.1125	**	69.8800 ± 3.5275	92.0174 ± 4.6467	**	14.5194 ± 0.4788	16.4878 ± 0.4517	**
Day 60	46.6873 ± 3.3859	50.3890 ± 0.5494	NS	82.5294 ± 6.3013	94.1468 ± 0.4696	*	17.3408 ± 1.6363	16.9264 ± 0.4695	NS
Day 90	47.0703 ± 0.5476	49.6223 ± 1.9194	NS	83.7152 ± 1.0994	92.4028 ± 4.1320	*	17.5392 ± 0.2223	16.5792 ± 0.3096	*
Day 120	46.8310 ± 0.0175	48.6231 ± 2.1984	NS	83.0802 ± 0.4510	90.8189 ± 4.9577	NS	17.6097 ± 0.1516	16.3289 ± 0.5126	*
Day 150	42.2328 ± 0.7441	46.9009 ± 2.8279	*	74.5232 ± 1.2590	89.7843 ± 3.7344	**	15.6867 ± 0.0368	15.7740 ± 0.7338	NS
Day 180	43.8924 ± 0.0759	47.3615 ± 2.4193	NS	77.1622 ± 0.2490	88.6747 ± 5.6653	*	16.3665 ± 0.6179	15.9641 ± 0.6504	NS

	αC18:3			γC18:3		
	Model	**Control**	**Sign.**	**Model**	**Control**	**Sign.**
Day 1	2.3301 ± 0.0287	2.2824 ± 0.4181	NS	0.9433 ± 0.3113	0.0000 ± 0.0000	**
Day 15	2.4956 ± 0.1610	2.4101 ± 0.0979	NS	0.8716 ± 0.0720	0.0000 ± 0.0000	***
Day 30	2.3828 ± 0.1180	2.5579 ± 0.1394	NS	0.8871 ± 0.2968	0.0000 ± 0.0000	***
Day 60	2.8114 ± 0.1258	2.6247 ± 0.0182	NS	1.2302 ± 0.0565	0.0000 ± 0.0000	***
Day 90	2.8741 ± 0.0586	2.4378 ± 0.1929	*	1.2105 ± 0.0948	0.0000 ± 0.0000	***
Day 120	2.7719 ± 0.1734	2.5567 ± 0.0937	NS	1.2227 ± 0.0904	0.0000 ± 0.0000	***
Day 150	2.5730 ± 0.0718	2.4881 ± 0.1273	NS	1.1071 ± 0.1039	0.0000 ± 0.0000	***
Day 180	2.8614 ± 0.1242	2.4886 ± 0.0955	*	1.2354 ± 0.0291	0.0000 ± 0.0000	***

NS - Not significant; * - P < 0.05, ** - P< 0.01; *** - P < 0.001

Table 3. Statistical analysis of the free fatty acids released in matured Cheddar cheese with *Debaryomyces hansenii* and *Yarrowia lipolytica* as adjunct starters compared to the free fatty acids released in the control cheese during ripening (mg/g cheese).

	C10:0		Sign.	C12:0		Sign.	C14:0		Sign.
	Model	**Control**		**Model**	**Control**		**Model**	**Control**	
Day 1	0.0000 ± 0.0000	0.0692 ± 0.0021	***	0.0135 ± 0.0012	0.0756 ± 0.0033	***	0.0397 ± 0.0017	0.2048 ± 0.0058	***
Day 15	0.0000 ± 0.0000	0.0327 ± 0.0029	***	0.0202 ± 0.0009	0.0355 ± 0.0044	**	0.0291 ± 0.0093	0.1079 ± 0.0054	***
Day30	0.0000 ± 0.0000	0.0632 ± 0.0021	***	0.0236 ± 0.0018	0.0699 ± 0.0008	***	0.0582 ± 0.0009	0.1858 ± 0.0117	***
Day 60	0.0000 ± 0.0000	0.0325 ± 0.0019	***	0.0273 ± 0.0008	0.0358 ± 0.0054	NS	0.0801 ± 0.0067	0.0823 ± 0.0012	NS
Day 90	0.0000 ± 0.0000	0.0514 ± 0.0018	***	0.0257 ± 0.0004	0.0570 ± 0.0030	***	0.0307 ± 0.0105	0.1459 ± 0.0038	***
Day 120	0.0000 ± 0.0000	0.0377 ± 0.0034	***	0.0261 ± 0.0003	0.0412 ± 0.0033	**	0.0673 ± 0.0006	0.1074 ± 0.0037	***
Day 150	0.0000 ± 0.0000	0.0511 ± 0.0010	***	0.0259 ± 0.0010	0.0549 ± 0.0016	***	0.0639 ± 0.0028	0.1590 ± 0.0035	***
Day 180	0.0000 ± 0.0000	0.0598 ± 0.0007	***	0.0200 ± 0.0031	0.0636 ± 0.0039	***	0.0559 ± 0.0053	0.1844 ± 0.0013	***

	C14:1		Sign.	C16:0		Sign.	C16:1		Sign.
	Model	**Control**		**Model**	**Control**		**Model**	**Control**	
Day 1	0.0000 ± 0.0000	0.0180 ± 0.0009	***	0.1143 ± 0.0070	0.6122 ± 0.0111	***	0.0000 ± 0.0000	0.0345 ± 0.0030	***
Day 15	0.0000 ± 0.0000	0.0178 ± 0.0012	***	0.0881 ± 0.0131	0.2886 ± 0.0126	***	0.0000 ± 0.0000	0.0248 ± 0.0040	***
Day30	0.0000 ± 0.0000	0.0161 ± 0.0010	***	0.2054 ± 0.0077	0.5333 ± 0.0334	***	0.0000 ± 0.0000	0.0359 ± 0.0026	***
Day 60	0.0000 ± 0.0000	0.0163 ± 0.0002	NS	0.3036 ± 0.0048	0.2164 ± 0.0033	***	0.0000 ± 0.0000	0.0274 ± 0.0008	***
Day 90	0.0000 ± 0.0000	0.0154 ± 0.0004	***	0.0995 ± 0.0436	0.3750 ± 0.0235	***	0.0000 ± 0.0000	0.0229 ± 0.0018	***
Day 120	0.0000 ± 0.0000	0.0156 ± 0.0005	***	0.1843 ± 0.0032	0.2639 ± 0.0184	***	0.0000 ± 0.0000	0.0189 ± 0.0012	***
Day 150	0.0000 ± 0.0000	0.0157 ± 0.0004	***	0.1788 ± 0.0047	0.4282 ± 0.0157	***	0.0000 ± 0.0000	0.0273 ± 0.0009	***
Day 180	0.0000 ± 0.0000	0.0154 ± 0.0003	***	0.1840 ± 0.0025	0.4458 ± 0.0346	***	0.0000 ± 0.0000	0.0295 ± 0.0006	***

	C18:0		Sign.	C18:1		Sign.	C18:2		Sign.
	Model	**Control**		**Model**	**Control**		**Model**	**Control**	
Day 1	0.0478 ± 0.0033	0.2456 ± 0.0226	***	0.1351 ± 0.0040	0.6040 ± 0.0048	***	0.0396 ± 0.0014	0.1171 ± 0.0045	***
Day 15	0.0490 ± 0.0033	0.1155 ± 0.0101	***	0.0815 ± 0.0069	0.3181 ± 0.0074	***	0.0191 ± 0.0009	0.0845 ± 0.0094	***
Day30	0.1075 ± 0.0026	0.2589 ± 0.0073	***	0.2305 ± 0.0193	0.5883 ± 0.0028	***	0.0583 ± 0.0052	0.1393 ± 0.0055	***
Day 60	0.1697 ± 0.0085	0.0923 ± 0.0088	***	0.3391 ± 0.0175	0.2326 ± 0.0117	***	0.0903 ± 0.0003	0.0563 ± 0.0061	***
Day 90	0.0284 ± 0.0047	0.1586 ± 0.0045	***	0.0593 ± 0.0038	0.3827 ± 0.0020	***	0.0225 ± 0.0020	0.0826 ± 0.0027	***
Day 120	0.0671 ± 0.0010	0.0998 ± 0.0003	***	0.1947 ± 0.0048	0.2863 ± 0.0149	***	0.0608 ± 0.0052	0.0631 ± 0.0014	NS
Day 150	0.0605 ± 0.0053	0.1530 ± 0.0019	***	0.1943 ± 0.0004	0.3821 ± 0.0087	***	0.0632 ± 0.0025	0.0928 ± 0.0024	***
Day 180	0.0857 ± 0.0011	0.1832 ± 0.0012	***	0.1852 ± 0.0032	0.4708 ± 0.0106	***	0.0528 ± 0.0030	0.1086 ± 0.0045	***

NS - Not significant; ** - P< 0.01; *** - P < 0.00

CHAPTER 7

THE INCORPORATION AND SURVIVAL OF PROBIOTICS IN MATURED CHEDDAR CHEESE

ABSTRACT

The health benefits of probiotic-containing products, the so-called functional foods, are becoming a key factor affecting consumer choice, and therefore the existing limited range of such products needs to be expanded. A limited number of studies have demonstrated different cheeses being able to support the viability of certain Bifidobacteria and *Lactobacillus* strains. Consequently, in this study *L. acidophilus* strain Ki and *Bifidobacterium bifidum* sp. strain Bo were incorporated into a South African matured Cheddar cheese. This was done without any alteration of the cheesemaking technology, which make the system attractive for commercial exploitation. The viability of these two organisms during long-term ripening and storage as well as their effect on cheese flavour and texture were determined. The chemical properties of the cheese were also determined. *L. acidophilus* strain Ki remained highly viable for at least 120 days of ripening, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days. The normal aerobic microflora of the cheese was not affected. Despite the indication that Cheddar cheese offers potential to be an effective vehicle for the delivery of the probiotic strains to the consumer and the survival of *L. acidophilus* at high numbers, the results indicated that the maturation time for matured Cheddar cheeses proved to be too long to support the survival of *Bifidiobacterium* species maintaining the probiotic minimum. Furthermore, the higher total population of starter organisms resulted in the development of sour off-tastes during ripening.

INTRODUCTION

Probiotics are described as a mono- or mixed culture of live microorganisms which, applied to man or animal (e.g. as dried cells or as a fermented product), beneficially effects the host by improving the properties of the indigenous microflora (Huis in't Veld and Havenaar, 1991). Probiotic bacteria assist with the maintenance and restoration of normal intestinal balance and are able to improve the symptoms of lactose intolerance in individuals with reduced ability to digest lactose into its component sugars, glucose and galactose (Sandine, 1979; Kim and Gilliland, 1983; Richardson, 1996). Some probiotic cultures have antitumorigenic activities resulting in a decreased risk of cancer (Richardson, 1996). They can also activate the hosts immune system and have the ability to produce vitamins (Richardson, 1996). In addition, a number of studies argued that some probiotic strains have the effect of lowering blood cholesterol levels (Gilliland and Speck, 1977; Marshall, 1996), but this health benefit remains a controversial topic. All these health benefits of probiotic-containing products, the so-called functional foods, are becoming a key factor affecting consumer choice. Fermented products should, however, contain > 10^6 cfu/ml of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cells at the time of consumption to be effective (Robinson, 1987; Rybka and Kailasapathy, 1995; Dave and Shah, 1997). It is therefore important to maintain viability of these organisms until consumption of the products in order to ensure any health aspects.

To date, the most popular food delivery systems for these cultures have been freshly fermented dairy foods, such as yoghurt, cultured milk, cultured buttermilk and powder preparations (Hoover, 1993; Ishibashi and Shimamura, 1993). The viability of bifidobacteria in products with a long shelf life at refrigeration temperatures is not satisfactory. They do not survive the low pH of fermented milks and the aerobic conditions of production and packaging (Rogers, 1991; Lourens and Viljoen, 2002). There are relatively few reports concerning cheese as a carrier of probiotic organisms to the human gastro- intestinal tract, although Cheddar cheese may offer certain advantages as a delivery system for live probiotic cultures (Stanton *et al*., 1998). Having a higher pH than the more traditional probiotic foods, it may provide a more stable milieu to support the long-term survival of probiotic organisms. Furthermore, the matrix and high fat content of the cheese may offer protection to the organisms

during passage through gastro-intestinal tract (Stanton *et al*., 1998). The oxygen toxicity problem experienced in milk may be overcome by introducing *Bifidobacterium* during a later stage of cheese making, such as milling or salting (Dinakar and Mistry, 1994). Such an expansion of the probiotic product range may offer a big marketing advantage to cheese industries in South Africa.

Despite the increased interest in probiotic dairy products, introducing bifidobacteria into the dairy food chain has proved to be difficult. The survival of bifidobacteria is determined by metabolic interactions with lactic acid bacteria starters, fermentation conditions and the storage and preservation temperatures of the dairy products (Modler *et al*., 1990). *B. bifidum* sp. strain Bo and *L. acidophilus* strain Ki survived well in Gouda cheese (Gomes *et al*., 1995), but had a significant effect on cheese flavour after nine weeks of ripening. Some strains of *Lactobacillus* survived well in Cheddar cheese during the ripening period (Gardiner *et al*., 1998; Stanton *et al*., 1998), while Bifidobacteria maintained its viability for at least six months in Cheddar cheese (Dinakar and Mistry, 1994). Bifidobacteria have also been used as the sole probiotic adjunct to produce cottage cheese (Blanchette *et al*., 1995; Blanchette *et al*., 1996; Riordan and Fitzgerald, 1998), Crescenza cheese (Gobbetti *et al*., 1998) and white brined cheese (Ghodussi and Robinson, 1996). Their survival ability varied between poor, moderate, acceptable, strain dependent and very good.

The use of Bifidobacteria and *L. acidophilus* as a probiotic mixture adjunct and as starter was only reported for semi-hard goat (Gomes and Malcata, 1998), Gouda (Gomes *et al*., 1995) and Argentinian Fresco (Vinderola *et al*., 2000) cheeses. The viability of the probiotic microorganisms was satisfactory in all these cheeses. Consequently, the objectives of this study were to explore the possibility of introducing *B. bifidum* and *L. acidophilus* into a South African commercial matured Cheddar cheese to study their viability during long-term ripening and storage. The effect of these two organisms on cheese flavour and texture as well as the chemical properties of the cheese was determined.

MATERIALS AND METHODS

Bacterial test strains

Ten units of the lactic acid bacteria starter strains (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus salivarius* spp. *thermophilus*), RAO 21 (Rhodia Foods, France) were used as starter culture for the production of matured Cheddar cheese. *Lactobacillus acidophilus* strain Ki and *Bifidobacterium bifidum* sp. strain Bo in the form of starter concentrates were obtained from CSK Food Enrichment BV (Leeuwarden, The Netherlands) and were added to the milk in the cheese vat 25 min after the addition of the starter culture during processing. The RAO 21 lactic acid starter (Rhodia Foods, France) was also applied for the production of a traditional matured Cheddar cheese as control.

Matured Cheddar cheese manufacture

Pasteurised cows milk (400 l) was used for the manufacture of matured Cheddar cheese at a pilot cheese plant in the Free State in South Africa. The procedure for cheese making was carried out as described by Kosikowski (1970a).

Sampling description

Samples were taken at selected points during the processing of matured Cheddar cheese – from the cheese vat, before renneting, at the end of the renneting process, after cutting, during and after cheddaring and after the salting process. The cheese was kept under controlled conditions (10-12°C) and sampled after 24 hrs, 4 d, 7 d, 10 d, 15 d and 20 d, followed by consecutive intervals of 10 d for 4 months. Similar samples were collected from the model cheese and the control cheese.

Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by opening the cheeses aseptically and cutting portions with a sterile knife. For each

sample, 10 g cheese were aseptically weighed and added to 90 ml of sterile Callichia's resuspension medium (CRM) (Callichia *et al*., 1993) into Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.). The samples were subjected to further serial decimal dilutions in 9 ml of sterile CRM (Callichia *et al*., 1993) and agitated by means of a vortex mixer. For the starter bacteria, the appropriate dilutions were plated in duplicate by the spread plate technique onto DeMann, Rugosa and Sharpe (MRS) plates (Oxoid, Basingstoke, U.K.) (selective for lactic acid bacteria) and incubated under aerobic conditions for 48 hrs at 25°C. For the probiotic bacteria, the appropriate dilutions were pour plated using the appropriate media. M-MRS agar (Hull and Roberts, 1984) was used for the differential enumeration of *Lactobacillus acidophilus* whereas NNLP agar (Laroia and Martin, 1991) was used for the enumeration of *Bifidobacterium bifidum.* Anaerobic incubation was carried out in anaerobic jars (Oxoid, Basingstoke, U.K.) at 37°C for 5 days for both the organisms. The anaerobic atmosphere was provided using Anaerocult A blocks (Merck, Darmstadt, Germany). An anaerobic indicator (Merck) was included in each jar.

Sample analysis

All plates containing between 25 and 150 colony forming units (cfu) on the highest dilution (or the highest number if below 25) were counted and the mean values determined. Results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions (2x2x3).

Physical and chemical analysis

Production of lactose, galactose, lactic acid, citric acid and acetic acid were determined by HPLC (Bouzas *et al*., 1991). Sampling for chemical analysis was performed as described for the microbial samples. On each sampling occasion, an additional 10 g of cheese were weighed into 10 ml of distilled water in Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.) for chemical analysis. The slurry was transferred to Eppendorf tubes with the addition of three drops of 30% H_2SO_4 and centrifuged at 5000 rpm for 5 min

The supernatant was then filtered using a 0.45 µm membrane filter (Millipore) into Eppendorf vials and stored at 4-5ºC until use (Roy *et al*., 1997). Sugar contents were measured by means of a Waters HPLC system with a biorad-aminex C42 Column and Refractive index detector (Bouzas *et al*., 1991). The organic acid contents were measured by means of a HPLC system equipped with a variable wavelength detector set at 220nm. 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 24ºC with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1970b).

Sensory analysis

The sensory quality of the model and control cheeses was judged by a panel of experts in the field of cheese evaluation, based on a standard protocol including openness, texture and taste at various intervals during the ripening period. Both cheeses were evaluated after 2 months and 4 months and the results compared.

RESULSTS AND DISCUSSION

Microbial populations

The lactic acid bacterial counts increased during the manufacturing of the cheese, from 1.46 x 10^7 cfu/g to a value of 2.24 x 10^8 cfu/g after the cheddaring process (Figs. 1 and 3). The lactic acid bacteria continued to grow during the ripening period and an increase of one logarithmic cycle was observed, reaching maximum viable numbers of 1.12 x 10^9 cfu/g after 50 days of ripening. This was followed by a gradual decrease in numbers to reach a final value of 1.46 x 10^8 cfu/g after 120 days of ripening (Figs. 1 and 3). The viability profile of the model cheese corresponds with the profile observed in the control cheese (Fig. 1). The presence of the probiotic cultures therefore did not affect the normal aerobic microflora of the cheese, although *Bifidobacterium bifidum* exhibits antibacterial activity (Anand *et al*., 1984). The normal survival of the starter culture corresponds with the work of Gobbetti *et al.* (1998) who reported that the addition of bifidobacteria did not change the gross composition of Crescenza cheese, nor did it affect the normal aerobic microflora of

the cheese. Dinakar and Mistry (1994) who added bifidobacteria to Cheddar cheese also reported that the presence of bifidobacteria in the cheese did not affect the normal aerobic microflora of the cheese.

Incorporation of bifidobacteria into the food chain can be difficult. These organisms are anaerobes and prefer to grow at temperatures of 37-41ºC and pH values of 6.5-7.0 (Scardovi, 1986). *B. bifidum* counts increased from 2.90 x 10^4 cfu/g in the milk to a value of 9.80 x 10^5 cfu/g after the cutting process (Figs. 2 and 3). The initial increase was followed by a decrease to a value of 8.80 x 10^4 cfu/g after salting of the curd. This decrease could be due to rigorous conditions such as cheddaring, salting and the presence of rapidly multiplying and metabolically active lactic acid bacteria (Dinakar and Mistry, 1994). During the first 24 h of ripening the viable counts increased again to reach a value of 1.83 x 10^6 cfu/g. The counts continued increasing during the subsequent 40 days until a value of 1.15 x 10^7 cfu/g was reached. The counts remained in the 10^6 log cycle for 70 days but then decreased to a value of 1.68 x 10^5 cfu/g after 120 days of ripening. Vinderola *et al*. (2000) reported bifidobacteria counts of 10^6 cfu/g after 60 days in Argentinian Fresco cheese. They found the survival of the bacteria being strain dependent. When *B. bifidum* sp. strain Bo was used in combination with *Lactobacillus acidophilus* strain Ki as the starter in Gouda cheese manufacture (Gomes *et al*., 1995), the final numbers after nine weeks (63 days) were still in excess of the suggested minimum of 10^6 cfu/g (Robinson, 1987; Rybka and Kailasapathy, 1995; Dave and Shah, 1997). According to Dave and Shah (1997) several factors have been claimed to affect the viability of bifidobacteria in fermented milk products. Acidity, pH, temperature of storage, oxygen content and the concentration of lactic and acetic acids may be responsible for reduced viability of these organisms.

The *L. acidophilus* strain grew and sustained high viability in cheese during the ripening period. The viable cell numbers of the organism increased during the manufacturing of the cheese, from 1.42 x 10^6 cfu/g in the milk to a value of 1.03 x 10^8 cfu/g after salting of the curd (Figs. 2 and 3). The counts continued increasing until a maximum count of 1.20 x 10^9 cfu/g was reached after 60 days of maturation. This was followed by a gradual decrease in cell numbers to a value of 1.11 x 10^8 cfu/g after 120 days of ripening. *L. acidophilus* could therefore survive for at least four months

in matured Cheddar cheese at a level higher than 10^6 cfu/g and consequently satisfy the criteria for a probiotic food product. Gomes *et al*. (1995) and Vinderola *et al*. (2000) reported decreases in numbers of some *L. acidophilus* strains during cheese ripening, although the final numbers were still above the therapeutic minimum after 60 days of ripening.

Physical and chemical analysis

During the processing of matured Cheddar cheese, the pH declined from 6.65 in the raw milk to a value of 5.32 after salting of the curd (Table 1; Fig. 4). This decrease in the pH value is attributed to the production of lactic acid by the mesophilic lactococci applied as starters, as well as by *Lactobacillus acidophilus* and *Bifidobacterium bifidum* applied as probiotic adjuncts (Kandler and Weiss, 1986; Scardovi, 1986). The production of small amounts of acetic acid by the probiotic microorganisms (Kandler and Weiss, 1986; Scardovi, 1986) also contributed to the decrease in pH. After this rapid decline, the pH decreased gradually to a value of 5.11 after 40 days of ripening followed by a further decrease to a value of 5.08 after 120 days of ripening. In the control cheese a pH of 5.40 was reached after salting of the curd followed by a decrease to a value of 5.28 after 40 days of ripening (Table 1). The pH of the control cheese remained constant for the remaining period of ripening reaching a final value of 5.29 after 120 days of ripening. The pH of the probiotic-inoculated cheese was therefore much lower compared to the control cheese throughout the ripening period. The lower pH is attributed to the presence of *L. acidophilus* and *B. bifidum* enhancing the production of organic acids in the model cheese (Table 1).

Lactose is the major sugar present in dairy products (Scott, 1981) being a disaccharide that is hydrolysed to glucose and galactose before entering the catabolic pathway for hexoses (Schlegel, 1993). All the lactose present in the milk was hydrolysed to glucose and galactose, with the lactose content being depleted during the cheddaring process (Table 1; Fig. 5). Both *B. bifidum* and *L. acidophilus* utilise lactose (Kandler and Weiss, 1986; Scardovi, 1986) resulting in an enhanced depletion of lactose compared to the control cheese (Table 1). In a study by Gomes *et al*. (1995) who used a starter entirely composed of *B. bifidum* sp. strain Bo and *L. acidophilus* strain Ki for the production of Gouda cheese, the lactose was virtually

exhausted 24 hrs after cheese production started. The depletion of lactose can therefore be attributed to the lactic acid bacteria as well as the probiotic starter adjuncts.

The glucose derived from the breakdown of lactose was utilised rapidly, whereas the galactose content of the cheese initially accumulated (Table 1; Fig. 5). The galactose content of 0.37% after cutting increased to a value of 1.20% after salting of the curd. At this stage, the utilisation of galactose was initiated, resulting in a gradual decrease and being depleted after 30 days of ripening (Table 1; Fig. 5). The control cheese still retained 0.11% galactose after 120 days of ripening. Both *L. acidophilus* and *B. bifidum* have the ability to utilise galactose (Kandler and Weiss, 1986; Scardovi, 1986) and therefore attributed to its depletion in the model cheese.

The production of lactic, acetic and citric acids was strongly related to the consumption of lactose and galactose during the ripening period. The lactic acid content increased rapidly from 0.07% in the milk to a value of 1.63% after the salting process (Table 1; Fig. 4). A further gradual increase in the lactic acid content of the cheese was observed until a value of 2.40% was reached after 120 days of ripening. This was higher than the 1.68% observed in the control cheese at that time and therefore contributed towards the lower pH and the development of a sour taste in the model cheese. At least half of the fermentation end products of *L. acidophilus* is lactic acid (Kandler and Weiss, 1986) while one third of the fermentation end products of *B. bifidum* comprises of lactic acid (Scardovi, 1986).

The acetic acid content varied between 0.03% and 0.2% during the ripening period (Table 1; Fig. 4). *B. bifidum* forms acetic- and lactic acid in the molar ratio of 3:2 (Scardovi, 1986). *B. bifidum* sp. strain Bo therefore actively contributed to the acidification of the cheese. The amount of acetic acid formed at any given time was not related to the growth of the organism. This corresponds with the findings of Desjardins *et al*. (1990) who observed the unrelatedness of growth and acid production by *B. bifidum*. They reported that the production of 70-75% of total acids by Bifidobacteria takes place in the stationary phase of growth, which may suggest metabolic activity by injured cells. Gomes *et al*. (1995) reported that a synergistic effect seems to exist between *Bifidobacterium* sp. strain Bo and *L. acidophilus* strain

Ki because the rate of acid production of the mixed culture is higher than the sum of the rates of production when the two strains are employed independently with the same inoculum sizes in cultured milk. The citric acid content of the cheese varied between 0.07% and 0.15% during the ripening period (Table 1; Fig. 4).

Sensory analysis

In Cheddar cheese, where probiotic bacteria have been incorporated, *Lactobacillus paracasei* (Gardiner *et al*., 1998; Stanton *et al*., 1998) and *Bifidobacterium. bifidum* (Dinakar and Mistry, 1994) were found to cause no adverse effects on sensory criteria while others (Broome *et al*., 1990; McSweeney *et al*., 1994; Lynch *et al*., 1996; Gardiner *et al*., 1999) found that probiotic adjuncts improved the flavour of Cheddar cheese. In a study by Gomes *et al*. (1995) however, Gouda cheese containing *Bifidobacterium* sp. srain Bo and *L. acidophilus* strain Ki was found to have organoleptic defects, probably due to the formation of acetic acid and added milk protein hydrolysate. In this study, matured Cheddar cheese with *L. acidophilus* and *B. bifidum* applied as starter adjuncts developed sour off-tastes. This is attributed to overacidification of the cheese by the higher number of fermenting microorganisms and to the formation of small amounts of acetic acid by *B. bifidum*.

CONCLUSION

The so-called ‘functional’ foods are becoming a key factor affecting consumer choice, and therefore the existing limited range of such products needs to be expanded. Incorporation of the probiotic adjuncts *Lactobacillus acidophilus* and *Bifidobacterium bifidum* into matured Cheddar cheese, as conducted in this study can be achieved without alteration of the cheesemaking technology, making this system attractive for commercial exploitation. A limited number of studies have demonstrated different cheeses being able to support the viability of certain Bifidobacteria and *Lactobacillus* strains. The data presented here, suggest that *L. acidophilus* strain Ki, remain highly viable for at least 120 days of ripening, while the numbers of *B. bifidum* sp. strain Bo remained above the therapeutic minimum for 70 days. The normal aerobic microflora of the cheese was not affected, but the higher number of fermenting organisms in the model cheese resulted in overacidification of the cheese with the resulting development of a sour taste. Cheddar cheese however, still offers potential to be an effective vehicle for the delivery of probiotic strains to the consumer, but more strains need to be evaluated.

Table 1. Analytical data of matured Cheddar cheese during processing and ripening over a period of 120 days.

Sampling time	pH	pH (control)	Sugars		Sugars (control)		Organic acids			Organic acids (control)		
			Lactose (%)	Galactose (%)	Lactose (%)	Galactose (%)	Lactic Acid (%)	Acetic acid (%)	Citric acid (%)	Lactic acid (%)	Acetic acid (%)	Citric acid (%)
Cheese vat (start)	6.65	6.62	4.73	0.00	4.72	0.00	0.07	0.08	0.09	0.58	0.00	0.15
Cheese vat (end)	6.58	6.59	4.63	0.00	4.83	0.00	0.33	0.19	0.13	0.60	0.00	0.15
Renneting (start)	6.58	6.59	4.31	0.00	4.85	0.09	0.49	0.14	0.11	0.69	0.00	0.15
Renneting (end)	6.54	6.57	3.57	0.00	4.69	0.17	0.45	0.10	0.09	0.62	0.00	0.15
Cutting	6.33	6.53	0.94	0.37	1.03	0.57	1.70	0.04	0.11	1.60	0.00	0.21
Cheddaring	5.70	6.00	039	0.97	1.05	0.97	1.96	0.03	0.16	1.24	0.00	0.20
Cheddaring (end)	5.37	5.48	0.00	1.16	1.06	1.61	1.79	0.07	0.13	1.20	0.00	0.19
Curd after salting	5.32	5.40	0.00	1.20	1.00	1.42	1.63	0.04	0.11	1.17	0.00	0.19
24 hours	5.31	5.28	0.00	0.82	0.91	1.13	1.59	0.05	0.11	1.11	0.00	0.16
4 days	5.18	5.27	0.00	0.86	-	-	2.10	0.06	0.15	-	0.00	-
7 days	5.11	5.26	0.00	0.79	-	-	1.81	0.03	0.07	-	0.00	-
10 days	5.10	5.25	0.00	0.65	0.04	1.06	1.54	0.03	0.06	1.14	0.00	0.15
15 days	5.12	5.26	0.00	0.35	0.00	1.00	1.98	0.03	0.08	-	0.00	-
20 days	5.11	5.27	0.00	0.39	0.00	-	1.81	0.03	0.09	-	0.00	-
30 days	5.12	5.29	0.00	0.00	0.00	0.56	1.98	0.04	0.08	1.31	0.00	0.16
40 days	5.11	5.28	0.00	0.00	0.00	-	1.82	0.03	0.07	-	0.00	-
50 days	5.01	5.27	0.00	0.00	0.00	-	2.08	0.05	0.08	-	0.00	-
60 days	5.02	5.27	0.00	0.00	0.00	0.49	2.48	0.12	0.12	1.58	0.00	0.18
70 days	5.08	5.26	0.00	0.00	0.00	-	2.33	0.15	0.11	-	0.00	-
80 days	5.03	5.27	0.00	0.00	0.00	-	2.25	0.06	0.11	-	0.00	-
90 days	5.10	5.26	0.00	0.00	0.00	0.13	2.49	0.06	0.11	1.64	0.00	0.20
100 days	5.08	5.27	0.00	0.00	0.00	-	2.75	0.06	0.10	-	0.00	-
110 days	5.03	5.27	0.00	0.00	0.00	-	2.39	0.04	0.10	-	0.00	-
120 days	5.08	5.29	0.00	0.00	0.00	0.11	2.40	0.07	0.08	1.68	0.00	0.21

- Data are the means of 3 repetitions

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ADDENDUM

CHAPTER 8

THE INCORPORATION AND SURVIVAL OF PROBIOTICS IN GOUDA CHEESE

ABSTRACT

As consumers become more familiar with probiotics, the demand for probiotic containing food products will grow. Manufacturers will have to respond by incorporating value added probiotic products in the market. The majority of probiotic foods already on the market are fresh products such as yogurt, which are consumed within days or weeks of manufacture. A limited number of studies have demonstrated different cheeses with longer ripening and storage periods as being able to support the viability of certain *Lactobacillus* and *Bifidobacterium* strains. Consequently, Gouda cheese was produced with *B. bifidum* (BLC-1 DSF) and *L. acidophilus* (LAC-1 DSF) as a mixture probiotic adjunct. This was done without altering the technological steps of the manufacturing process, therefore making the system attractive for commercial application. The viability of these two organisms during ripening and storage as well as their effect on cheese flavour and texture were determined. The chemical properties of the cheese were also determined. *L. acidophilus* remained highly viable for at least 120 days, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days. Antagonistic activities towards the lactic acid bacteria in the probiotic containing cheese were observed, but the effect was not significant. The probiotic starter adjunct did not affect the texture or appearance of the cheese compared to those of the control cheese, but contributed to the development of a bitter taste. The high numbers of *L. acidophilus* also resulted in the overacidification of the cheese. The results of the present study indicated that Gouda cheese offers potential to be an effective vehicle for the delivery of probiotic strains to the consumer, but the need to search for probiotc strains with no adverse effects on the sensory criteria of the cheese and probably the exclusion of *L. acidophilus* as an adjunct starter, remain.

INTRODUCTION

Probiotics are described as a single or mixed culture of live microorganisms and when applied to man or animal, beneficially effects the host by improving the properties of the indigenous microflora (Huis in't Veld and Havenaar, 1991). Human beings are daily exposed to potential health hazards and factors such as stress, unbalanced diet and antibiotic therapy, all factors that can alter the composition of the intestinal microflora (Rogelj *et al*., 2002). Probiotic bacteria are therefore important in the maintenance and restoration of the normal intestinal balance (Richardson, 1996). Other than the above, probiotics improve lactose intolerance and digestibility of milk products in people with a reduced ability to digest lactose into its component sugars, glucose and galactose (Sandine, 1979; Kim and Gilliland, 1983; Richardson, 1996) and have antitumorigenic activities reducing the risk of cancer (Richardson, 1996). Some strains also have the potential to enhance the human immune system and to produce vitamins (Richardson, 1996). Furthermore, claims are strong that certain probiotic strains have the ability to lower blood cholesterol levels (Gilliland and Speck, 1997). Foods containing such bacteria fall within the category of functional foods and are described as foods claiming to have a positive effect on health. In a modern world of health oriented nutritional habits, such products are rapidly gaining more popularity. The market for functional foods is presently very buoyant and is predicted to expand substantially in the coming decade (Stanton *et al*., 2001).

Probiotic foods currently consist of fresh products such as yogurt and fermented milk drinks which are generally consumed within days or weeks of manufacture (Ross *et al*., 2002). In contrast, a product such as cheese can have long ripening times and therefore the development of a probiotic cheese requires careful examination of the viability of the particular probiotic strains during ripening and storage of the product. For probiotic cultures to be effective in a product, they should maintain their viability in the food until the time of consumption and be present at levels of 10^6 cfu/g in the product (Robinson, 1987; Rybka and Kailasapathy, 1995; Dave and Shah, 1997). To perform their claimed benefits, the organisms must have the ability to survive passage through the gastrointestinal tract, survive the food manufacturing process and have the capacity to grow and survive during the ripening/storage period (Stanton *et al.*, 1998). Furthermore, they must not adversely affect the composition, texture or sensory

criteria of the cheese (Stanton *et al*., 1998). Some important characteristics of strains to consider include their degree of acid and oxygen sensitivity (Desjardins *et al*., 1990; Gomes and Malcata, 1998). Other factors affecting the viability of bifidobacteria in fermented foods include the pH, storage temperature and the concentration of lactic and acetic acids (Roy *et al*., 1997). These organisms are anaerobes and prefer to grow at temperatures of 37-41ºC and pH values of 6.5-7.0 (Scardovi, 1986). Cheese may offer certain advantages as a delivery system for live probiotics to the gastrointestinal tract like a denser matrix and high fat content, which may offer protection to the organisms during passage through the gastrointestinal tract. Furthermore, having a higher pH than the more traditional probiotic foods, cheese may provide a more stable milieu to support the long-term survival of probiotic organisms (Stanton *et al*., 1998).

Although an increased interest in probiotic dairy products occurred during the past few years, introducing bifidobacteria into the food chain has proved to be difficult (Modler *et al*., 1990). Limited studies have evaluated cheese as a carrier of bifidobacteria species. *Bifidobacterium bifidum* remained viable in Cheddar cheese for 24 weeks and did not affect the sensory criteria of the cheese (Dinakar and Mistry, 1994). McBrearty *et al*. (2001) demonstrated Cheddar cheese to be a suitable food system for the delivery of some commercially available bifidobacteria strains to the consumer. Bifidobacteria were also used as starter adjuncts to produce fresh cheese (Roy *et al*., 1997), white-brined cheese (Ghoddusi and Robinson, 1996), cottage cheese (Blanchette *et al*., 1996) and Crescenza cheese (Gobbetti *et al*., 1998). Variable ability to survive was observed in the different studies. *Lactobacillus paracasei* was found to sustain high viability in cheese during ripening, without any negative impact on cheese quality (Gardiner *et al*., 1998; Stanton *et al*., 1998). Rogelj *et al*. (2002) demonstrated that the *L. acidophilus* LF 221 strain remained viable during ripening and could be detected at levels above the therapeutic minimum for six weeks.

The use of Bifidobacteria and *L. acidophilus* as a probiotic mixture adjunct was only reported for Gouda (Gomes *et al*., 1995), Argentinian Fresco (Vinderola *et al*., 2000), and semi-hard goat (Gomes and Malcata, 1998) cheeses. When *L. acidophilus* strain KI and *Bifidobacterium* sp. strain Bo were incorporated as a mixture adjunct for the

manufacturing of matured Cheddar cheese (Chapter 7), *L. acidophilus* remained viable for at least 120 days of ripening, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days. The numbers of *B. bifidum* however, decreased significantly during the rigorous cheddaring process. The probiotic adjuncts also had a negative influence on the flavour and aroma of the cheese causing overacidification. Consequently, the objectives of this study were to incorporate *B. bifidum* (BLC-1 DSF) and *L. acidophilus* (LAC-1 DSF) into a South African commercial Gouda cheese to study their survival during the shorter ripening and storage period. The effect of these two organisms on cheese flavour and texture as well as the chemical properties of the cheese was also determined.

MATERIALS AND METHODS

Bacterial test strains

Twenty five units of the lactic acid bacteria starter strains (*Lactococcus lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus*), RAO 26 (Rhodia Foods, France) were used as starter culture for the production of Gouda cheese. *Lactobacillus acidophilus* (LAC-1 DSF) and *Bifidobacterium bifidum* (BLC-1 DSF) in the freeze-dried form were obtained from DSM Food Specialties (Australia) and were added to the milk in the cheese vat 10 min after the addition of the starter culture during processing. The RAO 26 lactic acid starter (Rhodia Foods, France) was also applied for the production of a traditional Gouda cheese as control.

Matured Cheddar cheese manufacture

Pasteurised cows milk (1000 l) was used for the manufacture of Gouda cheese at a cheese plant in the Free State in South Africa. The procedure for cheese making was carried out as described by Kosikowski (1970a).

Sampling description

Samples were taken at selected points during the processing of Gouda cheese – from the cheese vat, before renneting, at the end of the renneting process, after cutting, after the rinsing process, before and after pressing and after brining (24hrs).. The cheese was kept under controlled conditions (10-12°C) and sampled after 5 d, 10 d, 15 d and 20 d, followed by consecutive intervals of 10 d for 4 months. Sampling of the control cheese started after brining (24hrs) and was taken at the same intervals as for the model cheese.

Sampling procedure

Duplicate cheese samples were prepared for microbial analysis on each occasion by opening the cheeses aseptically and cutting portions with a sterile knife. For each sample, 10 g cheese were aseptically weighed and added to 90 ml of sterile Callichia's resuspension medium (CRM) (Callichia *et al*., 1993) into Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.). The samples were subjected to further serial decimal dilutions in 9 ml of sterile CRM (Callichia *et al*., 1993) and agitated by means of a vortex mixer. For the starter bacteria, the appropriate dilutions were plated in duplicate by the spread plate technique onto DeMann, Rugosa and Sharpe (MRS) plates (Oxoid, Basingstoke, U.K.) (selective for lactic acid bacteria) and incubated under aerobic conditions for 48 hrs at 25°C. For the probiotic bacteria, the appropriate dilutions were pour plated using the appropriate media. M-MRS agar (Hull and Roberts, 1984) was used for the differential enumeration of *Lactobacillus acidophilus* whereas NNLP agar (Laroia and Martin, 1991) was used for the enumeration of *Bifidobacterium bifidum.* Anaerobic incubation was carried out in anaerobic jars (Oxoid, Basingstoke, U.K.) at 37°C for 5 days for both the organisms. The anaerobic atmosphere was provided using Anaerocult A blocks (Merck, Darmstadt, Germany). An anaerobic indicator (Merck) was included in each jar.

Sample analysis

All plates containing between 25 and 150 colony forming units (cfu) on the highest dilution (or the highest number if below 25) were counted and the mean values determined. Results are the means of duplicate plate samples originated from duplicate cheese samples manufactured on three occasions (2x2x3).

Physical and chemical analysis

Production of lactose, galactose, lactic acid, citric acid and acetic acid were determined by HPLC (Bouzas *et al*., 1991). Sampling for chemical analysis was performed as described for the microbial samples. On each sampling occasion, an

additional 10 g of cheese were weighed into 10 ml of distilled water in Whirl Pak bags (Nasco, U.S.A.) and homogenised for 2 min using a Colworth 400 Stomacher (London, U.K.) for chemical analysis. The slurry was added to Eppendorf tubes together with three drops of 30% H_2SO_4 and centrifuged at 5000 rpm for 5 min. The supernatant was then filtered using a 0.45 µm membrane filter (Millipore) into Eppendorf vials and stored at 4-5ºC until use (Roy *et al*., 1997). Sugar contents were measured by means of a Waters HPLC system with a biorad-aminex C42 Column and Refractive index detector (Bouzas *et al*., 1991). The organic acid contents were measured by means of a HPLC system equipped with a variable wavelength detector set at 220nm. 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 24ºC with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1970b).

Sensory analysis

The sensory quality of the model and control cheeses were judged by a panel of experts in the field of cheese evaluation. The cheeses were evaluated for the characteristics "flavour/aroma" and "body/texture". Both cheeses were evaluated after 30 d, 50 d and 70 d and the results compared.

RESULTS AND DISCUSSION

Microbial populations

The lactic acid bacterial numbers increased during processing from 8.95 x 10^6 cfu/g in the milk to a value of 4.05 x10^8 cfu/g after pressing of the curd (Figs. 1 and 3). The numbers continued to increase until a maximum value of 1.25 x 10^9 cfu/g was reached after 40 days. At this stage the lactic acid bacterial numbers started to decrease slightly and declined to a value of 1.36 x 10^8 cfu/g after 120 days. The numbers of lactic acid bacteria in the control cheese increased throughout the ripening and storage period and reached a value of 2.2 x 10^8 cfu/g after 40 days (Fig. 1). The numbers continued to increase and reached a final value of 5.6 x 10^8 cfu/g after 120 days. The reason for the higher number of lactic acid bacteria in the control cheese may be due

to antagonistic activities exhibited by the probiotic bacteria towards the lactic acid bacteria in the model cheese. Antagonism is caused by bacteriocins, which are peptides or proteins exhibiting antibiotic properties (De Vuyst and Vandamme, 1994; Dodd and Gasson, 1994). Furthermore, the ability to produce bacteriocins is often discussed as a desirable property of probiotics (Salminen *et al*., 1996). The lactic acid bacterial numbers in both the model and control cheeses, however, remained in the 10^8-log cycle (Fig. 1) and therefore the degree of antagonism towards the lactic acid bacteria in the model cheese was not significant.

According to literature, the addition of bifidobacteria as a starter adjunct in Cheddar cheese may not be successful, considering the aerobic conditions of cheese making, the rigorous conditions of cooking and cheddaring and the presence of rapidly multiplying and metabolically active lactic acid bacteria (Dinakar and Mistry, 1994). During Gouda-making, however, with less rigorous conditions, the numbers of *Bifidobacterium bifidum* slowly increased during processing, reaching a value of 5.0 x 10^5 cfu/g after pressing of the curd (Figs. 2 and 3). *B. bifidum* continued to proliferate and reached a number of 1.25 x 10^6 cfu/g after 5 days of ripening. The numbers remained above the therapeutic minimum for 70 days followed by a decrease to reach a value of 7.1 x 10^4 cfu/g after 120 days. According to Dave and Shah (1997), several factors have been claimed to affect the viability of probiotic bacteria in fermented milk products. These include acidity, pH, hydrogen peroxide, temperature of storage, oxygen content and the concentration of lactic- and acetic acids. Furthermore antagonistic activities produced by lactic acid bacteria like benzoic acid and biogenic amines have also been indicated as possible inhibiting agents (Sieber *et al*., 1995; Desmazeaud, 1996; Weber, 1996; Leuschner *et al*., 1998). *B. bifidum* is an anaerobe and prefers to grow at temperatures of 37-41ºC and pH values of 6.5-7.0 (Scardovi, 1986). Despite the presence of the above-mentioned inhibiting factors, *B. bifidum* remained viable at levels above the therapeutic minimum for 70 days, which are the recommended shelf life of Gouda cheese in South Africa.

Lactobacillus acidophilus strains remained viable at numbers exceeding the therapeutic minimum at all times. The numbers increased during processing, reaching a value of 3.65 x 10^8 cfu/g after pressing of the curd (Figs. 2 and 3). The numbers continued to increase and reached a value of 1.57 x 10^9 cfu/g after 20 days of

ripening. This was followed by a slight decrease, but the numbers of *L. acidophilus* remained in the 10^8 log order for at least 120 days and consequently satisfied the criteria for a probiotic food product. Earlier studies (Gomes *et al*., 1995; Roy *et al*., 1997; Gardiner *et al*., 1998; Stanton *et al*., 1998 and Vinderola *et al*., 2000) also reported the viability of different probiotic *Lactobacillus* strains in various dairy products.

Physical and chemical analysis

During processing of Gouda cheese, the pH decreased from 6.82 in the pasteurised milk to a value of 5.22 after pressing of the curd (Table 1; Fig. 4). This decrease is attributed to the production of organic acids, especially lactic acid, by the thermo-mesophilic bacteria applied as starter cultures and *Lactobacillus acidophilus* and *Bifidobacterium bifidum* applied as probiotic adjuncts. The pH remained constant for the initial 50 days followed by an increase to reach a value of 5.40 after 120 days. This increase could be due to contaminating microorganisms utilising the lactic acid, considering the shelf life of Gouda cheese only being 70 days. In the control cheese, a pH of 5.20 was reached after brining (Table 1). The pH also remained constant for the first 50 days, followed by an increase to a value of 5.48 after 120 days. The addition of the probiotic bacteria as adjuncts during cheesemaking therefore did not have any significant effect on the pH of the cheese.

The major sugar present in dairy products, lactose (Scott, 1981), was hydrolysed to its component sugars glucose and galactose being depleted after pressing of the curd (Table 1; Fig. 5). Both *B. bifidum* and *L. acidophilus* utilise lactose (Kandler and Weiss, 1986; Scardovi, 1986) and therefore contributed to the depletion of the lactose content. Gomes *et al*. (1995) who use a starter entirely composed of *L. acidophilus* and *B. bifidum* for the production of Gouda cheese reported that lactose was virtually exhausted 24 hours after cheese production.

The glucose derived from the breakdown of lactose was rapidly utilised, whereas the galactose content initially accumulated in the cheese (Table 1; Fig. 5). The galactose content of 0.01% in the milk remained constant but increased after the cutting process to reach a value of 0.77% after pressing of the curd. At this stage the utilisation of

galactose was initiated, resulting in a rapid decrease being depleted after 5 days of ripening (Table 1; Fig. 5). Both *L. acidophilus* and *B. bifidum* have the ability to utilise galactose (Kandler and Weiss, 1986; Scardovi, 1986) and therefore attributed to its depletion. The sugar consumption in the control cheese corresponded to those in the model cheese (results not shown).

The production of lactic acid was strongly related to the depletion of the sugars during the ripening period. The lactic acid content increased from 0.12% in the milk, to a value of 2.20% after pressing of the curd (Table 1; Fig. 4). A further gradual increase was observed until a value of 2.88% was reached after 60 days followed by a decrease to reach a value of 2.33% after 120 days. Since at least half of the fermentation end products of *L. acidophilus* (Kandler and Weiss, 1986) and one third of *B. bifidum* (Scardovi, 1986) are lactic acid, both species contributed to the acidification of the cheese. This is also confirmed by the lactic acid content of the control cheese only being 1.49% after 120 days (Table 1). Gomes *et al*. (1995) reported that a synergistic effect seems to exist between *Bifidobacterium* sp. strain Bo and *L. acidophilus* strain Ki, because the rate of acid production of the mixed culture is higher than the sum of the rates of production when the two strains are employed independently with the same inoculum sizes in cultured milk.

The acetic acid content varied between 0.02% and 0.08% during the first 70 days of ripening and storage (Table 1; Fig. 4) and therefore did not affect the flavour, texture or appearance of the cheese during the shelf life period of the cheese. It increased to a value of 0.17% after 120 days. *B. bifidum* forms acetic- and lactic acid in the molar ratio of 3:2 (Scardovi, 1986) and therefore contributed to the acidification of the cheese, although it was not evident of any vigorous metabolic activity. The lack of metabolic activity can be attributed to low temperature ripening, low pH values due to the accumulation of lactic acid and to competition with other metabolically active microorganisms in the cheese. Furthermore, the amount of acetic acid formed at any given time, was not related to the growth of the organism. This corresponds with the findings of Desjardins *et al*. (1990) who observed the unrelatedness of growth and acid production by *B. bifidum*. They reported that the production of more than 70% of the total acids by bifidobacteria take place in the stationary phase of growth, which may suggest metabolic activity by injured cells. The citric acid content of the cheese

varied between 0.01 and 0.12% during ripening and storage of the cheese (Table 1; Fig. 4).

Sensory analysis

In Cheddar cheese, whith the addition of probiotic bacteria like *Lactobacillus paracsei* (Gardiner *et al*., 1998; Stanton *et al*., 1998) and *Bifidobacterium. bifidum* (Dinakar and Mistry, 1994), no adverse effects on sensory criteria were detected while others (Broome *et al*., 1990; McSweeney *et al*., 1994; Lynch *et al*., 1996; Gardiner *et al*., 1999) found that probiotic adjuncts improved the flavour of Cheddar cheese. In a study by Gomes *et al*. (1995), however, Gouda cheese containing *Bifidobacterium* sp. strain Bo and *L. acidophilus* strain Ki was found to have organoleptic defects, probably due to the formation of acetic acid and added milk protein hydrolysate. In this study, Gouda cheese with high levels of *L. acidophilus* and lower levels of *B. bifidum* applied as starter adjuncts was found to develop a bitter taste after 50 days. Bitter tastes usually develop in dairy products due to the formation of biogenic amines, which involves the decarboxylation of the carboxyl group of an amino acid and the liberation of the corresponding amine (Joosten, 1988). Furthermore, higher levels of lactic acid was detected in the model cheese compared to the control, probably due to the high numbers of *L. acidophilus* contributing to the overacidification of the cheese (Kandler and Weiss, 1986). The results indicated the need to search for probiotic strains with no adverse effects on the sensory criteria of cheese and probably the exclusion of *L. acidophilus* as an adjunct starter.

CONCLUSION

Cheese offers advantages as a food delivery system for probiotic bacteria to the human gastrointestinal tract. A limited number of studies have demonstrated that cheese can support the viability of certain *Bifidobacterium* and *Lactobacillus* strains. Results obtained proved the effectiveness of adding *B. bifidum* (BLC-1 DSF) and *L. acidophilus* (LAC-1 DSF), two commercially available probiotic strains, during the manufacture of Gouda cheese without any alteration of the cheesemaking technology. *L. acidophilus* remained highly viable for at least 120 days, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days. Considering the shelf life of Gouda cheese only being 70 days, the survival of both organisms satisfied the criteria for a probiotic food product. However, despite a similar texture and appearance, a bitter taste developed in the model cheese probably due to the high numbers of *L. acidophilus*, which also resulted in the overacidification of the cheese. The results indicate that Gouda cheese offers potential to be an effective vehicle for the delivery of probiotic strains to the consumer, but the need to search for probiotc strains with no adverse effects on the sensory criteria of the cheese and probably the exclusion of *L. acidophilus* as an adjunct starter, remain.

Table 1. Analytical data of Gouda cheese during processing and ripening over a period of 120 days.

Sampling time	pH	pH (control)	Sugars		Organic acids			Organic acids (control)		
			Lactose (%)	Galactose (%)	Lactic acid (%)	Acetic acid (%)	Citric acid (%)	Lactic acid (%)	Acetic acid (%)	Citric acid (%)
Cheese vat (start)	6.82	-	4.70	0.01	0.12	0.02	0.00	-	-	-
Addition of probiotics	6.80	-	4.60	0.01	0.12	0.02	0.00	-	-	-
Renneting (start)	6.74	-	4.06	0.01	0.09	0.02	0.00	-	-	-
Renneting (end)	6.74	-	4.15	0.01	0.12	0.02	0.00	-	-	-
Cutting end (whey off)	6.69	-	3.85	0.02	0.14	0.02	0.00	-	-	-
Rinse end (water off)	6.52	-	1.49	0.14	0.46	0.02	0.00	-	-	-
Before press	6.36	-	1.21	0.19	0.57	0.02	0.00	-	-	-
After press	5.22	-	0.02	0.77	2.20	0.02	0.00	-	-	-
After brining (24 hrs)	5.23	5.20	0.00	0.22	2.21	0.02	0.01	1.63	0.01	0.06
5 days	5.22	5.22	0.00	0.00	2.44	0.02	0.01	2.42	0.01	0.04
10 days	5.22	5.24	0.00	0.00	2.35	0.02	0.01	2.39	0.01	0.02
15 days	5.23	5.24	0.00	0.00	2.54	0.02	0.01	2.31	0.01	0.08
20 days	5.20	5.24	0.00	0.00	2.85	0.02	0.01	2.19	0.01	0.10
30 days	5.19	5.23	0.00	0.00	2.80	0.03	0.05	2.25	0.01	0.12
40 days	5.22	5.22	0.00	0.00	2.90	0.03	0.09	1.48	0.01	0.07
50 days	5.24	5.26	0.00	0.00	2.88	0.06	0.08	1.56	0.02	0.08
60 days	5.30	5.34	0.00	0.00	2.88	0.07	0.12	1.83	0.03	0.09
70 days	5.45	5.33	0.00	0.00	2.38	0.08	0.11	1.33	0.02	0.15
80 days	5.46	5.44	0.00	0.00	2.44	0.13	0.09	1.53	0.04	0.20
90 days	5.40	5.52	0.00	0.00	2.19	0.14	0.09	1.40	0.03	0.20
100 days	5.47	5.51	0.00	0.00	2.32	0.12	0.10	1.64	0.04	0.19
110 days	5.44	5.50	0.00	0.00	2.16	0.13	0.08	1.87	0.06	0.30
120 days	5.40	5.48	0.00	0.00	2.33	0.17	0.10	1.49	0.05	0.31

* Data are the means of 3 repetitions

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ADDENDUM

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

A worldwide market increase in the demand for and consequent production of cheese and cheese products evolved since the early 1900's. This resulted in reduced cheese manufacturing times and increased milk throughput which consequently intensified the demand for improved starter cultures. Numerous studies have been conducted to accelerate cheese ripening, urged by the economic advantages of a rapid development of stronger cheese flavour in a shorter time (Law, 1984). Since lactic acid bacterial starters are inhibited by unfavourable environmental conditions such as low pH-values (McSweeney *et al*., 1994) and high salt concentrations (McSweeney *et al*., 1994; Laubscher and Viljoen, 1999) the inclusion of yeasts as adjunct starter cultures was proposed (Devoyod, 1990; Fleet, 1990; Welthagen and Viljoen, 1998; Laubscher and Viljoen, 1999; Suzzi *et al*., 2001).

Yeasts grow under conditions unfavourable to many bacteria (Fleet and Mian, 1987; Seiler and Busse, 1990; Fleet, 1992). The proteolytic and lipolytic activity of certain yeast species, possible microbial interactions, their inhibitory activity against spoilage organisms (Loretan *et al*., 1998), their ability to produce vitamins (Purko *et al*., 1951) and amino acids (Wyder *et al*., 1999) and the utilisation of lactic acid with a resulting higher pH (Lenoir, 1984; Wyder *et al*., 1999), all make yeasts potentially viable organisms for use as starter cultures in the dairy industry. Consequently, it was considered relevant to study the potential of different yeast species as agents for accelerated ripening of matured Cheddar cheese.

In addition to accelerated cheese production, consumers are becoming more concerned about nutrition and health properties, and therefore a health promoting niche was established with the introduction of functional foods. Probiotics, as part of the functional foods, are a mono- or mixed culture of live microorganisms which, applied to human or animal, beneficially effect the host by improving the properties of the indigenous microflora (Huis in't Veld and Havenaar, 1991). Yoghurt and fermented milks have received considerable attention as carriers of live probiotic cultures. With the growing consumer awareness of the importance of balanced and

varied diets for the maintenance of good health, a demand for new food products with proven health claims was pursued. Cheddar cheese may offer certain advantages as a carrier system for live probiotics to the gastro-intestinal tract of humans. If probiotic containing cheese can be produced with little or no alteration to the cheese making technology, it would make the development of probiotic cheeses attractive for commercial exploitation. The successful incorporation of probiotic cultures into cheese will expand the probiotic product range whereas the cheese industries will benefit from a marketing advantage such as value-added probiotic containing cheeses.

1. The role of *Debaryomyces hansenii* in the ripening of matured Cheddar cheese

The possibility of including *Debaryomyces hansenii* as part of the starter culture for cheese production was proposed due to the species ability to stimulate cheese ripening by means of lipolytic and proteolytic activities (Devoyod, 1990; Fleet, 1990). Welthagen and Viljoen (1998) and Laubscher and Viljoen (1999) suggested further research on the possibility of including *Debaryomyces hansenii* as part of cheese starter cultures due to its great resistance against high salt concentrations, low temperatures and proliferating activities. The species control the pH of the cheese by utilising the lactic and acetic acid available (Devoyod, 1990; Fleet, 1990) and has the ability to inhibit the germination of undesired microorganisms in cheeses (Fatichenti *et al*., 1983).

In this study, the potential of *D. hansenii* as an agent for the accelerated ripening of matured Cheddar cheese was evaluated. The interaction between the yeast and the lactic acid bacteria starter cultures as well as the physical and chemical properties of the cheese were determined. The species had the ability to grow and compete with other naturally occurring yeasts in the cheese as well as with the lactic acid bacteria without any inhibition of the starter culture. The yeast contributed to the very good texture and body of the cheese, although a slightly bitter taste developed. This was a major constraint and was partly due to the addition of insufficient salt, which could have resulted from continued hydrolysis of protein and fat with resulting bitter flavours (Thomas and Pearce, 1981).

2. The role of *Yarrowia lipolytica* in the ripening of matured Cheddar cheese

Yarrowia lipolytica occurs frequently in milk products and the species has the ability to predominate in real system over the naturally occurring yeasts (Guerzoni *et al*., 1998). Its compatibility with starter cultures and possible stimulating action when co-inoculated have been evidenced by Guerzoni *et al*. (1998) and Van den Tempel and Jakobsen (2000). The species is further known for its exceptional strong proteolytic and lipolytic activity (Choisy *et al*., 1987; Roostita and Fleet, 1996; Wyder and Puhan, 1999a, b; Guerzoni *et al*., 2001; Suzzi *et al*., 2001**).** Based on these features, it was possible to accelerate cheese ripening and to improve the quality of cheese by the addition of the species (Lenoir *et al*., 1985; Devoyod, 1990) and therefore *Y. lipolytica* is regarded as a good candidate as a ripening agent in cheese (Guerzoni *et al*., 1998).

In this study, the potential of *Y. lipolytica* as an agent for the accelerated ripening of matured Cheddar cheese was evaluated. The interaction between the yeast and the lactic acid bacteria as well as the physical and chemical properties of the cheese were determined in order to evaluate the contribution of the yeast to cheese production. The yeast grew and competed with the lactic acid bacteria without any inhibition of the starter culture but died off after 5 months of maturation. The yeast contributed to the accelerated formation of the desired Cheddar flavour. Although a slightly fruity flavour was detected, the body, flavour and texture of the matured cheese granted it with a very good overall impression. *Y. lipolytica* may be regarded as a co-starter for cheesemaking.

3. Co-inoculation of *Debaryomyces hansenii* and *Yarrowia lipolytica* as potential starter cultures in the making of matured Cheddar cheese

The possibility of using *Debaryomyces hansenii* and *Yarrowia lipolytica* as adjunct starter cultures for cheese production was proposed due to the species positive attributes to cheese ripening (Devoyod, 1990; Fleet, 1990). Both species stimulate cheese ripening by means of their proteolytic and lipolytic activities (Fleet and Mian, 1987; Wyder and Puhan, 1999 a, b). They also control the pH by the utilisation of the lactic and acetic acid present (Devoyod, 1990; Fleet, 1990) thereby encouraging the

growth of the lactic acid starter bacteria essential for cheese ripening. According to a study by Wyder *et al*. (1999), the combination of *D. hansenii* and *Y. lipolytica* might have a positive effect on flavour development in cheese. Furthermore, a synergistic effect between lactic acid bacteria and *D. hansenii* with a resulting prolonged survival of the lactic acid bacteria has been reported by Yamauchi *et al*. (1975), whereas *Y. lipolytica* exerts possible stimulating action when co-inoculated with lactic acid bacteria (Guerzoni *et al*., 1998).

Consequently in this study, we endeavoured to apply both species, *D. hansenii* and *Y. lipolytica*, as part of the starter culture for the production of matured Cheddar cheese on industrial scale. The growth profiles of the two yeasts and the lactic acid bacteria were monitored, as well as the physical and chemical properties of the cheese in order to evaluate the contribution of the yeasts to cheese production. Both yeast species had the ability to grow and compete with each other, with the other natural occurring yeasts in the cheese and with the lactic acid bacteria without inhibition of the starter culture. A remarkable viability and actual increase in the lactic acid bacterial counts was observed throughout the ripening period, encouraged by the growth of yeasts, providing growth factors to the starter bacteria. The cheese had a good, strong flavour after 4 months of ripening with a very good texture and body attributed to the enhanced growth rate of the lactic acid bacteria. The model cheese retained this good flavour and texture after nine months, while the control cheese at that time, developed off flavours. The use of *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese, with resulting accelerated ripening, was proposed.

4. Yeasts as adjunct starters in matured Cheddar cheese

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In this chapter, results obtained from the previous chapters (Chapter 2, 3 and 4) were summarized and compared to evaluate the contribution of using different combinations of yeasts and lactic acid bacteria. Previous results indicated that yeasts could be included as part of starter cultures for the manufacturing of cheese, enhancing flavour development during the maturation. The potential of *D. hansenii*

and *Y. lipolytica* as agents for accelerated ripening of matured Cheddar cheese has been evaluated during four cheese treatments. The interaction between the two yeast species and the lactic acid bacteria was surveyed incorporating (i) *D. hansenii*, (ii) *Y. lipolytica* and (iii) both species as adjuncts to the starter culture and (iv) a control cheese without any additions for the production of matured Cheddar cheese. The physical and chemical properties of the cheeses during all combinations were compared to evaluate the contribution of the yeasts as single species or when co-inoculated to cheese maturation. The yeasts grew in association with the lactic acid bacteria without any inhibition. The yeasts species, when individually added, contributed to the development of bitter and fruity flavours despite accelerated development of strong Cheddar flavours. When both species were incorporated as part of the starter culture, the cheese had a good strong flavour after a reduced ripening period. The cheese retained this good flavour and aroma nine months after production. The simultaneous application of *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese is proposed.

5. Changes in fatty acid contents present in matured Cheddar cheese with the addition of *Debaryomyces hansenii* and *Yarrowia lipolytica* as adjunct starters

Based on the positive contribution of *Debaryomyces hansenii* and *Yarrowia lipolytica* in dairy products, these two strains were incorporated together as part of the starter culture for the production of matured Cheddar cheese. An enhanced development of a strong Cheddar flavour was observed in the model cheese when it was compared to those of the control cheese. The cheese had a clean, slightly sweet, pleasant taste and retained its good strong flavour after nine months, while the control cheese developed a bitter and slightly impure taste at that time. Free fatty acids (FFA) are important components in the flavour of many cheese types and are also precursors of aroma compounds such as methyl ketones, secondary alcohols, lactones and esters (Choisy *et al*., 1986; Schrödter, 1990; Ha and Lindsay, 1991; Molimard and Spinnler, 1995). Excessive quantities of FFA are, however, associated with hydrolytic rancidity flavour defects in cheese (McDaniel *et al*., 1969; Arnold *et al*., 1975; Bynum *et al*., 1984).

Consequently, the aim of this study was to determine the profile of the total lipids as well as the FFA present in a matured Cheddar cheese co-inoculated with both *D. hansenii* and *Y. lipolytica* as adjunct starters. These data were statistically compared with the control cheese produced by the standard procedure under similar conditions to establish the effect of the application of yeasts on the lipid profiles of the cheese. Significant lower levels of FFA in the model cheese compared with the control cheese indicated the significance of lipolysis and FFA breakdown by the yeasts. Furthermore, all of the C14:1 and C16:1 in the model cheese were transformed into their relative aroma compounds, while the presence of γC18:3 only in the model cheese, designates the enzymatic activities of the two yeast species. The lipolytic activity of the yeasts probably contributed to the enhanced development of a matured taste in the model cheese when compared to the control cheese.

6. The incorporation and survival of probiotics in matured Cheddar cheese

The beneficial effects of probiotic bacteria have been well established (Rybka and Kailasaphathy, 1995) and probiotic-containing foods are becoming a key factor affecting consumer choice. To date, the most popular food delivery systems for these cultures have been freshly fermented dairy foods (Hoover, 1993; Ishibashi and Shimamura, 1993). Bifidobacteria, however, failed to remain viable to reach the therapeutic minimum of 10^6 cfu/ml at the time of consumption of these products. Cheddar cheese may offer certain advantages as a carrier system for live probiotic cultures. The higher pH, and the matrix and high fat content of cheese may offer protection to the organisms and support their long-term survival (Stanton *et al*., 1998). The oxygen toxicity problem experienced in milk may be overcome in cheese (Dinakar and Mistry, 1994). Such an expansion of the probiotic product range may offer a big marketing advantage to cheese industries in South Africa.

The objectives of this study were to explore the possibility of introducing *Bifidobacterium bifidum* sp. strain Bo and *Lactobacillus acidophilus* strain Ki into a South African commercial Cheddar cheese to study their viability during long-term ripening and storage. The effect of these two organisms on cheese flavour and texture as well as the chemical properties of the cheese was determined. This was done

without alteration of the cheesemaking technology, which makes the system attractive for commercial exploitation. *L. acidophilus* remained highly viable for at least 120 days of ripening, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days. The normal aerobic microflora of the cheese was not affected, but the higher number of fermenting organisms in the model cheese resulted in overacidification of the cheese, with the resulting development of a sour taste. Cheddar cheese, however, still offers potential to be an effective vehicle for the delivery of probiotic strains to the consumer, but more strains need to be evaluated.

7. The incorporation and survival of probiotics in Gouda cheese

For probiotic cultures to be effective in a product, they should maintain their viability in the food until the time of consumption and be present at levels of 10^6 cfu/g in a product (Robinson, 1987; Rybka and Kailasapathy, 1995; Dave and Shah, 1997). Factors affecting the viability of probiotic strains in fermented foods include the pH, storage temperature, the concentration of lactic and acetic acids and the quantity of oxygen present (Scardovi, 1986; Roy *et al*., 1997).

The objectives of this study were to incorporate *B. bifidum* (BLC-1 DSF) and *L. acidophilus* (LAC-1 DSF) into a South African commercial Gouda cheese to study their survival during the shorter ripening and storage period. The effect of these two organisms on cheese flavour and texture as well as the chemical properties of the cheese was determined. *L. acidophilus* remained highly viable for at least 120 days, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days. Considering the shelf life of Gouda cheese only being 70 days, the survival of both organisms satisfied the criteria for a probiotic food product. The probiotic starter adjunct did not affect the texture or appearance of the cheese compared to those of the control cheese, but a bitter taste developed. The results indicated that Gouda cheese offers potential to be an effective vehicle for the delivery of probiotic strains to the consumer, but the need to search for probiotic strains with no adverse effects on the sensory criteria of the cheese and probably the exclusion of *L. acidophilus* as an adjunct starter, remain.

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CHAPTER 10

SUMMARY

A literature review dealing with various aspects of cheesemaking and ripening is given in Chapter 1. With respect to starter cultures, the criteria for strain selection as well as the taxonomy of different starter strains were discussed. Cheese ripening is a complex system that involves numerous chemical, physical and bacteriological changes and can be accelerated by various techniques. Special reference was made to yeasts associated with dairy products, their characteristics as well as their contribution in the dairy industry. Yeasts play a very important role in the ripening and manufacturing of a wide variety of cheeses and can cause spoilage or have beneficial effects on cheese ripening. Furthermore, the properties of probiotic bacteria were discussed including their therapeutic value, their survival in bio-products and the possible expansion of the probiotic product range. Cheese offers certain advantages as a delivery system for live probiotic bacteria to the human gastro-intestinal tract.

Debaryomyces hansenii and *Yarrowia lipolytica* are typical foodborne yeast species frequently associated with dairy products and capable of predominating the yeast composition in such systems. The two species fulfil a number of criteria to be regarded as co-starters for cheese making. They are known for their proteolytic and lipolytic activity as well as their compatibility and stimulating action with the lactic acid starter cultures when co-inoculated. Recent studies indicated that yeasts could be included as part of starter cultures for the manufacturing of cheese, enhancing flavour development during the maturation. The potential of *D. hansenii* and *Y. lipolytica* as agents for accelerated ripening of matured Cheddar cheese has been evaluated during four cheese treatments. The interaction between the two yeast species and the lactic acid bacteria was surveyed incorporating (i) *D. hansenii*, (ii) *Y. lipolytica* and (iii) both species as adjuncts to the starter culture and (iv) a control cheese without any additions for the production of matured Cheddar cheese. The physical and chemical properties of the cheeses were monitored in order to evaluate the contribution of the yeasts to cheese maturation. The yeasts grew in association with the lactic acid bacteria without any inhibition. An enhanced viability of the lactic acid starter

bacteria was observed. The yeasts species when individually added contributed to the development of bitter and fruity flavours despite accelerated development of strong Cheddar flavours. When both species were incorporated as part of the starter culture, the cheese, however, had a good strong flavour after a reduced ripening period. The cheese retained the good flavour and aroma for nine months after production. Furthermore, the significant differences in the fatty acid contents of the model and control cheeses designated enhanced enzymatic activities in the model cheese, attributed to the presence of the two yeast species in the model cheese. The simultaneous application of *D. hansenii* and *Y. lipolytica* as part of the starter culture for the production of matured Cheddar cheese is proposed.

The health benefits of probiotic-containing products are becoming a key factor affecting consumer choice, and therefore the existing limited range of such products needs to be expanded. Cheese may offer certain advantages as a carrier system for live probiotic organisms to the human gastro-intestinal tract. The possibility of introducing strains of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* into South African commercial Cheddar and Gouda cheese were explored. The organisms viability during long-term ripening and storage, their effect on cheese flavour and texture as well as the chemical properties of the cheese were determined. *L. acidophilus* remained highly viable for at least 120 days of ripening, while the numbers of *B. bifidum* remained above the therapeutic minimum for 70 days in both cheeses, satisfying the criteria for probiotic foods in Gouda cheese with its shorter shelf life. The viability profile of the lactic acid starter bacteria was not affected and a normal good cheese texture and appearance were retained. Both Cheddar and Gouda cheese offer potential to be effective vehicles for the delivery of probiotic strains to the consumer despite the development of sour and bitter tastes. Additional strains need to be selected and evaluated to prevent these constraints.

Keywords: *Debaryomyces hansenii*; *Yarrowia lipolytica*; co-inoculum; accelerated cheese ripening; fatty acids analysis; *Lactobacillus acidophilus*; *Bifidobacterium bifidum*; functional foods