MICROBIAL DEVELOPMENT AND INTERACTION IN BLUE VEINED CHEESES

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MICROBIAL DEVELOPMENT AND INTERACTION IN BLUE VEINED CHEESES

by

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'All we know is still infinitely less than all that still remains unknown'

William Harvey, 1578-1657, English physician
(*De Motu Cordis et Sanguinis,* 1628)

This dissertation is dedicated to my dad, John David Myles Knox.

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LIST OF ABBREVIATIONS

a_w:	water activity
cfu:	colony forming units
Fig(s):	Figure(s)
g:	gram
h:	hour
HPLC	high-performance liquid chromatography
hrs:	hours
l:	litres
min:	minute(s)
ml:	milliliter
mM:	millimol
pH	hydrogen ion concentration
w/v:	weight per volume
w/w:	weight per weight

LIST OF PUBLICATIONS

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Chapter 1

Introduction and literature review

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1. INTRODUCTION

Cheese production and consumption can be dated back to Biblical times (Fox, 1993). From a simple means of preserving milk, cheese has evolved to become a highly nutritious food often associated with *haute cuisine* (Kosikowski, 1977; Law, 1981; Scott, 1986).

The ripening of cheese involves the interaction of a large number of microorganisms, each inducing very typical flavours to the cheese. Yeasts are found mostly associated with the surface microflora of cheese, however little is known about their contribution to the ripening of the cheese and their interactions with the starter cultures and other moulds present in the cheese. Due to their ability to tolerate low pH, temperatures and water activity values, as well as their tolerance against increased salt concentrations, conditions considered unfavourable to many bacteria (Fleet and Mian, 1987), the occurrence of yeasts in cheese is not unusual (Fleet, 1990a). Yeast counts of 10^5-10^6 cells/g and even as high as 10^7-10^8 cells/g have been reported in some varieties of cheeses (Fleet, 1990a). Depending on the type of cheese, yeasts can contribute negatively to the spoilage of the cheese or may have a positive contribution, by improving flavour development during the maturation stages (Fleet, 1990a; Fleet and Mian, 1987).

The ability of some yeasts to: inhibit undesired microorganisms, support the starter culture due to lipolytic and proteolytic activity, utilise the lactic acid present, thereby increasing the pH, produce growth factors that support the starter cultures and gas which leads to the openness of the curd are all positive aspects of the role of yeasts in cheese. However, yeasts also have a negative contribution to cheese in terms of spoilage organisms. Typical problems include the excessive production of gas, a fruity flavour, increased acidity, changes in texture, as well as the production of bitter and rancid flavours (Horwood *et al.*, 1987; Walker, 1988).

Very little attention has been focused on the possible pathogenic effects of yeasts in dairy products. While there is a vast amount of literature on the pathogenic yeasts found in other foods, little is known about the pathogenic yeasts in dairy products, more specifically in cheeses. The origin of sources of yeasts that may cause contamination in cheese include the raw milk (Fleet and Mian, 1987), cheese factories, the working environment and the workers (Welthagen and Viljoen, 1998), starter culture inoculums, the brine (Viljoen and Greyling, 1995) as well as the rennet (Martínez *et al.*, 1986).

An additional problem encountered with South African blue-mould cheeses is the presence of *Brevibacterium linens*. This bacterium causes an orange-reddish discolouration on the outer layer of cheeses (Chapman and Sharpe, 1981). In addition, lipases produced by *B. linens* release volatile fatty acid components from the triacylglycerols resulting in an off-flavour (Hosono, 1986). Although most European countries favour the growth of this bacterium and the resulting taste, South African consumers reject cheeses with this flavour and discolouration. Hence *B. linens* needs to be eliminated from the final cheese products.

2. AIMS

- Evaluation and selection of a medium suited for the isolation and enumeration of yeasts in the presence of moulds.
- Study of the general microbial diversity present in blue veined cheeses, including the isolation and identification of all yeasts present in the cheese at the time of investigation.
- Investigation into the problems associated with *Brevibacterium linens* involving trials with radical water, Antibac B and Diverson products, with the aim of reducing or removing *B. linens* from the final cheese product.
- Possible bio-control of *B. linens* by investigating the stimulatory and inhibitory effects of selected yeasts species and other microorganisms on *B. linens*.
- The inoculation of blue-mould cheese with the yeast *Debaryomyces hansenii*. Ferreira and Viljoen (2003) reported a number of positive effects the yeast had when co-inoculated with the starter culture in mature Cheddar cheese. In addition to an improvement in taste the maturation time was also shortened, thus allowing the product to reach the market in a shorter time.

3. THE CHEESE MAKING PROCESS

The making of cheese is an age-old process that came about as a means of preserving milk by the decreasing of pH and water activity (Shaw, 1986). The production of cheese involves three main steps, namely, coagulation of the milk, separation of the curd and whey and ripening of the curd (Davis, 1965). The typical industrial process for the production of blue-mould cheese is illustrated in Fig. 1.1.

Mould-ripened cheeses have their origins in Europe and include the well-known white-mould cheese types like Camembert and the blue-mould types like Roquefort, Stilton and Gorgonzola. South African blue-mould cheeses are based on similar varieties compared to their European counter parts, comprising Simonzola (Gorgonzola-type), Creamy Blue (Danablu-type) and Blaaukrantz (Roquefort-type). Mould-ripened soft cheeses differ from other cheeses in that the curd is not scalded, the cheese is not pressed and the cheese is ripened for varying time intervals to achieve the unique flavour and aroma characteristic of mould-ripened cheeses. As a result, the cheese being subjected to other biological induced changes (Fig. 1.3) apart from that of the lactic acid bacteria to acquire its distinctive taste (Shaw, 1986).

The making of cheese can be subdivided into two main stages, namely, production and maturation. Although all cheeses are produced via the same basic production process (Fig. 1.1) the maturation process differs significantly from type to type. The unique blue-mould cheeses are produced via the incorporation of mould spores (usually a species of *Penicillium*) directly into the milk. Additional mould spores can be added directly to the cut curd, brine or on the surface of the cheese after the salting process.

The first step of cheese manufacture is acidification of the milk by the addition and growth of a starter culture, usually representatives of lactic acid bacteria. Rennet and other proteolytic enzymes are added to convert the liquid milk into

a very weak jelly or soft coagulum. This solidification is due to a small change in the structure of casein, the most abundant milk protein. The coagulum, now called the curd, is cut into many small cubes, allowing the water and water-soluble components to be separated as whey. The strength of the curd formation is affected by the strength of the rennet gel, including amount of milk components such as the calcium and casein, pH, rennet source, enzyme activities and heat treatment of milk (Fox, 1987). The firm curd is finely milled, salted and packed into hoops or moulds were it is subjected to considerable pressure (Robinson, 1995). Blue-mould cheese manufacture (Fig. 1.2.) differs only slightly from the process describe above. The first major difference being the addition of *Penicillium* starter cultures with the lactic acid bacteria starters. Secondly, after the scooping of the curd into the relevant moulds these moulds are turned five times at 15 minute intervals and subsequently subjected to dry salting. Depending on the type of blue-mould cheese being manufactured, days 2 - 6 involve a combination of dry salting and brining ending with the cheese in the first maturation stages. For the weeks that follow the cheeses are matured first at 9°C then at 2°C, followed by grading and packaging.

Cheese production is essentially a dehydration process, in which the fat and casein components of the milk are concentrated to between 6- and 12-times, depending on the varieties (Fox, 1993). At the end of the curd production phase most cheeses are bland, white and unpalatable and thereafter require a period of ripening, to enhance the sensory characteristic of the final product (Walstra, *et al.*, 1999; Fox *et al,* 2000).

4. MILK

The primary constituent of cheese is milk, which consists of water, fats, carbohydrates, proteins and trace amounts of vitamins, minerals as well as organic acids (Table 1.1) (Fox, 2002). Cheese making evolved as a method for preserving milk for longer time intervals, by lowering the pH and water

activity of the milk (Shaw, 1986) and thereby separating the solid part of the milk (the curd) form the watery part (the whey). The different compounds in milk vary among breeds of the same species, most substantially between mammalian species, and as a result, variations in the quality of cheese do occur, depending on the type of milk used.

4.1. Casein

The main milk protein, casein, is not a single protein, but consists of a combination of several different types of molecules such as α_{s1}-, α_{s2}-, β- and κ-caseins (with varying proportions). Alpha$_{s1}$-casein is the major component of the caseins, while alpha$_{s2}$-casein is a minor component, which is the most highly and variably phosphorylated casein (Creamer, 2002). Beta-casein is the most hydrophobic of the intact caseins. Kappa-casein constitutes 10 - 12% of the whole casein and plays a crucial role in stabilising the casein micelles in milk (Creamer, 2002). In addition to amino acids, residues of phosphates and glucides like hexsoses and sialic acid are also present. The casein is aggregated to form casein micelles, which are composed of other proteins, enzymes (lipases and proteases), mineral (Mg^{2+}, Na^{+}, K^{+}), citrate, α_{s1}-, α_{s2}-, β- and κ-caseins, calcium phosphate (8g per 100g casein), and water (Walstra *et al.*, 1999). The micelles have a roughly spherical structure, consisting of subunits approximately 10 - 12 nm diameter (Mulder and Walstra, 1974). A small part of the casein, notably β-casein, is not present in the micelles, but only in the serum.

The non-casein proteins fraction of milk is the whey protein fraction and comprises approximately 2% of the total protein. Whey proteins consist of four major proteins, β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins, which comprise more than 95% of the non-casein proteins (Ng-Kwai-Hang, 2002).

4.2. Fat

The second dominant component of milk is the lipid fraction, also known as milk fat (or butterfat), which has a very complicated composition and structure, even more complicated than most other natural fats (Mulder and Walstra, 1974). The milk fat content of different species varies from 5.8 - 9.0% in sheep's milk, to 3.5 - 5.0% in cow's milk and 2.8 - 6.5% in goat's milk (Tamime *et al.*, 1991). The lipid fraction of a cow's milk is present as small globules ranging in size from 0.1 to 20 μm in diameter (Mulder and Walstra, 1974). Milk fat is relatively rich in low molecular weight fatty acids including: butyric, caproic and capric. These fatty acids are released on hydrolysis and contribute to the cheese flavour due to their volatile nature (Fox and Cameron, 1982). Milk fat is composed primarily of triglycerides (or triacylglycerides), which account for 98% of the total milk fat with small amounts of other milk lipids constituting the remaining 2%. These include diacylglycerides (0.25 - 0.48%); monoacylglycerides (0.02 - 0.04%); phospholipids (0.6 - 1.0%); cholesterol (0.2 - 0.4%); glycolipids (0.006%); and free fatty acids (0.1 - 0.4%). The triacylglycerides are chemically the most inactive and appolar lipids in milk, additionally they are quantitatively the most important lipids since they act as a solvent for many other lipids (Muller *et al.,* 1974). In raw milk they are present as globules that are protected from enzymatic degradation by a membrane. Milk fat becomes susceptible to lipolysis if excessive shear forces disrupt this membrane.

Lactic acid bacteria produced very limited, if any, lipases and thus have no influence on fat hydrolysis during cheese ripening (Stadhouders and Mulder, 1958). Further lipolysis of fatty acids results in 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, 1996) (see Section 12.2 for full details on lipolysis).

4.3. Lactose

Lactose is the dominant carbohydrate present in milk, also known as milk sugar and is dispersed throughout the milk serum. Lactose is a disaccharide composed of one molecule of galactose linked to the hydroxyl group on carbon 1 in a β-glycosidic and one molecule of glucose linked to the hydroxyl group of carbon 4 (Cogan and Hill, 1993).

4.4. Citrate

Milk has a low concentration of citrate, 8-10 mM (Fox *et al.*, 1990) contributing to its neutral pH. This provides an ideal environment for bacteria to proliferate and dominate, consequently fungi are rarely a problem (Cousin, 1982). Yeast counts in pasteurised and fresh milk are generally reported at low numbers of less than 10^3 cells.ml^{-1} (Fleet, 1992). The occurrence of yeasts in cheese cannot be eliminated and are of significance because they can cause spoilage, effect desirable and undesirable biochemical 1and may adversely affect human health (Fleet and Mian, 1987).

5. RENNET AND SALT

The action of rennet in cheese is rapid and can be detected by the production of α_{s1}-I peptide and the hydrolysis of α_{s1}-I casein (Gripon, 1987). The rennet, traditionally obtained from the fourth stomach of young milk fed claves, consists of 80% chymosin and 20% pepsin (Chiosy *et al.*, 1986; Walstra *et al.*, 1999). The chymosin specifically cleaves the caseinomacropeptide (CMP) from the κ-casein. As the CMP dissolves into the whey, it leaves behind the para-κ-casein bound in the micelle. The removal of the CMP reduces the electrostatic repulsion between the micelles, making them prone to aggregation. Coagulation of the milk will take place when 70% of the κ-casein have been split, provided that there are sufficient Ca^{2+} ions present in solution to neutralise the remaining negative charges on the micelles and to aid with

salt bridge formation between the micelles (Walstrá *et al.*, 1999). Most of the lactose is lost into the whey, while small amounts of fat globules and some whey proteins remain bound in the gel like suspension. The residual rennet will continue to degrade the curd proteins into the initial stages of maturation (Harper and Kristoffersen, 1956; Holmes *et al.*, 1977; Stadhouders *et al.*, 1977).

Salt is a traditional food preservative that inhibits microbial growth, by lowering the water activity. In addition to flavour contribution, salt also plays an important role in the rind formation. Salt brings about physical changes in the cheese proteins, which influences the texture and protein solubility. This may influence the rate of maturation and the final quality of the cheese. The salt present in the cheeses has a number of positive functions, like the suppression of unwanted microorganisms including the pathogenic ones, regulation of the growth of the desired organisms, such as lactic acid bacteria, the promotion of the desired physical and chemical changes during production and maturation of the cheese as mentioned above, and influences the final flavour both directly and indirectly by affecting changes in the protein structure of the cheese (Sutherland, 2002).

Salt may be introduced into the cheese in three ways. The first involves the immersion of the cheese into concentrated salt solutions or brines. The second is the direct rubbing of salt into the surface of the formed cheese and the third is the mixing of the dry into the curd prior to moulding/pressing. The salt concentration in cheese can vary from 0.7 to 7.0% (w/w) (Beresford *et al.*, 2001) and as Table 1.2. indicates, blue cheeses have one of the highest salt contents (Fox *et al.*, 2000). The time required for salting depends largely on the level of salt expected in the final product. However, a number of other factors, including the brine temperature, which affects the rate of diffusion, the cheese dimension, as well as the pH and moisture content of the cheese play a role in determining the final level of salt present in the cheese (Sutherland, 2002).

6. STARTER CULTURES

Traditionally, raw (non-pasteurised) milk was used for cheese making. This milk contained a number of wild type starter cultures and thus required no supplementation. These included mesophilic lactic streptococci (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactic* subsp *lactis*), *Streptococcus faecalis* (*Enterococcus faecalis*), *S. faecalis* var. *liquefaciens* (*E. faecalis* var. *liquefaciens*), *Leuconostoc dextranicum, Leuconostoc mesenteroides, Lactobacillus casei* and *Lactobacillus plantarum* (Devoyod, 1969). However, with increasing public concern surrounding health and the associated risks of using raw milk, pasteurised milk is now used for all cheese productions. During the pasteurisation process all wild type starter cultures are killed and thus supplementation is essential.

As early as the end of the 19th century, selected starter cultures have been obtained from specialised laboratories (Petterson, 1988). Bacteria, yeasts, moulds or combinations of these are the microorganisms involved in the fermentation of milk during cheese production (Robinson, 1981). The primary role of the starter bacteria is to ferment lactose to lactic acid thereby reducing the pH of milk. The reduction in pH not only has a preservative effect on the milk, but more importantly it affect a number of aspects of cheese manufacturing and ultimately cheese composition and quality. As a secondary reaction the starter cultures aid in the coagulation of casein miscelles. The growth of various microorganisms, as well as the activity of the enzymes involved in cheese ripening, is a direct result of acidification since the rate and extent of pH reduction determines the buffering capacity of the cheese (McSweeney *et al.*, 2000).

Starter cultures can be grouped into either mesophilic starter cultures or thermophilic starter cultures. Mesophilic lactic cultures grow in the temperature range of 10 to 40°C with an optimum temperature of approximately 30°C. In contrast thermophilic starter cultures have a

temperature growth range of 40 to 50°C, with an optimum of approximately 45°C. Depending on the cheese being made either mesophilic cultures are used as in the production of Cheddar, Gouda, Edam, Blue-mould and Camembert cheeses, while thermophilic cultures are used in Swiss and Italian variations (Fox, 1993).

6.1. Lactic acid bacteria

Bacteria starters for milk fermentations are mostly mesophilic lactic starter cultures that include members of the genera *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc* and *Enterococcus* (Beresford *et al.*, 2001). *Streptococcus salivarius* subsp. *thermophilus* (*Str. thermophilus*), has an optimum temperature of approximately 37°C and rapidly ferments lactose to lactic acid and is a Gram-positive coccus that appears as long chains when growing in milk (Robinson, 1995).

Axelsson (1998) reported that the type of lactic acid bacteria could contribute to the final flavour of the cheese, depending on how they metabolise the glucose and galactose. The selection of the starter culture depends on a number of criteria (Mäyrä-Mäkinen and Bigret, 1998), the most important being the rapid production of acid in milk (reduction of the milk pH to less than 5.3 in 6 hours), (Beresford *et al.*, 2001).

After curd production the lactic acid bacteria dominate the microbial population (up to 10^9 cfu/g) and will continue to grow, fermenting the lactose in the curd until a pH of 4.5-5.0 is obtained.

6.2. Mould starter cultures

Mould starter cultures are the primary determinants of the final texture, flavour and appearance of mould-ripened cheeses and contribute to the production of aromatic and flavour compounds by catabolism of the free fatty acids and

amino acids. Starter cultures should be non-toxigenic. The majority of the *Penicillium* species produce toxic mycotoxins, whereas *Penicillium roqueforti* and *P. camemberti* are not able to produce toxic metabolites (Leistner, 1990).

6.2.1. *Penicillium*

Penicillium, being the main characterising microorganism of mould-ripened cheeses, plays an important role in terms of its growth and development, as well as its interactions with other dairy associated microorganisms. The growth of *P. roqueforti* appears as a blue-green mould that grows rapidly at low oxygen and high carbon dioxide levels (Cousin, 2002). The piercing of the cheese plays a pivotal role in the development of *P. roqueforti* by allowing sufficient air to allow the growth and sporulation of the *Penicillium* inside the curd. This usually occurs 2 to 3 weeks after manufacture (Gripon, 1987). *P. roqueforti* is a psychrotroph that grows well at low temperatures, but not above 35°C, making it ideal for growth in blue-mould cheeses, which are matured at low temperature (<10°C).

The proteolytic and lipolytic activities of the *Penicillium* play an important role in flavour development (Hansen and Jakobsen, 1996). *P. roqueforti* produces proteases that degrade the α- and β-caseins to ammonia, aldehydes, acids, alcohols, amines and other compounds (see Section 12.3 and Fig. 1.8). In addition, *P roq*ueforti produces lipases responsible for the degradation of the fats to methyl ketones, secondary alcohols and free fatty acids, all of which play an important role in the flavour and aroma formation of blue cheese (see Section 12.2 and Fig. 1.6). *P. roqueforti*, in combination with the residual rennet present in the cheese after production, catalyses proteolysis and so alter the texture of the cheese. Approximately 10 to 15 days after manufacturing the *Penicillium* has utilised all the residual lactic acid present in the cheese. This leads to an increase in pH and promotes the growth of other microorganisms such as *Brevibacterium linens*, which prefers a more neutral pH (Lenoir, 1963; Richard and Zadi, 1983). *P. roqueforti* and *P. camemberti*

both have antimicrobial activities associated with them. *P. roqueforti* has especially activity against *Escherichia coli* and *L. monocytogenes* (Geisen *et al.*, 1988; Leistner, 1990; Laporte *et al.*, 1992).

7. DAIRY ASSOCIATED YEASTS

Despite raw milk being the primary constituent of most dairy products, minimal attention has been given to the importance of yeasts in cheese flavour and texture formation. 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, 1990a). 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. 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. Since primarily bacteria ferment milk, they 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).

Yeasts are frequently found within the microflora of a wide variety of cheeses (Lenoir, 1984; Fleet and Mian, 1987; Devoyod, 1990; Fleet, 1990a; Seiler and Busse, 1990; Viljoen and Greyling, 1995; Roostita and Fleet, 1996) and play a significant role in the acceptability of foods as they can effect undesirable fermentations or cause spoilage (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).

Yarrowia lipolytica, Debaryomyces hansenii, Kluyveromyces marxianus, Saccharomyces cerevisiae, Torulaspora delbrueckii, Rhodotorula glutinis, Cryptococcus albidus, R. minuta and several *Candida* species are reported as the dominant species isolated from cheeses (Lenoir, 1984; Brocklehurst and Lund, 1985; Engel, 1986; Fleet and Mian, 1987; Nooitgedagt and Hartog, 1988; Devoyod, 1990; Fleet, 1990a; Roostita and Fleet, 1996; Welthagen and Viljoen, 1998; Welthagen and Viljoen, 1999; Viljoen *et al.*, 2003). All of these yeasts exhibit similar properties (Table 1.3), which facilitate growth in milk and cheeses. The yeast species most frequently isolated from blue-mould cheeses include; *Debaryomyces hansenii, Kluyveromyces marxianus, Kluyveromyes lactis, Yarrowia lipolytica* and *Candida* spp. (de Boer and Kuik, 1987; Besançon *et al.*, 1992; Roostita and Fleet, 1996; Barth and Gaillardin, 1997; van den Tempel and Jakobsen, 1998). These yeast species form an integral part of the microflora of blue-mould cheeses and positively contribute towards ripening by the production of aroma compounds (Hanssen *et al.*, 1984; Martin *et al.*, 1999), flavour formation through lipolytic and proteolytic activity (Coghill, 1979; Wyder and Puhan, 1999a and b), the excretion of growth factors (Jakobsen and Narvhus, 1996), the production of gas which leads to curd openness (Jakobsen and Narvhus, 1996), and assists with *Penicillium roqueforti* development (Seiler, 2002). In addition, Martin *et al.* (1999) found that the sensory profiles indicated that the yeasts influenced the development of specific fruity odours, especially when associated with bacteria. Although some yeasts can survive the pasteurisation process (Fleet, 1990b), the primary sources of yeast contamination in milk and cheese processing include the brine and factory surfaces, including the floors, walls and equipment (Seiler and Busse, 1990; Welthagen and Viljoen, 1999). To a lesser extent post-pasteurisation contamination sources include, the air, the workers hands and aprons (Viljoen and Greyling, 1995). The ability of yeasts to grow and survive in dairy products, with special emphasis on the unique

chemical and physical properties of blue cheese varieties, is summarised in Table 1.4.

7.1. *Debaryomyces hansenii*

Debaryomyces hansenii, the perfect form of *Candida famata*, with its ability to grow at low temperatures (5 - 10°C) (Davenport, 1980), in extremely high salt concentrations (up to 15% NaCl) and at water activities as low as 0.84 - 0.89 (Seiler and Busse, 1990; Jermini and Schmidt-Lorenz, 1987), has been repeatedly isolated from mould ripened cheese and from salt brines (Mrak and Bonnar, 1939; Seiler and Busse, 1990; Fleet 1992) and is predominate 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 high numbers of *D. hansenii* in cheeses is due to the species ability to grow at 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* strains have varying abilities to produce proteolytic and lipolytic enzymes (Schmidt *et al.*, 1979) and also have the ability to utilise lactic acid, citric acid, glucose and galactose. The utilisation of organic acids results in an increase in the pH, rendering the environment more favourable for other microorganisms. Furthermore, Yamauchi *et al.* (1975) reported a synergistic effect between lactic acid bacteria and *D. hansenii* resulting in a prolonged survival of the lactic acid bacteria. *D. hansenii* also inhibits the germination of undesired microorganisms like *Clostridium butyricum* and *C. tyrobutyricum* in cheese brines (Fatichenti *et al.*, 1983). High numbers were also reported in yoghurt (Sruyarachci and Fleet, 1981) and raw milk (Fleet and Mian, 1987).

7.2. *Yarrowia lipolytica*

The ascomycetous yeast *Yarrowia lipolytica* (originally classified as *Candida lipolytica*) is readily isolated from dairy products and other foods (McKay,

1992; Barth and Gaillardin, 1997; Guerzoni *et al.*, 1998). This yeast is considered as non-pathogenic and aerobic in nature (McKay, 1992; Barth and Gaillardin, 1997). Van Heerikhuizen *et al.* (1985) reported that the high GC content, unusual structure of the rDNA genes, and the lack of the RNA polymerase I consensus sequences of this species, suggest it may have diverged considerably from other ascomycetous yeast. *Y. lipolytica* strains have potent extracellular lipases, as well as acid and alkaline proteases (Guerzoni, *et al.*, 1993; Glover *et al.,* 1997; Heard and Fleet, 1999; Guerzoni *et al.*, 2001; Suzzi *et al.*, 2001), resulting in the production of significantly higher levels of free fatty acids and amino acids. Wyder and Puhan (1999a) reported *Y. lipolytica* as the yeast species with the strongest proteolytic activity whereas Choisy *et al.* (1986) considered it as the most predominant species contributing to lipolytic activity. In addition, Alford and Pierce, 1961 reported *Y. lipolytica* strains to have strong proteolytic and lipolytic activities at temperatures below 0°C.

Guerzoni *et al.* (1998) and van den Tempel and Jakobsen (2000) reported on this yeast compatibility with starter cultures and the possible stimulating action when co-inoculated. Enzymatic browning is the main cause of spoilage in mould-ripened cheeses. *Y. lipolytica* has been implicated as the main causative agent (Nicol *et al.*, 1996; Eliskases-Lechner and Ginzinger, 1999; Ross *et al.*, 2000; Carreira *et al.*, 2001). Martin *et al.* (1999) reported that *Y. lipolytica* produces more volatile compounds, most notably cheesy flavours, than both *D. hansenii* and *K. lactis*. *Y. lipolytica* is regarded as a good candidate as a ripening agent in cheese (Guerzoni *et al.*, 1998), since it fulfils a number of the above specific criteria to be applied as co-starter in cheese making (Guerzoni *et al.*, 2001; Suzzi *et al.*, 2001).

7.3. *Saccharomyces cerevisiae*

The brewer's yeast *Saccharomyces cerevisiae* is well known in the food and beverage industries for its fermentative properties. Due to this species ability

to utilise a large variety of carbon sources, its natural occurrence is widespread and can often result in the spoilage of foods and beverages due to the formation of gas and yeasty or fruity flavours in dairy products (Walker, 1988). Strains of *S. cerevisiae* are sensitive to high levels of salt concentrations (Roostita and Fleet, 1996) and lack the ability to utilise lactose and citric acid and to produce lipases or proteases (Fleet and Mian, 1987).

7.4. *Rhodotorula* species

These basidiomycetous species lack fermentative abilities and are generally not recognised as typical spoilage microorganisms. However, they are frequently associated with dairy product spoilage as they are thermo-tolerant yeasts that are capable of growing at sub-zero temperatures and at pH values as low as 2.4 (Pitt and Hocking, 1985). The ability of these yeasts to peptonise casein and attack butterfat can be detrimental to milk and milk product quality (Fleet and Mian, 1987).

7.5. *Kluyveromyces marxianus*

The presence of this yeast in dairy products is not uncommon, due to its ability to ferment and assimilate lactose, lactic acid and citric acid. In addition, the production of proteases and lipases that could hydrolyse milk casein and fat favour their growth in dairy products (Fleet and Mian, 1987). Although, *Kluyveromyces marxianus* may proliferate in the interior of cheeses due to its ability to grow at low oxygen levels, it is well know for its ability to grow on the surface of cheeses as well.

7.6. *Kluyveromyces lactis*

According to literature *Kluyveromyces lactis* is a strong producer of aroma compounds responsible for the fruity flavours such as alcohols (isoamyl alcohol, isobutyl alcohol, and 2-phenyelethanol), aldehydes

(2-phenylacetadehyde), ester (ethylacetate, and 2-phenylacetate) as well as monoterpenes (Hanssen *et al.*, 1984; Lee and Richard, 1984; Martin *et al.*, 1999). These compounds play a major role in the final development of the cheese flavour and aroma (Fig. 1.7).

8. THE ROLE OF DAIRY ASSOCIATED YEASTS

There are a great number of yeasts frequently associated with cheeses, however two distinct groups can be identified. The first and most logical group comprises those yeasts that are capable of survival and reproduction within the environment created by the cheese making process (Deàk and Beuchat, 1996). The second group encompasses those yeasts, which do not possess the characteristics of the former group. These yeasts rely solely on dissemination for survival. Thus, in order to establish a complete overview of all yeasts present during the production and maturation of blue-mould cheese the population should be followed over time.

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, 1990a). In addition, the formation of alkaline metabolism products, such as ammonia from amino acids deamination (Fleet, 1990a), further aid in the deacidification of the cheese further promoting the growth of bacteria such as *B. linens*. Yeasts also have proteolytic and lipolytic activity (Coghill, 1979; Wyder and Puhan, 1999 a and b), excrete growth factors, like B-vitamins, pantothenic acid, niacin, riboflavin and biotin which promote the growth of lactic acid bacteria (Purko *et al.*, 1951; Lenoir, 1984; Fleet, 1990a; Jakobsen and Narvhus, 1996) and produce gas that leads to curd openness (Choisy *et al.*, 1986; Jakobsen and Narvhus, 1996). Yeasts have been reported for their ability to improve the quality of cheeses through their lipolytic activity (Proks *et al.*, 1959; Mahmoud *et al.*, 1979; Masek and Zak, 1981; Choisy *et al.*, 1986).

9. YEASTS AS SPOILAGE ORGANISMS

Yeasts are generally not regarded as a significant component of the microflora of cheeses. They are usually found in cheese due to natural contamination from the surrounding environments and the favourable conditions created during the ripening process (see Section 7). The role of yeasts as spoilage organism is directly linked to their nutritional requirements, growth at low temperatures, low pH values, low water activates and high salt concentration (Davenport, 1980; Seiler and Busse, 1990). Yeasts that produce undesirable changes in foods during the fermentation process are regarded as spoilage yeasts (Fleet, 1990a; Fleet, 1992; Deàk and Beuchat, 1996). Spoilage may occur as pellicle or turbidity in liquids, or as a slimy or powdery coating on solid surfaces (Fleet, 1992). Other undesirable changes include the production of metabolic products resulting in fruity, bitter or yeasty-off flavours, as well as a gassy open texture in semi-hard to hard cheese.

The yeasts responsible for food spoilage are often well-known species (Fleet, 1990a) and begin to multiply and grow when the conditions are favourable. Despite the many attempts applied to inhibit yeast growth during the production and post-production of foods, the ability of yeasts to grow and adapt to a wide range of environmental conditions allow their continued growth. However, the losses caused by yeasts are considered minimal in comparison to the losses caused by bacteria and moulds (Marth, 1987; Fleet, 1990a). This can be attributed to the slower growth rate of yeasts in comparison to bacteria, as well as their tendency to be overgrown by indigenous bacteria present in foods (Fleet, 1990b; Fleet, 1992; Deàk and Beuchat, 1996). The presence of spoilage yeasts in food has to date not resulted in food poisoning (Fleet and Mian, 1987; Fleet, 1990b; Fleet, 1992), since the metabolic products of yeasts are considered non-toxic. Despite some of the yeasts species being pathogenic, none are known to be responsible for infections or poisoning as in the case of certain bacteria and fungal species (Peppler, 1976; Fleet, 1992; Deàk, 1994).

10. OTHER DAIRY ASSOCIATED MICROORGANISMS

Although yeasts are frequently associated with dairy products, several other microorganisms have been identified (de Boer and Kuik, 1987). These include amongst others *Brevibacterium linens* and *Penicillium.*

10.1. *Brevibacterium linens*

Brevibacterium linens, one of the main microorganisms associated with the surface flora of mould-ripened cheeses, contributes to the final surface flavour, colour and aroma due to its strong proteolytic activity as well as through the production of methanethiol (Cuer *et al.*, 1979b; Bikash, *et al.*, 2000). *Brevibacterium* species are Gram-positive, non-motile, non-ramifing and non-lipophilic small rods. This bacterium is strictly aerobic, non-motile, do not form endospores and has an optimum growth temperature of 20 - 30 or 37°C (varies from species to species), can tolerate salt concentrations of up to 15%, is capable of growth at 10°C, does not reduce litmus and is non-thermoduric (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000). The classification of this bacterium has posed a number of problems for taxonomists due to it similar morphology to other genera, such as *Arthrobacter, Caseobacter, Corynebacterium* and *Rhodococcus* (Rattray and Fox, 1999) and consequently the classification has altered numerous times over the last two decades (Fiedler *et al.*, 1981). Breed (1953) first described the genus *Brevibacterium* with its type species *B. linens. Brevibacterium* (sub-order of *Micrococcineae*, order of *Actinomycetales*, subclass of *Actinobacteridae*, class of *Actinobacteria*) was first proposed to accommodate short gram-positive, non-sporulating bacilli (Euzéby, 1997). The seventh edition of *Bergy's Manual of Determinative Bacteriology* classifies the genus *Brevibacterium* with the type species as *B. linens* and includes the species *B. iodinum, B. casei* and *B. epidermidis.* The *incertae sedis* group, *Brevibacterium incertum, B. acetylicum, B. oxydans, B. halotolerans, B. frigritolerans* and *B. rufescens*, contains the *Brevibacterium*

for which there is insufficient data to allow for their reclassification with confidence (Rattray and Fox, 1999).

In addition, *Brevibacterium* spp. have a unique morphology. During a normal growth cycle the cell morphology ranges from rod shaped during the exponential phase to coccid-shaped during the stationary phase (Rattray and Fox, 1999). *Brevibacterium* spp. are obligate aerobes, hence their presence on the outer surface of mould-ripened cheeses, with only slight or no acid production from glucose, produce extracellular proteinases, do not hydrolyse aesculin, urea or starch, are catalyse-positive with oxidase and nitrate reductase varying according to species (Euzéby, 1997). The pepidoglycan present in the cell walls contains mesodiaminopimelic acid (DAP) as the diamino acid, arabinose and mycolic acids are absence from the cell walls, and dehydronated menaquinone are present in large numbers (Euzéby, 1997; Rattray and Fox, 1999; Bergey's Manual [7th ed]).

Boyaval and Desnazeaud (1983) extensively studied the physiology, biochemistry and enzymology of *B. linens*. One of its most distinguishing characteristics is it yellow/orange to red colour, which intensifies with exposure to light (Rattray, 2002). Some varieties of mould-ripened cheese are made with *B. linens*, these include the true French Brie, Limburger or Romadour cheeses. However, in most other cheeses, such as the blue-mould cheese, this aerobic bacterium causes a reddish discoloration (Chapman and Sharpe, 1981) and an off-taste that is not favoured by all consumers. The lipases produced by *B. linens* release volatile fatty acid components from the triacylglycerols which influence the characteristic taste of ripened cheeses (Hosono, 1986). *B. linens* produces extracellular aminopeptidases which require a neutral/basic pH (7.0 - 9.5) and are active on the leucine at the N-terminal of peptides as well as extracellular proteinases, such as serine proteinases, which are highly active on α-s1 and β-casein (Rattray, 2002). This bacterium also produces various bacteriocins and anti-microbial substances, the properties of which appear to be strain

dependant. Some of these bacteriocins have been shown to be inhibitory towards certain foodborne pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* (Rattray, 2002).

Although the presence of *B. linens* in cheese is not harmful to humans, the South African consumer in general demands cheese devoid of the taste/aroma and/or the off colour caused by *B. linens*.

11. THE CHEMISTRY OF BLUE CHEESE DURING MARURATION

The ripening phase is the most significant phase of cheese production, since starter and non-starter bacteria, indigenous milk enzymes, and chymosin interact to develop the organoleptic and textural properties of the cheese. There are a number of complex reactions that are involved in the flavour formation during the maturation of cheese since both microorganisms and enzymes play a role (Fig. 1.2). These reactions can be divided into primary and secondary reactions. The major constituents, proteins, carbohydrates and fats in milk, undergo a variety of chemical and physical changes during these reactions and are degraded to primary and secondary products. The primary reactions include glycolysis, proteolysis and lipolysis and are detailed in Fig. 1.7. The final end products of all these reactions interact to form the aroma and flavour characteristics of cheese. Further metabolism of the degraded products gives rise to a multitude of substances each of which can further affects the body, flavour and aroma of the cheese (Scott, 1981). The secondary reactions involve catabolism, some of which results in the release of volatile flavour compounds. Additional secondary reactions include the degradation of the curd, which influences the texture as well as the flavour development. In surface-ripened cheese, the surface microflora are responsible for much of the flavour development, since bacteria and most notably the coryneform bacteria produce volatile sulphur compounds (Martin *et al.*, 1999). In addition, Martin *et al.* (1999) reported on the importance of

yeasts in the development of specific fruity flavours especially when associated with bacteria.

11.1. Glycolysis

Lactic acid bacteria will continue to ferment the residual lactic acid in the fresh curd until depletion of the lactose the resulting pH of the curd will range from 4.5 to 5.0. In addition, any trace amounts of citrate will be metabolised to flavour compounds such as acetate, diacetyl, acetone, 2,3-butanediol and 2-butanone (Fig. 1.5.) (McSweeney and Sousa, 2000).

Glycolysis can be divided into two main reactions, namely lactose fermentation and citrate metabolism.

11.1.1. Lactose fermentation

Lactose formation contributes positively towards the ripening of cheese. The rate and amount of lactic acid formed, which represses harmful and undesirable bacteria is controlled by the active growth of microorganisms as well as the biochemical reactions in blue-mould cheese varieties.

Lactose is metabolised via the glycolytic- (most starter bacteria) or phosphoketaloase (*Leuconostoc* spp.) pathway (Fig. 1.4). The principle products of lactose metabolism are L- or D-lactate or a racemic mixture of both. Some strains, such as *Leuconostoc* spp., produce other products including ethanol (Vedamuthu, 1994). *Lactoccus lactis* subsp. *cremoris* and *Lc. lactis* are the most common starter cultures used in cheese production. Lactic acid bacteria ferment lactose to lactic acid via the hexose diphosphate pathway (Cogan and Hill, 1993). Lactate contributes to the flavour of acid-curd cheeses and probably also contributes to the flavour of ripened cheese varieties, particular early in maturation. Lactate can be oxidised *in vitro* to acetate and carbon dioxide by components of the non-starter lactic acid bacteria (NSLAB) present in cheeses (Fox *et al.*, 1995). Acetate, an important

flavour compound in many cheeses, may also be formed as a result of citrate and lactate metabolism, or as a product of the catabolism of amino acids (McSweeney *et al*, 1999). Residual lactose in the fresh curd will continue to be fermented into lactic acid by starter bacteria. Once the lactose is exhausted, the pH of the cheese will be between 4.5 and 5.0. In addition to the residual lactose, there is a small amount of citrate retained in the curd. Depending on variety, lactate may also be further metabolised by a number of pathways to various compounds that contribute to the cheese flavour.

11.1.2. Citrate metabolism

Milk initially contains *ca.* 8 mmol.L^{-1} citrate and *ca.* 94% is present in the soluble phase of the milk and thus is lost during cheese making. The low concentrations of citrate in cheese curd (10 mmol.kg^{-1}) are of great importance since it may be metabolised to a number of volatile flavour compounds by certain mesophilic starters.

Citrate is metabolised by lactic acid bacteria into flavour compounds (Fig. 1.5) (McSweeney *et al.,* 2000). The major flavour compounds are acetate, acetaldehyde, diacetyl, acetoin and 2,3-butanediol, while CO_2 contributes indirectly to the texture (Hugenholtz and de Felipe, 2002). Acetate is produced form citrate in equimolar concentrations and diacetyl is usually produced only in small amounts (1 - 10 µg.ml^{-1} in milk), but acetoin is generally produced in much higher quantities (10 - 50 fold higher than diacetyl concentrations). Diacetyl is an important aroma compound and can be converted to acetoin and 2,3-butanediol and 2-butanone, which are also important flavour compounds in some cheese varieties (Dimos *et al.*, 1996). Production of 2,3-butanediol by starters has not been studied in detail. Acetaldehyde is a very potent aroma compound is extremely volatile (Hugenholtz and de Felipe, 2002).

Due to the number of complex interactions that take place between the aroma bacteria in cheese maturation, it is relatively difficult to stimulate only citrate fermentation (Hugenholtz and de Felipe, 2002). Additional citrate is favourable since it increases the buffering capacity of the milk resulting in lower water retention and decrease acidification. This increase in pH promotes the growth of secondary microflora such as *B. linens*, which are essential for the final flavour and aroma of surfaced ripened smear cheeses. Additionally, extra citrate will result in the withdrawal of Ca^{2+} ions from the cheese, inhibiting the normal process of casein coagulation. This is favourable in preventing the eye formation characteristic (Hugenholtz and de Felipe, 2002).

11.2. Lipolysis

Lipolysis is important for the development of the typical flavour of cheese and is particularly extensive in bacteria surface ripened cheeses and blue-mould cheeses. Extensive lipolysis is considered undesirable because high levels of fatty acids lead to rancidity (Fox *et al.*, 1993).

Lipids play a major role in the solubilisation and retention of hydrophobic flavour compounds thus being important for typical flavour development (Walstra *et al.*, 1999). Acid and alkaline lipases secreted by *P. camemberti*, *P. roqueforti* and *G. candidum* are primary responsible for lipolysis in mould-ripened cheeses since calf rennet and commercial rennet lack lipolytic activity. The level of lipolysis is very high in mould-ripened cheeses, with blue-mould cheeses undergoing the highest level of lipolysis. The lactic acid bacteria present are only weakly lipolytic, but do possess esterases which release low levels of fatty acids over a long maturation period (Fox, 1993).

Lipolysis involves the enzymatic hydrolysis of triglycerides to free fatty acids, glycerol and mono- and diglycerides and at a later stage during maturation, deacidification of the curd (Fig. 1.9) (Law, 1984, Fox *et al.*, 1990; Fox and Law, 1991; McSweeney *et al.*, 1999). Enzymes responsible for the breakdown

of lipids in milk or cheese are lipases or glycerol ester hydrolases (EC 3.1.1.3). These enzymes are widely distributed in animals, plants, and microorganisms responsible for the hydrolysis of insoluble fats and oils (triacylglycerol, diacylglycerol and in some cases monoacylglycerols).
Two types of lipases are found in milk, both of which are inactivated by heat. The bacterial produced lipases hydrolyse mono- and diacylglyseride to fatty acids and glycerol (Kilara, 2002), with a very weak activity towards triglyceride. The bacterial lipases are divided into two types based on the positional specificity for the primary ester. The specificity of the lipases influences the development of the cheese flavour. The second milk lipases act rapidly on the fat, in the event of the lipoprotein lipase enzyme disrupting the globule membrane (Paltuaf and Wagner, 1976). Although milk contains indigenous lipases, in addition to a number of esterases, lipases from contaminating bacteria such as *Pseudomonas fluorescens* and *Pseudomanas fragi* may also be present and can survive ńormal milk heat treatment and continue to hydrolyse the fat in the finished product. Lipases in cheese originate from seven sources: milk, rennet preparation, starters, adjunct starters, non-starter bacteria, psychrotrophes and, if used exogenous lipases.

The simplest lipids are fatty acids. Fatty acids are components of many complex types of lipids such as triacylglycerol, glycerophospholipids and sphingolipids. The lipids are composed primary of fat although there are also small amounts of phospholipids, sterols, fat-soluble vitamins A and D, carotene and xanthophylls.

11.3. Proteolysis

Proteolysis is very intense in blue-mould cheeses, since the β-casein is degraded more extensively, with the intensity decrease from the inner core to the outer surface layer. This may suggest that the high amounts of sodium chloride present on the surface of the mould-ripened cheeses inhibit proteolytic action (Gripon, 1987). The final products of proteolysis are small

peptides and free amino acids. These contribute only in a small way to the flavour flair. The agents that catalyse these biochemical reactions include the indigenous milk proteinases, exogenous proteinases, peptidases, extracellular enzymes, residual rennet, starter bacteria and moulds, and the intracellular enzymes of all microorganisms that are released after cell lysis (Law, 1984; Fox, 1989; Fox *et al.*, 1990).

Proteolysis plays a vital role in the development of textural changes in the cheese curd (Coghill, 1979) due to the breakdown of the protein network, decrease in the a_w through water binding by liberated carboxyl and amino groups, and increase in pH. In addition, the liberation of amino acids from secondary catabolic changes such as deamination, decarboxylation, transamination, desulphuration, catabolism of aromatic compounds including phenylalanine, tyrosine, tryptophan and reactions of amino acids with other compounds aid in the texture development (Fig. 1.8). Proteolysis contributes to the taste of cheese by the breakdown of proteins into peptides (small-, medium- and large peptides) and amino acids, and the sapid flavour components generally partition into the soluble fraction on extraction of cheese with water (Sousa *et al.*, 2001). Large peptides (water insoluble) do not contribute directly to cheese flavours, but are important for the development of the correct texture. Small peptides and amino acids are water-soluble.

Proteolysis can also lead to bitter peptides (hydrophobic peptides), which accumulate in cheese causing bitterness (Ney, 1976). Bitter peptides isolated from whole casein and cheese, originate mostly from α_{s1} and β-casein (Gomez *et al.*, 1997). The amino acids derived from protein breakdown are precursors that are absolutely the key to cheese flavour. The primary phase during proteolysis influences the background brothy flavour, perhaps via peptide and glutamic acids. The sweet background flavours are influenced by the amino acids such as proline.

Enzymatic proteolytic and peptidolytic reactions are two of the biochemical and chemical processes occurring during cheese ripening that lead to characteristic mature body and texture. Cheese proteolysis involves the action of proteolytic enzymes, such as residual chymosin (in coagulant) and plasmin (in milk), intact casein in the cheese curd (primary phase of ripening), the further breakdown of large peptides and oligopeptides into small peptides and amino acids by the cell-envelope proteinase (CEP) and peptidases of lactic acid bacteria (secondary phase of ripening) (Fig. 1.8) (Visser, 1993; Sousa *et al*, 2001). The first reaction necessary for the production of amino acids from casein (Fig. 1.8) involves proteinases (Fox *et al.*, 1993). Proteolytic enzymes from lactic acid bacteria, serine proteinases, play an important role in the degradation of casein and peptides leading to the production of free amino acids. The peptidases from *Lactococcus*, lactococcal proteinases, degrade the casein to polypeptides, which are then further hydrolysed by peptidases, resulting in peptides and amino acids (Fox *et al.*, 1993).

11.4. Biochemical reactions during maturation

The objective of cheese manufacture is to produce a product with a flavour, aroma and texture of the intended variety, free of defects and in the shortest time possible (Fox *et al.*, 1993). Flavour is a sensation that includes taste, aroma and texture (Walstra and Jenness, 1984). Maturation involves the formation of flavour and texture compounds brought about by the action of enzymes from rennin and microorganisms (Harper and Kristoffersen, 1956). These reactions include the metabolism of lactic acid, deamination, transamination, decarboxylation, desulpheration and demethiolation of amino acids, oxidative degradation of fatty acids to methyl ketones and reduction to the corresponding secondary alcohols, and esterification of fatty acids with alcohols or sulphydryl groups (Fig. 1.7). Microbial proteases can reduce bitterness by hydrolysing bitter peptides formed in the cheese (Martinez-Cuesta *et al.*, 2001). For example, methional converted from

methionine. Another route is partial microbial metabolism, or biochemical conversions of amino acids as seen in the conversion of phenylalanine to phenethylamine, and 2-methylbutyric acids from isoleucine.

In addition, catabolism of lactic acid, amino acids and free fatty acids by microorganisms brings about further textural changes in the curd and generates volatile and non-volatile compounds responsible for the finer aspects of cheese flavour and aroma (Fig. 1.3.). Many flavour-producing substances such as peptides, ketones, alcohols, esters, diacetyl and ammonia have been isolated from cheese.

Calcium ions play an important role in the determination of the final texture of mould-ripened cheese. The rigid structure of cheese is created by the cross-linking effect cause by the calcium ions between proteins in the cheese matrix (Guinee, 2002). As the cheese ages the calcium ions are depleted from the core of the cheese (Fig. 1.6) resulting in a softening of texture due to a breakdown of calcium cross-linkings (Karahadian and Lindsay, 1987).

The main breakdown products of cheese are illustrated in Fig. 1.7 and include fatty acids, ketones, methyl ketones, alcohols, lactones, sulphur compounds, aldehydes and amines (Molimard and Spinnler, 1996). Theses compounds all interact with one another to produce the final texture and flavour of the cheese. Despite extensive research (Karahadian *et al.*, 1985; Molimard and Spinnler, 1996; McSweeney and Sousa, 2000; Yvon and Rijnen, 2001) the contribution of the individual flavour and aroma compounds has not been quantified, although certain flavours are characteristic of specific cheese types.

11.4.1. Fatty acids

Fatty acids are aromatic compounds that serve as precursors for alcohols, methyl ketones, lactones and esters. They are either saturated or

unsaturated. Table 1.5 listed some of the most common fatty acids. If the carbon of a fatty acid chain binds the maximum possible number of hydrogen atoms, the fatty acid is saturated, if the number of hydrogen atoms bound by the carbons is less than the possible maximum, the fatty acid is unsaturated.

Hydrolysis of fat, especially in blue-mould cheeses plays an important role in the ripening of cheese (Molimard and Spinnler, 1996). The fat in milk comprises 98% glyceride neutral lipids (Taylor and MacGibbon, 2002a) with the fatty acid composition of bovine milk (Table 1.5.) being more varied than any other natural product (Taylor and MacGibbon, 2002b).

Triacylglycerol or neutral lipids (also known as simple lipids), commonly found in cells as storage fats and oils, are so called because at cellular pH they bear no charged groups. Since triacylglycerol have no charge (i.e. the carboxyl group of each fatty acid is joined to glycerol through a covalent bond) they are sometimes referred to as neutral fats. Generally, they are completely non-polar, with no affinity for water. Almost all neutral lipids are a combination of fatty acids with the alcohol glycerol.

11.4.2. Methyl ketones and ketones

Methyls are the most abundant aromatic compounds in the surface of mould-ripened cheeses, and hence contribute significantly towards the final flavour of the cheese (Schwart and Parks, 1963; Dumont *et al.*, 1974). Methyl ketones are formed in a metabolic pathway that is directly connected to the β-oxidation pathway (Molimard and Spinnler, 1996). Methyl ketones can be reduced to the corresponding alcohols via reductase activity. It is interesting to note that some strains of *B. linens* are able to convert methyl ketones to secondary alcohols (Anderson and Day, 1966; Kinsella and Hwang, 1976). The strong peppery flavours that are associated with blue cheese are due to the presence of methyl ketones such as alkan-2-ones (Gallois and Langlois, 1990).

11.4.3. Alcohols

Primary and secondary alcohols play an important role in the aroma formation of cheese (Molimard and Spinnler, 1996). Lactose metabolism results in the formation of ethanol and butandiol (Adda, 1984). Alcohol flavours include mild, ethery (ethanol), mushroom (oct-1-en-3-ol), rose, floral (phenol) and fruity alcohol (3-methylbutanol) (Le Quéré and Molimard, 2002).

11.4.4. Lactones

Hydroxylated fatty acids, which are derived from normal fatty acid catabolism, are the precursors for lactone synthesis. The main lactones present in cheese are γ-decalactone, δ-decalactone, γ-dodecalactone and δ-dodecalactone (Le Quéré and Molimard, 2002). These lactones are characterised by fruity flavours such as peach, apricot and coconut. Hydroxyacids are usually present as triacylglycerols in milk and can be liberated by lipases (Le Quéré and Molimard, 2002).

11.4.5. Esters

A large percentage of the microorganisms present during the ripening of cheese are able to produce esterification enzymes, including carboxylesterases and arylesterases. However, yeasts are the main organisms involved in esterification (Molimard and Spinnler, 1996). Many enzymes are involved in esterification and most of these have a wide range of substrates. Caroxylesterases and arylesterases are two of the most common enzymes involved in cheese ripening (Le Quéré and Molimard, 2002).

11.5.6. Sulphur compounds

These compounds arise from the cleavage of the bonds between the carbon and sulphur by the methioninedemethiolase enzymes during methionine

degradation, mainly executed by lactococci (Alting *et al.*, 1995; Molimard and Spinnler, 1996; Yvon and Rijnen, 2001). Methanethiol is metabolised to sulphides such as dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide, as well as thioesters such as methylthioacetate, methylthiobutanoate, methylthiopentanoate (Cuer *et al.*, 1979a and b ; Martin *et al.*, 1999). The enzymes involved in methionine metabolism include cystathionine-γ-lase and cystathionine-β-lyase. The coryneform bacteria, especially *B. linens* are considered to be the main organisms responsible for sulphur compound production in cheese (Molimard and Spinnler, 1996; Yvon and Rijnen, 2001). The typical flavours associated with the sulphur compound production include cabbage, cauliflower, garlic and ripe cheesey flavours (Molimard and Spinnler, 1996; Le Quéré and Molimard, 2002).

11.4.7. Amines

Decarboxylation of amino acids results in the production of carbon dioxide and free amines. In addition, amino acid deamination results in the formation of ammonia, where *B. linens* plays a major role (Karahadian and Lindsay, 1987), contributing to the final flavour and aroma of the cheese. The amines present in dairy products include:

(a) Volatile amines such as methylamine, ethylamine, *N*-propylamine, isopropylamine, *N*-butylamine, 1-methylpropylamine, *N*-amylamine, isoamylamine, anteiso-amylamine, *N*-hexylamine, ethanolamine, dimethylamine, diethylamine, dipropylamine and dibutylamine (present at levels of up to 1.6 $m.kg^{-1}$ in blue cheeses) (Le Quéré and Molimard, 2002) and

(b) Non-volatile amines such as tyramine, histamine, tryptamine and putrescine. Amines are rarely the final end products since they are usually converted to aldehydes via oxidative deamination (Molimard and Spinnler, 1996).

11.4.8. Aldehydes

Aldehydes originate from amino acids either by transamination or by degradation. During ripening these simple reactions can take place without enzymatic catalysation (Molimard and Spinnler, 1996). Aldehydes function as transitory products, since they are rapidly converted to alcohols and their corresponding acids (Kinsella and Hwang, 1976; Dunn and Lindsay, 1985; Molimard and Spinnler, 1996). The main aldehydes found in cheese are hexanal, heptanal, nonanal, 2-methylpropanal, 2-methylbutanal, 3-methybutanal and benzaldehyde (Le Quéré and Molimard, 2002).

12. CLOSING STATEMENTS AND CONCLUSIONS

It is evident from the literature that there are many complex interactions and reactions involved in the maturation of cheese. Not only do the starter cultures play a significant role, but also the non-starter microorganisms influence the final flavour and aroma to a great extent. Despite an abundance of literature referring to the presence and growth of yeasts within blue cheeses (Devoyod and Sponem, 1970; Law, 1978; Noomen, 1983; Rohm *et al.*, 1992), little work has been conducted on the source of contaminating yeasts or the growth and development of these yeasts during processing and ripening. In addition the presence of pathogenic yeasts in dairy products, especially cheeses have received minimal attention. To this end, in part of this project we endeavoured to establish the sources of contaminating yeasts and to evaluate their growth and effects on South African blue-mould cheese during processing and maturation.

In European mould-ripened cheeses, *B. linens* growth is encouraged as this contributes to the flavour and texture of the cheese resulting in a unique cheese that is well accepted by the consumer. However, the South African consumer has rejected the presence of this bacterium in the mould-ripened cheeses. Despite the best control methods *B. linens* still proliferates on the

surface of mould-ripened cheeses, especially blue-mould cheeses. Thus one of the main aims of this project became the biological control of *B. linens* during the production and manufacture of blue-mould cheese.

Table 1.1. The basic composition of bovine milk, typical for milk of lowland breeds.

Component	Average %	Range (%)
Water	87.1	85.3 - 88.7
Lactose	4.6	3.8 - 5.3
Fat	4.0	2.5 – 5.5
Protein	3.3	2.3 – 4.4
Casein	2.6	2.3 – 4.4
Mineral substances	0.7	0.57 – 0.8
Calcium	0.08	0.06 – 0.1
Organic acids	0.17	0.12 – 0.21
Ash	0.7	0.2 – 1.2

Adapted from Tamime and Robinson, 1985; Fox and McSweeny, 1998 and Walstra *et al.*, 1999.

Table 1.2. Average percentage of salt (w/w) present in various cheese types.

Variety of cheese	Salt (%)
Emmental	0.7
Cheddar	1.7
Camembert and Brie	1.9-2.5
Gouda	2.5
Blue cheese	3.5-4.5
Feta	5-6

Adapted from Fox *et al.*, 2000, Beresford *et al.*, 2001 and Sutherland, 2002.

Table 1.3. Some growth properties that allow certain dominate yeasts to grow in milk and cheese products.

Growth properties	***Candida* species**	***Yarrowia lipolytica***	***Debaryomyces hansenii***	***Saccharomyces cerevisiae***	***Cryptococcus flavus***	***Kluyveromyces marxianus***	***Rhodotorula glutinis***
Growth at 5°C (low temperatures)	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Fermentation: Lactose	Yes	No	No	No	No	Yes	No
Sucrose	Yes	No	Yes	Yes	No	Yes	No
Assimilation: Lactose	No	No	Yes/No	No	Yes	Yes	Yes
Sucrose	Yes	No	Yes	Yes	Yes	Yes	Yes
Hydrolysis of: Casein	Yes	Yes	Yes	No	No	Yes	No
Fat	Yes	Yes	Yes	No	Weak	Yes	Yes
Growth in elevated salt concentrations	Yes	Yes	Yes	Yes (Sensitive)	Yes	Yes	Yes
Assimilation of: Acetic and citric acids	Yes	No	Yes	Yes (Limited)	No	Yes	Yes
Growth at low pH levels ($pH < 4$)	Yes	Yes	Yes	Weak	Yes	Yes	Yes

Adapted from Ahearn *et al.*, 1968; Walker and Ayres, 1970; Fleet and Mian, 1987; Jermini and Schmidt-Lorenz, 1987; Barnett *et al.*, 1990 and Roostita and Fleet, 1996.

Table 1.4. The unique characteristics of yeasts that allow their proliferation in blue-mould cheeses.

Growth at	Range	Reference
High salt concentrations	4.0 – 5.0% (w/w)	Mrak and Bonar, 1939
Low a_w values	0.84 – 0.89	Tilbury, 1980
Low temperatures	<10°C	Alford and Pierce, 1961; Davenport, 1980
High fat concentrations	23 – 30% (w/w)	Fleet and Mian, 1987
High protein concentrations	19 – 24% (w/w)	Fleet and Mian, 1987
High concentrations of lactic acid	1.0 – 1.6% (w/w)	Fleet and Mian, 1987
Low pH values	1.3 – 1.7	Pitt, 1974; Deàk and Beuchat, 1996
Other characteristics:		
The ability to produce extracellular proteases and lipases	Varies from species to species	Fleet and Mian, 1987
Resistance to benzoate	Varies from species to species	Fleet and Mian, 1987

Table 1.5. Common fatty acids (FA) found in milk.

Lipids	Name of lipids
C4:0	Butyric acid
C6:0	Hexanoic acid
C8:0	Caprylic acid
C10:0	Caproic acid
C12:0	Auric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid (an essential FA)
C18:3	Linolenic acid (an essential FA)

Adapted from Jensen and Newburg, 1995.

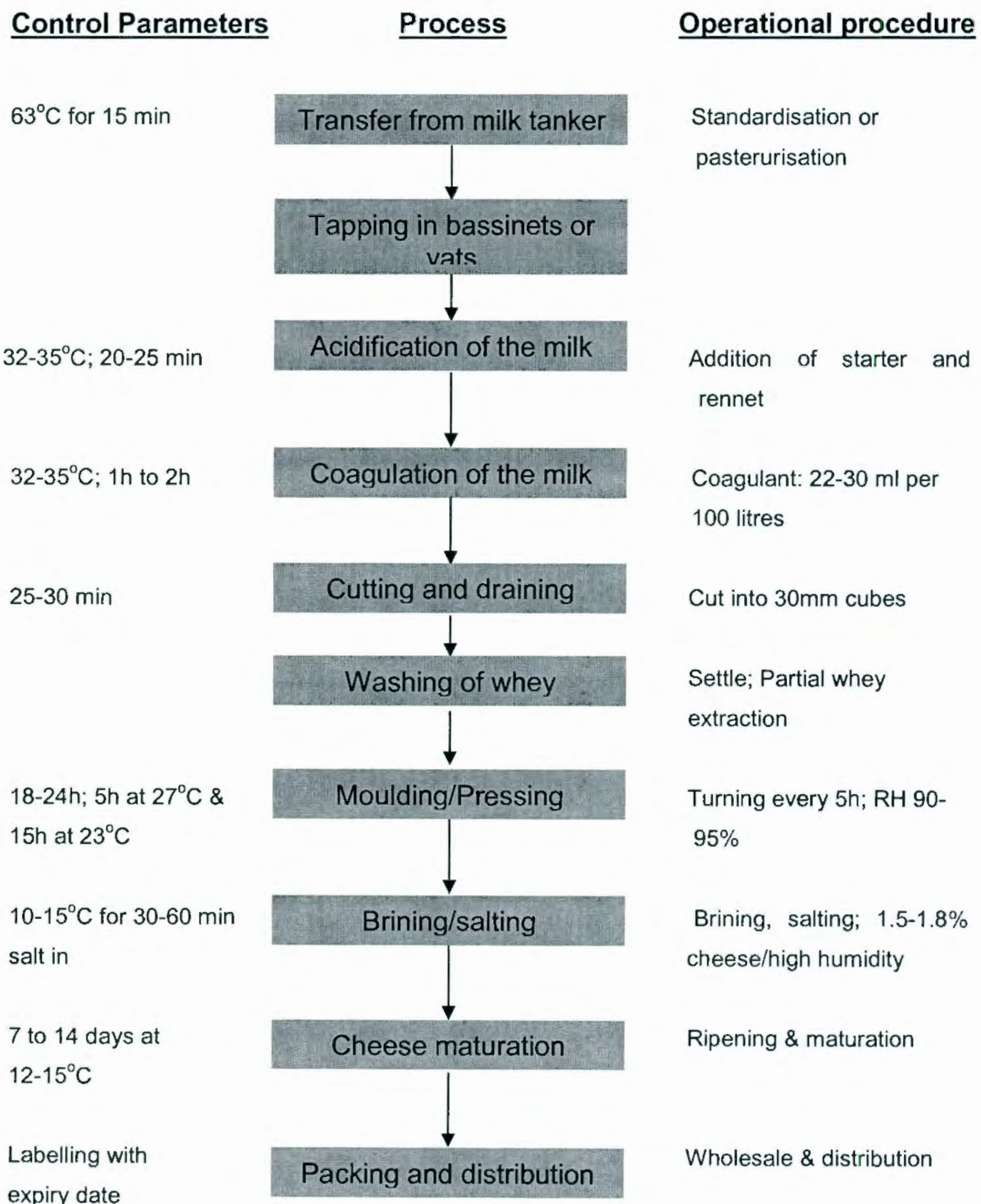

Fig. 1.1. The main steps involved in cheese manufacture. Adapted from Shaw, 1986 and Walstra *et al.*, 1999.

Process	Blaaukrantz	Creamy blue	Simonzola
Transfer from milk tanker			
Milk into vat(s)	±30 min; 34°C	±30 min; 32°C	±15 min; 33°C
Acidification and coagulation of the milk	±75 min; 34°C Lactic acid bacteria, *Penicillium* starter and rennet addition	±75 min; 34°C Lactic acid bacteria, *Penicillium* starter and rennet addition	±75 min; 34°C Lactic acid bacteria, *Penicillium* starter and rennet addition
Cutting and start stirring	±15 min; 33 - 34°C; Stirring, ±2h 15 min	±15 min; 31 - 32°C; Stirring, ±2h 15 min[a]	±45 min; 32 - 33°C; Stirring, ±45 min[b]
Draining of whey	±15 min; 33.5°C	±15 min; 31.5°C	±15 min; 36°C
Scooping into moulds	±15 min; 32°C	±15 min; 30.5°C	±15 min; 35°C
Turn no. 1	30 min after scooping; 30°C	30 min after scooping; 28°C	30 min after scooping; 33°C
Turn no. 2	15 min after Turn no. 1; 29°C	15 min after Turn no. 1; 27°C	15 min after Turn no. 1; 32°C
Turn no. 3	30 min after Turn no. 2; 28°C	30 min after Turn no. 2; 26°C	30 min after Turn no. 2; 31°C
Turn no. 4	45 min after Turn no. 3; 27°C	45 min after Turn no. 3; 25°C	45 min after Turn no. 3; 30°C
Turn no. 5	60 min after Turn no. 4; 26°C	60 min after Turn no 4; 24°C	60 min after Turn no. 4; 29°C
Brining/Salting	Salting 15 min after Turn no. 5 up to Day 2; 24°C; Brine Day 3 - 5	Salting 15 min after Turn no. 5 up to Day 3; 24°C; Brine Day 3 - 5	Salting 15 min after Turn no. 5 up to Day 3; 24°C; Brine Day 3 - 5
Piercing	Day 6 < 15°C	Day 6 < 15°C	Day 5 < 15°C
Maturation	7 - 9 weeks pH 5.6-6.2; 2-9°C	11 - 13 weeks pH 5.6 - 6.2; 2-9°C	9 - 11 weeks pH 5.6 – 6.2; 2-9°C
Packaging and distribution	Ideally during the 8th – 10th week	Ideally during the 12th – 14th week	Ideally during the 10th – 12th week

[a] Pasteurized dairy water added at 45°C [b] Dry stirring and dry salting

Fig. 1.2. The detailed steps involved in cheese manufacture of three typical South African blue-mould cheeses. Adapted from Shaw, 1986; Walstra *et al.*, 1999 and Simonsberg cheese factory.

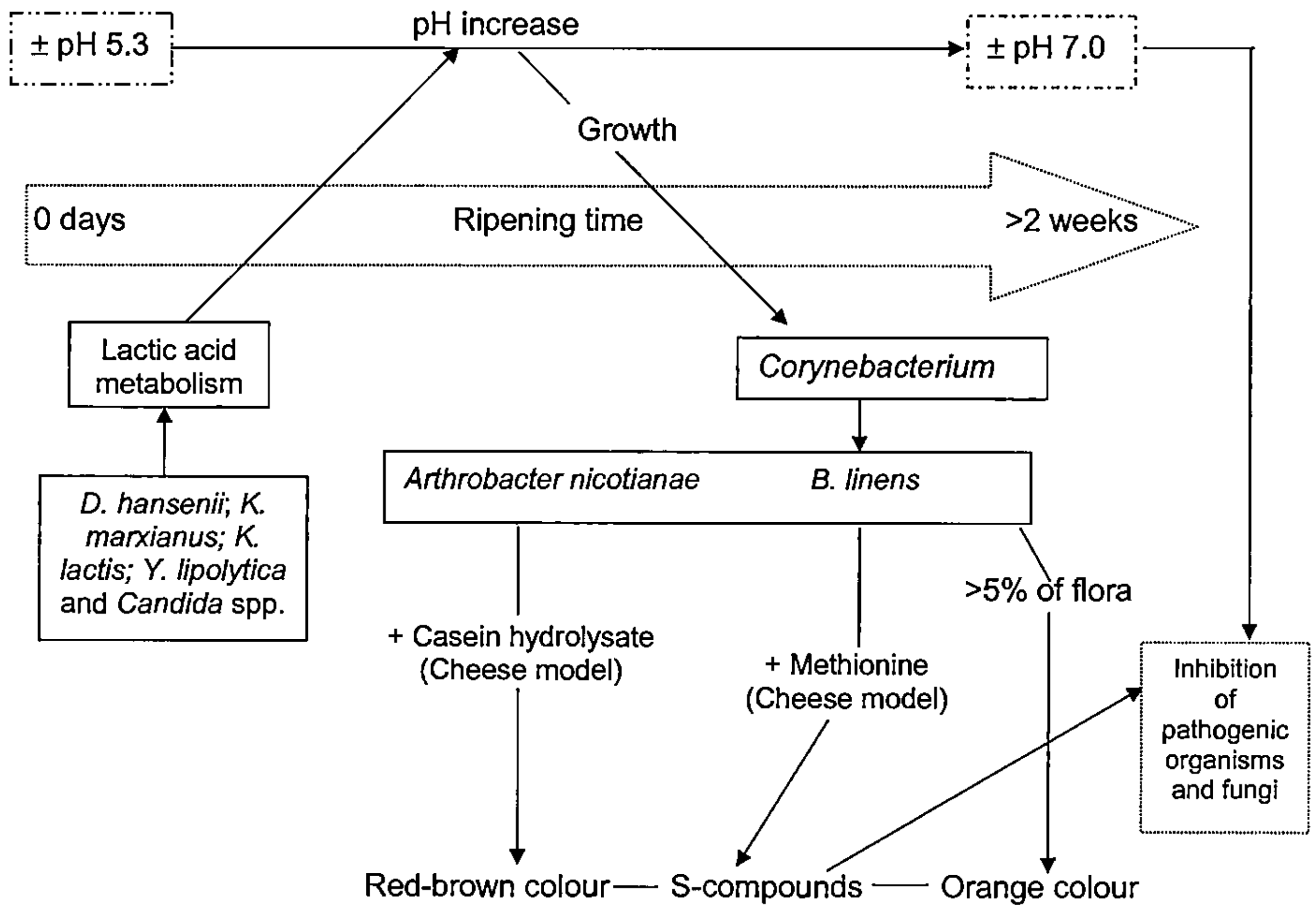

Fig. 1.3. A schematic representation of the growth and microbial changes that take place on the surface of mould-ripened cheese. Adapted from Bockelmann, 2002.

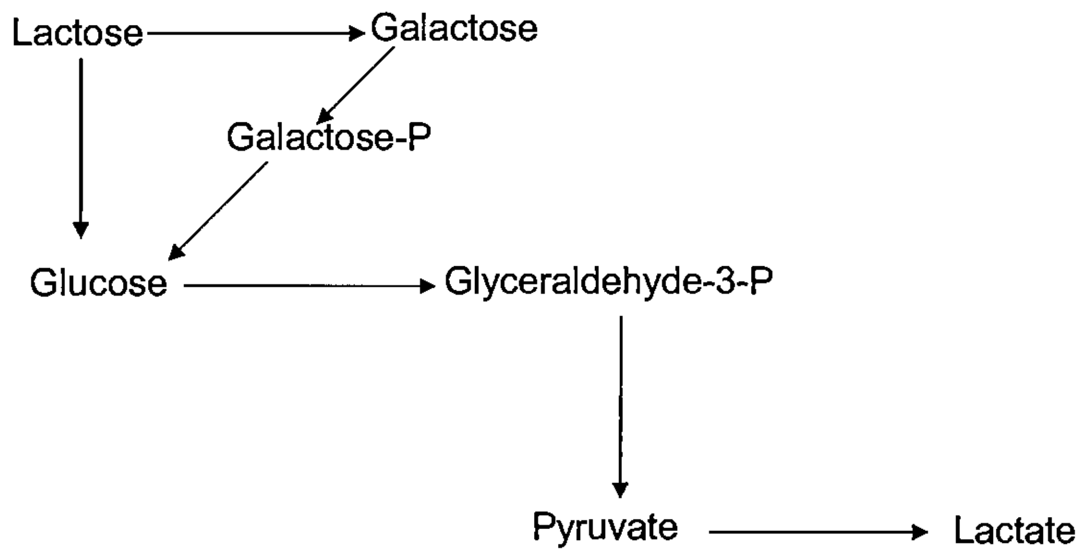

Fig. 1.4. A simplified schematic representation of lactose metabolism by lactoccci, leuconostocs and thermophilic cultutres.

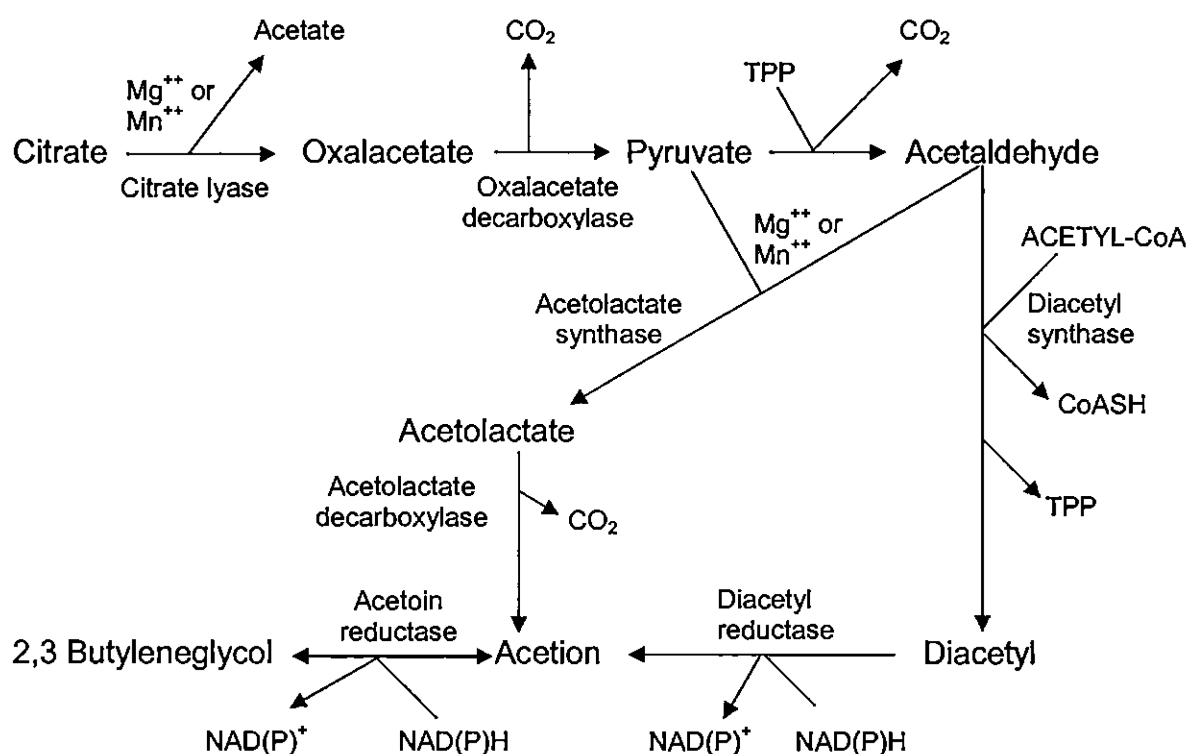

Fig. 1.5. The metabolism of citrate in *Leuconoctoc* sp and *Str. Lactic* subsp. *Diacetylactis*. Adapted from Fox *et al.* (1993) and Hugenholtz and de Felipe, 2002).

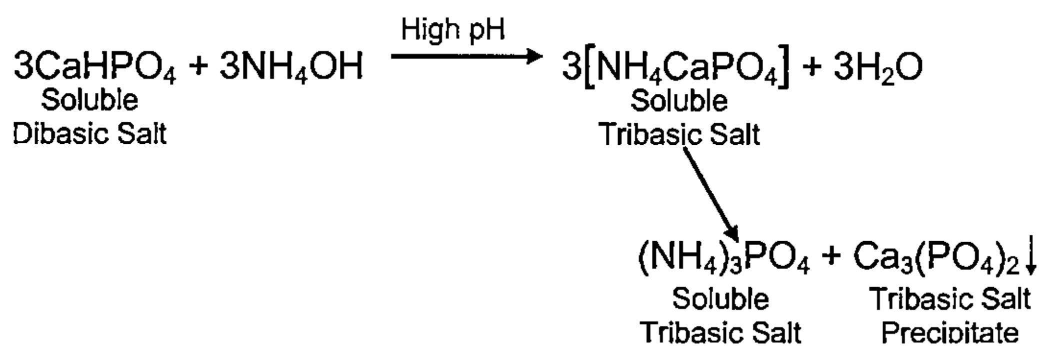

Fig. 1.6. The proposed model for the chemical reactions responsible for the redistribution of calcium and phosphorous during blue and white mould cheese ripening. Adapted from Karahadian and Lindsay, 1987.

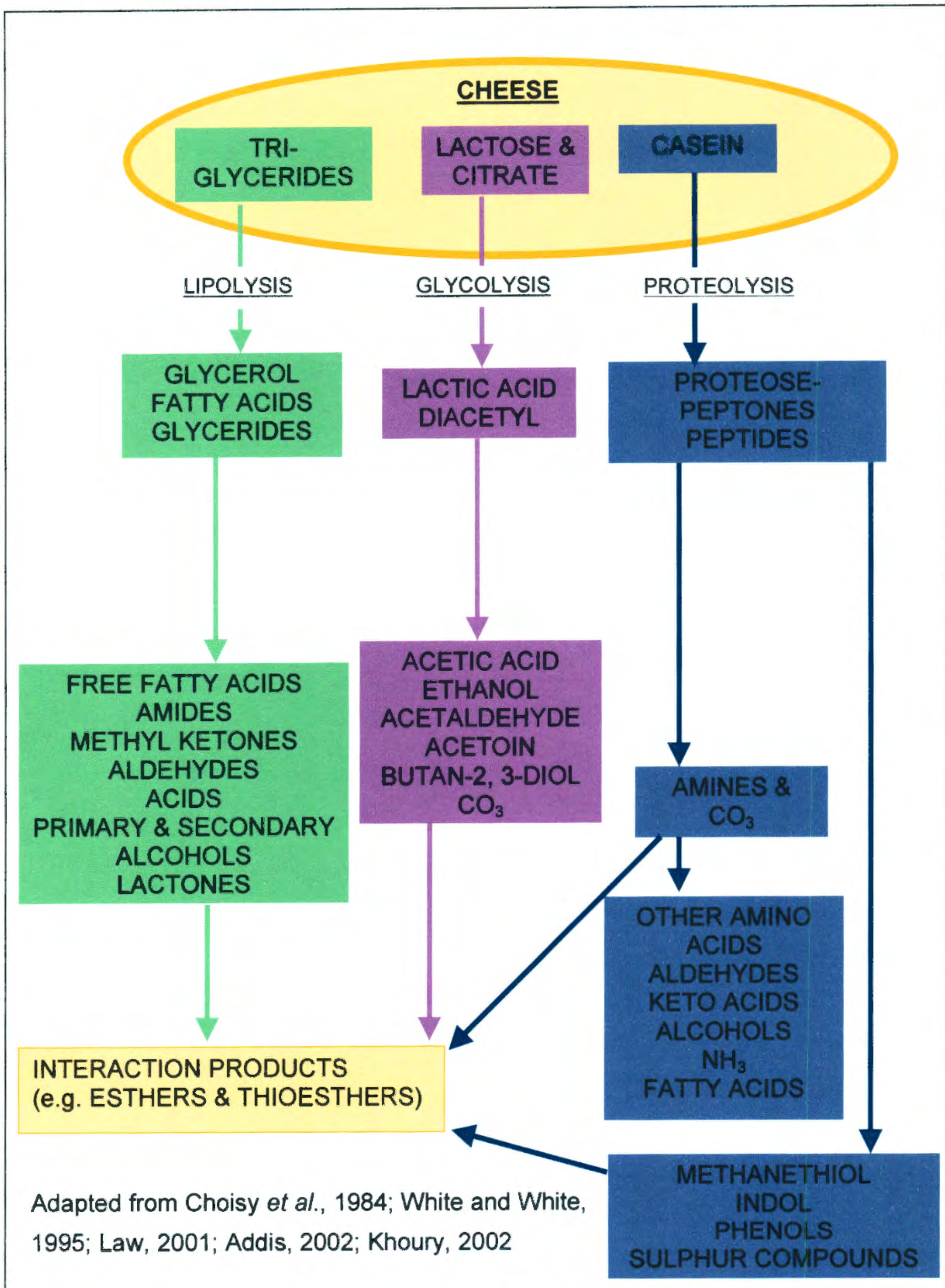

Fig. 1.7. The primary reactions involved in flavour development during cheese maturation.

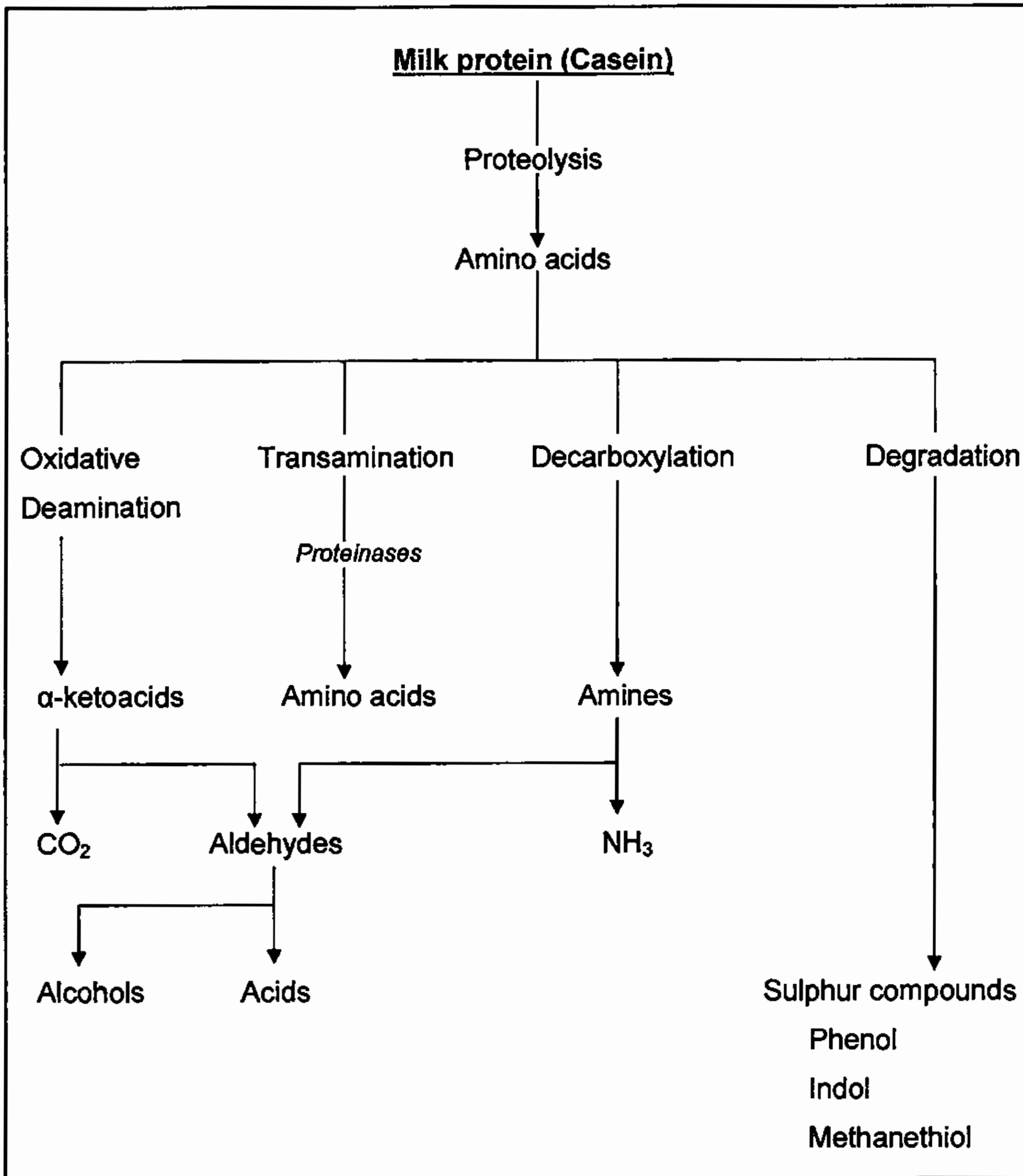

Fig. 1.8. The chemical reactions involved in the degradation of the milk proteins that contribute towards the final flavour and texture of the cheese. (Choisy *et al.*, 1984).

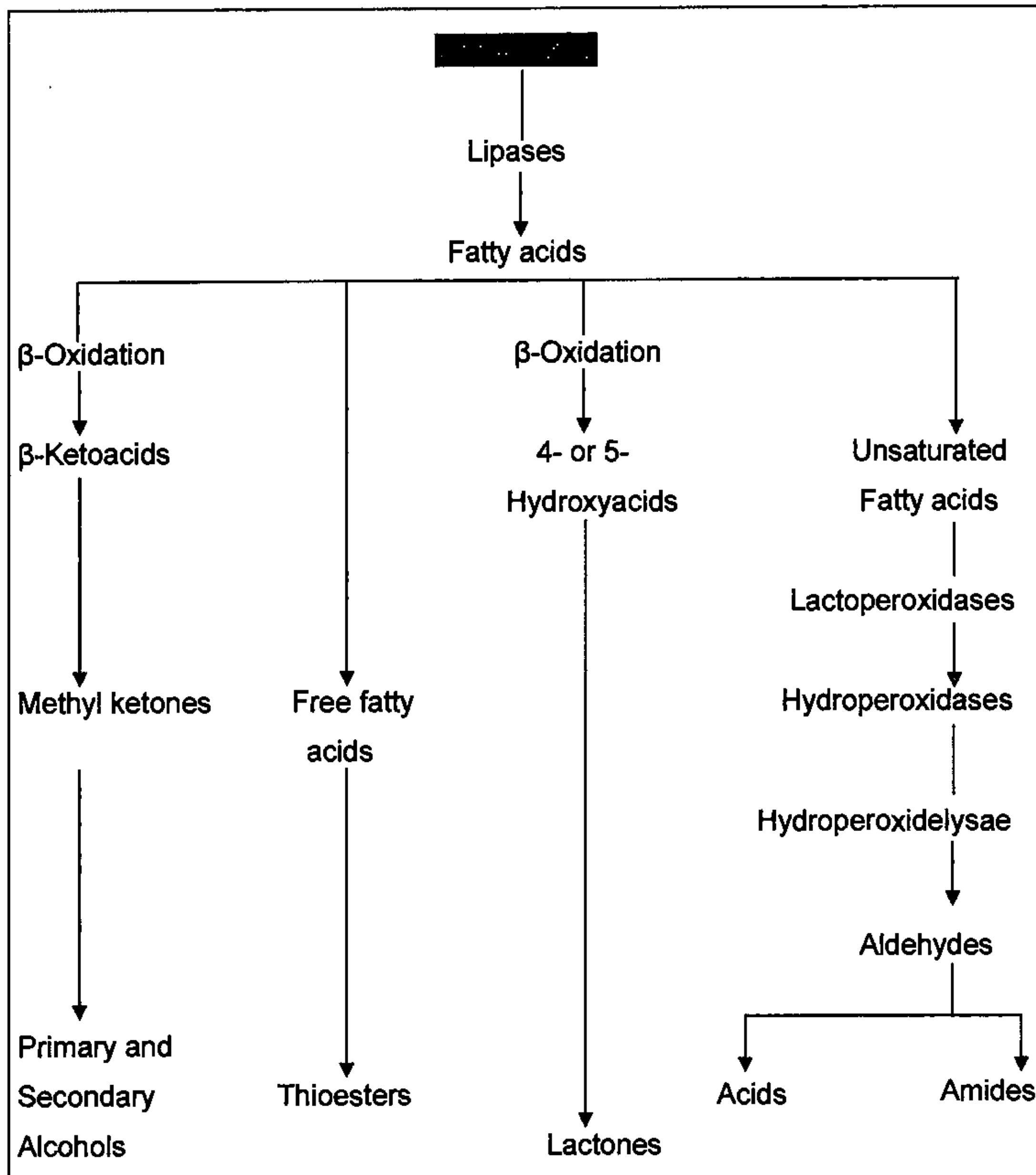

Fig. 1.9. The formation of flavour compounds from lipids (Dumont and Adda, 1978; Molimard and Spinnler, 1996; Fox, 2002).

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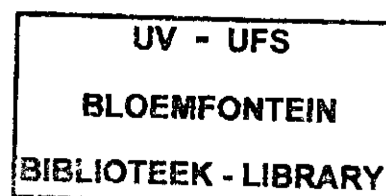
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Chapter 2

An inter-laboratory evaluation of selective media for the detection and enumeration of yeasts from blue-mould cheese

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ABSTRACT

Five countries representative of laboratories 1 to 5 evaluated eleven different selective media, designed to suppress mould and bacterial growth and support yeasts growth, for the recovery of yeast populations from blue veined cheeses. In addition, qualitative results were also incorporated. The yeast enumeration values were subjected to statistical analysis using analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test.

With the exception of Laboratory 3, none of the other laboratories was successful in recovering yeasts on all the media. No significant differences in quantitative data obtained on Rose-Bengal Chloramphenicol Agar (RBCA), Dichloran Rose-Bengal Chloramphenicol Agar (DRBC), Dichloran 18% Glycerol Agar (DG18), and Molybdate agar with 10% Sodium Propionate (MES) were detected by four of the collaborating laboratories whereas one laboratory found RBCA to be superior for yeast enumeration. DG18 and Malt Extract Agar with Biphenyl (MEB), however, were ranked superior based on qualitative results compared to the other media, attributed to distinctive individual yeast colonies and mould inhibition.

Keywords: Yeasts, blue-veined cheese, enumeration, selective media, and statistical significance.

1. INTRODUCTION

Mould ripened blue-veined cheeses are produced from pasteurised bovine milk to which lactic acid bacterial starters and a selected *Penicillium* species as secondary starter culture have been added. Yeasts are not traditionally added as part of the starter culture or as adjunct starter cultures, but are often reported to develop as natural contaminants to high numbers (> 10^6 cfu/g) in these cheeses (Addis *et al.*, 1998; Roostita and Fleet, 1996; van den Tempel and Jakobsen, 1998). The complicated interactions between these contaminating yeasts and the starter cultures have been investigated (Hansen and Jakobsen, 1996). The yeasts may affect the final product negatively by causing spoilage or uncontrolled fermentation reaction products that may affect the final flavour and texture. However, they may be desirable by contributing positively towards the chemical changes that take place during ripening resulting in improved sensory qualities of the product (Heard *et al.*, 1998; Jakobsen and Narvhus, 1996; Viljoen, 1998).

Since the survival of the yeast species and their interaction with other microorganisms are governed by unique environmental factors, it is imperative to select suitable media for surveying the yeast development during processing and maturation, and for strain isolation purposes. Factors that may contribute to unreliable yeast enumeration results include cheese storage at low temperature, high salt contents in the cheese, overgrowth of yeasts by predominantly moulds but also bacteria, and variable results between the inner and outer parts of the cheese (Addis *et al.*, 1998). The ideal medium would support the growth of all the yeasts nutritionally, while suppressing the growth of bacteria and moulds (Beuchat, 1993). A number of researchers have formulated media in this respect, mainly with the addition of fungal static chemicals (King *et al.*, 1986; Beuchat, 1993). However, in practice the moulds still tend to over grow and obscure the yeasts or the media did not support yeast growth sufficiently, resulting in reduced yeast colony counts. In addition yeasts are susceptible to structural and metabolic injury when

exposed to extreme environmental conditions including temperature, chemical and desiccation stress, conditions often prevailing during *in vitro* cultivations (Beuchat, 1984; Deák and Beuchat, 1996). Several previous studies have been performed to compare mycological media (Addis *et al.*, 1998; Welthagen and Viljoen, 1997; Deák *et al.*, 2001) but none proved to be totally adequate for the enumeration of yeasts in blue-veined cheeses. In an attempt to address this problem, the International Commission of Food Mycology initiated a collaborative study between five countries.

Since acidified media are not favoured for the enumeration of yeasts and moulds from most foods (Beuchat and Cousin, 2000) we omitted all acidified media, although some scientists continue to use them. Media containing antibiotics and/or various chemicals to suppress or inhibit mould growth are preferred (Beuchat *et al.*, 1998). Eleven different selective media were selected to statistically compare their suitability to isolate and enumerate the yeasts associated with blue-veined mould-ripened cheeses. The media included; Rose-Bengal Chloramphenicol (RBC) agar, Dichloran Rose-Bengal Chloramphenicol (DRBC) agar, Dichloran 18% glycerol (DG18) agar, Potato Dextrose agar with 7.5% sodium chloride (PDS), malt extract agar with biphenyl (MEB), Molybdate (MA) agar, Oxytetracycline Gentamicin Glucose Yeast extract (OGGY) agar, Malt Extract with 0.2% Ox-bile (MEO) and Molybdate agar with 10% sodium propionate (MEC), Yeast extract Glucose Chloramphenicol agar with Oligomycin (YGCO) and Yeast Extract agar with Eugenol (YEE).

2. MATERIALS AND METHODS

2.1. Sample preparation

A commercial blue-mould cheese sample was purchased in each country at local retail outlets and aseptically cut into three similar sub-samples and kept at 4°C until microbial analysis were completed. Ten-gram samples of each

sub-sample portion were removed, including both the inner and outer area of the sample to ensure complete microbial analysis. The weighed portions were homogenised in sterile sampling bags (Whirl-pack, Nasco) using a Colworth 400 stomacher (London, U.K.) with 90 ml of 0.1% (w/v) sterile peptone water (Biolab, Merck) for 2 min. After 3 min, allowing for settling, serial dilutions were prepared as required using sterile peptone water and aliquots of 0.1 ml spread plated in duplicate onto each of the selected media using a bent glass rod.

All plates were incubated in an upright position in the dark at 25°C for 5 days (± 2 h) without disturbing the plates. Selected plates bearing colonies between 15 – 150 colony forming units (cfu), or the highest number if below 15 were analysed.

2.2. Enumeration media

All media (Table 2.1) were prepared in accordance with the manufacturer's instructions and autoclaved at 121°C for 15 min. An aliquot of between 15 and 20 ml sterile media were dispensed into 90 mm Petri dishes and allowed to dry overnight at room temperature. In accordance with Deák *et al.*, (1998), all Dichloran Rose-Bengal Chloramphenicol (DRBC) agar plates (Biolab diagnostics, Merck, 100466), (King *et al.*, 1979) were kept in the dark to prevent Rose-Bengal from producing a toxic derivative which inhibits both the growth of the yeasts and the moulds. The remaining experimental media included: Rose-Bengal Chloramphenicol (RBC) agar (Biolab diagnostics, Merck, 100467), Dichloran 18% glycerol (DG18) agar (Oxoid, CM 729) (Hocking and Pitt, 1980), MES comprised malt extract agar (MEA, Merck, 105398) supplemented with 4% (w/v) NaCl (Merck, 6400) and 100 mg/l oxytetracycline (Merck 109877) to inhibit bacterial growth (Pitt *et al.*, 1992), malt extract agar with biphenyl (MEB), used MEA (Merck, 105398) supplemented with 0.05% (w/v) Biphenyl (Fluka, 14410) and filter sterilised 100 mg.l^{-1} oxytetracycline (Merck, 109877) to inhibit bacterial growth,

Molybdate (MA) agar (Atlas, 1993), Oxytetracycline Gentamycin Glucose Yeast extract (OGGY) agar (Mossel *et al.*, 1975) included OGY (Merck, 110877) supplemented with 0.1% (w/v) filter sterilised oxytetracycline solution (Merck, 109877) and a 0.05% (w/v) gentamycin sulphate (Fluka, 48760), Malt Extract agar (Merck, 105398) supplemented with 0.2% Ox-bile (Merck, 103756) (MEO) and Molybdate agar with 10% calcium propionate (MEC) (Rale and Vakil, 1984), Yeast extract Glucose Chloramphenicol agar (Biolab diagnostics, Merck, 116000), supplemented with Oligomycin (Merck, 116000) (YGCO) (Eliskases-Lechner and Prillinger, 1996) and Yeast Extract agar (Biloab diagnostics, Merck, 103750) supplemented with Eugenol (Fluka 46100) (YEE) (Vázquez *et al.*, 2001).

Table 2.1. Details the eleven media used in this study for the isolation and enumeration of yeasts from blue-mould cheese.

Media	Full Name/Description	Manufacturer	pH	Reference
DRBC	Dichloran Rose-Bengal Chloramphenicol	Merck, 100466	5.6	King *et al.*, 1979
RBCA	Rose-Bengal Chloramphenicol Agar	Merck, 100467	7.2	Jarvis, 1973
DG18	Dichloran 18% Glycerol Agar	Oxoid, CM 729	5.6	Hocking & Pitt, 1980
MES	Malt Extract Agar with 4% NaCl	Merck, C10 & Sigma 0-5750	6.0	Addis *et al.*, 1996; Jakobsen & Narvhus, 1996
MEB	Malt Extract Agar with Biphenyl	Merck, C10 & Sigma 0-5750	5.4	Addis *et al.*, 1996; Beech & Carr, 1995
MA	Molybdate agar	Sigma	5.3	Atlas, 1993
OGGY	Oxytetracycline Gentamycin Glucose Yeast Extract with 0.1% Gentamycin solution	Merck, 10877 & Sigma, G-6896	7.0	Mossel *et al.*, 1975
MEO	Malt Extract Agar with 0.2% Ox-bile	Merck, C10 & Oxoid, L50	5.4	
MEC	Molybdate agar with 10% Calcium propionate	Sigma		Rale & Vakil, 1984
YGCO	Yeast Extract Glucose Chloramphenicol with Oligomycin	Merck, 116000	6.6	Eliskases-Lechner & Prillinger, 1996
YEE	Yeast extract Agar with Eugenol	Merck, 103750 & Fluka, 46100	7.2	Vázquez *et al.*, 2001

2.3. Statistical analysis

Means and standard deviations of yeast counts (cfu/g) for all media and laboratories were tabulated in Table 2.2. Ease of counting colonies (EFC) was also indicated for each media. Only media where yeast enumeration was successful and/or easy for all laboratories were subjected to further statistical analysis. Analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test ($\alpha = 0.05$) were carried out to determine whether significant differences between yeast counts on the selected media existed within each laboratory (NCSS, 2001).

3. RESULTS AND DISCUSSION

Since each laboratory, representative of five different countries purchased their blue cheese sample in their corresponding countries, variable results regarding the enumeration of yeasts associated with these cheeses were expected. The survey, however, was not based on comparisons between cheeses of the different countries, instead the tendencies of accumulative results using eleven different selective media as applied in each laboratory was compared. In addition to the enumeration of yeast populations, subjective evaluations by the collaborating laboratories concerning the ease of counting the yeasts based on colony formation, and the inhibition of moulds and bacteria were also taken into consideration. Table 2.2 summarises the data obtained during the collaborative study.

The results as reflected in Table 2.2 indicated that only Laboratory 3 was successful in enumerating yeast populations on all 11 selective media. Laboratories 2 and 4 failed to recover any yeasts from MA, OGGY, MEC and YEE, whereas laboratories 1 and 5 failed to recover any yeasts from the MEC medium. Therefore, four laboratories (1, 2, 4 and 5) were unable to obtain any yeast enumeration results from MEC, and in addition Laboratory 3 also indicated difficulty of counting the yeasts due to mould overgrowth. Molybdate

agar was originally formulated for the isolation of clinical species, but modified with the addition of calcium propionate to prevent mould spreading aiding in the isolation of yeasts from mould-contaminated foods (Rale and Vakil, 1984). The addition of calcium propionate however, proved inadequate for the isolation and enumeration of yeasts (Table 2.2) from blue mould cheeses with high mould populations due to limited prevention of mould spreading. Addis *et al.* (1998) and Eliskases-Lechner and Prillinger (1996) also stressed on the limited inhibitive action of calcium propionate when applied for the isolation of yeasts from blue cheese. Therefore, the results obtained from the MEC medium were omitted from statistical comparisons. Similarly, results obtained from MA, OGGY and YGCO media were also omitted for the final statistical comparisons due to inconsistent enumeration results attributed to poor visibility of the yeasts overgrown by moulds. The antibiotic supplemented OGGY and Molybdate agar both proved to be unsuccessful in preventing mould spreading obscuring the yeasts and consequently difficult counting. Contrary to previous reports (Eliskases-Lechner and Prillinger, 1996), the addition of oligomycin to YGC was also found to be ineffective to prevent mould spreading.

In contrast, MEO and YEE contained 0.2% ox-bile and eugenol respectively as inhibitive substances for mould growth (Vazquez *et al.*, 2001), both media were however highly contaminated with bacterial loads making the enumeration of yeasts difficult or impossible (Table 2.2). Consequently, these two media were also omitted from statistical comparisons, as both proved inadequate for yeast enumeration in an environment with high numbers of moulds and bacteria. It was interesting to note, however that eugenol (the main component of glove oil) showed antifungal properties and should be evaluated with the addition of an antibiotic to prevent bacterial growth.

Differences in mean populations of yeasts recovered from cheeses by the collaborating laboratories on the five remaining media (RBCA, DRBC, DG18, MES and MEB) were small, but significant for Laboratories 1, 4 and 5

(Table 2.3). Laboratory 2 and 3 showed similar yeast enumeration results within each laboratory respectively for all five media, as the results yielded no significant differences when statistically analysed. On the contrary Laboratory 1 and 5 obtained significantly different values within their respective laboratory analyses, as both indicated that significant ($p<0.001$) lower enumeration values were recovered on MEB compared to the other media. Laboratory 4 obtained insignificant differences in yeast populations between MEB and DG18, and between DRBC and MES, but the latter showed significant ($p<0.01$) higher results than the former. This laboratory however, found the recovering of yeast populations from RBCA to be significantly ($p<0.01$) superior compared to the other media. Despite significant quantitative differences obtained between the five media, it must be noted that three laboratories indicated that superior qualitative results were obtained using malt extract agar with biphenyl. According to the results, the addition of biphenyl to MEB resulted in easy differentiation and isolation of yeast colonies since the growth of the moulds was substantially retarded. Although inferior to biphenyl, the addition of Rose-Bengal, Dichloran or NaCl, normally added as mould inhibitors (Addis *et al.*, 1998; Deàk and Beuchat, 1996), also proved to aid yeast enumeration in environments contaminated with high populations of moulds (Table 2.2 and Table 2.3). Laboratory 1 and 5 indicated that similar qualitative results compared to MEB were obtained from DG18.

In summary, based on the results obtained during the inter laboratory survey applying 11 different media, RBCA, DRBC, DG18, and MES were superior in supporting yeast colony development in samples obtained from blue veined cheeses. Other than Laboratory 4 who found RBCA to be superior for yeast enumeration, all the other laboratories found no quantitative distinction between the four media. DG18 and MEB, however, were ranked superior based on qualitative results compared to the other media, attributed to distinctive individual yeast colonies and mould inhibition.

Table 2.2. The mean yeast populations enumerated on 11 different selective media by each of five participating countries.

Laboratory number	[a]MEAN YEAST POPULATIONS (cfu/g)										
	RECOVERY MEDIA										
	RBCA	DRBC	DG18	MES	MEB	MA	OGGY	MEO	MEC	YGCO	YEE
1	$1.11x10^{5}$	$1.15x10^{5}$	$1.30x10^{5}$	$1.18x10^{5}$	$4.02x10^{4}$	$5.63x10^{7}$	$7.75x10^{4}$	$8.18x10^{4}$	0	$1.07x10^{5}$	$9.33x10^{7}$
SD	$0.07x10^{5}$	$0.36x10^{5}$	$0.39x10^{5}$	$0.42x10^{5}$	$1.3x10^{4}$	$3.04x10^{7}$	$2.23x10^{4}$	$0.98x10^{4}$	0	$0.56x10^{5}$	$4.3x10^{7}$
EFC	***	***	****	***	****	*	***	*	*	*	**
2	$2.62x10^{4}$	$3.33x10^{4}$	$5.67x10^{4}$	$4.03x10^{4}$	$3.40x10^{4}$	0	0	$5.58x10^{4}$	0	$5.57x10^{4}$	0
SD	$0.71x10^{4}$	$0.66x10^{4}$	$1.1x10^{4}$	$0.95x10^{4}$	$0.15x10^{4}$	0	0	$0.45x10^{4}$	0	$1.0x10^{4}$	0
EFC	***	***	***	***	***	NA	NA	***	NA	***	**
3	$1.26x10^{7}$	$4.22x10^{7}$	$1.26x10^{7}$	$1.34x10^{7}$	$8.73x10^{6}$	$3.59x10^{7}$	$2.31x10^{7}$	$1.92x10^{7}$	$1.80x10^{7}$	$1.32x10^{7}$	$3.62x10^{7}$
SD	$0.31x10^{7}$	$0.1x10^{7}$	$0.3x10^{7}$	$0.28x10^{7}$	$1.6x10^{6}$	$3.3x10^{6}$	$2.9x10^{6}$	$1.35x10^{7}$	$0.63x10^{7}$	$4.17x10^{6}$	$1.80x10^{7}$
EFC	***	***	***	***	****	*	***	**	*	*	**
4	$1.98x10^{4}$	$2.83x10^{3}$	$9.83x10^{3}$	$5.00x10^{3}$	$1.00x10^{4}$	0	0	$6.75x10^{3}$	0	$1.22x10^{4}$	0
SD	$0.45x10^{4}$	$2.8x10^{3}$	$2.02x10^{3}$	$2.29x10^{3}$	$0.28x10^{4}$	0	0	$3.18x10^{3}$	0	$0.4x10^{4}$	0
EFC	***	***	***	***	***	*	*	**	*	***	**
5	$4.00x10^{4}$	$2.50x10^{4}$	$3.75x10^{4}$	$3.43x10^{4}$	$8.03x10^{3}$	$1.58x10^{6}$	$1.05x10^{4}$	$3.03x10^{5}$	0	$1.85x10^{4}$	$3.37x10^{6}$
SD	$0.78x10^{4}$	$0.38x10^{4}$	$1.16x10^{4}$	$1.0x10^{4}$	$4.56x10^{3}$	$1.06x10^{6}$	$0.35x10^{4}$	$0.77x10^{4}$	0	$0.3x10^{4}$	$1.03x10^{6}$
EFC	***	***	****	***	****	*	*	**	*	*	**

RBCA - Rose-Bengal chloramphenicol agar;
DRBC - Dichloran Rose-Bengal chloramphenicol agar;
DG18 - Dichloran 18% glycerol agar;
MES - Malt extract agar supplemented with NaCl and oxytetracycline;
MEB - Malt extract agar with biphenyl;
MA - Molybdate (MA) agar;
OGGY - Oxtetracycline gentamycin glucose yeast extract agar;
MEO - Malt extract agar supplemented with 0.2% Ox-bile;
MEC - Molybdate agar with 10% calcium propionate;
YGCO - Yeast extract glucose chloramphenicol agar, supplemented with oligomycin;
YEE - Yeast Extract agar supplemented with eugenol.

a - Means of values obtained from three similar cheese samples, each sampled in duplicate (3 x 2);

0 – Indicates no colonies or colonies were too difficult to count; **NA** – Not applicable; **EFC** – Ease of counting colonies; **SD** – Standard deviation;

* – Very difficult to count, plates overgrown with moulds;
** – Difficult to count, too many bacteria present;
*** – Enumeration successful, mould and bacteria growth limited;
**** – Yeast enumeration easy, i.e. obtained the best results.

Table 2.3. Comparison of media for the enumeration of yeast populations associated with blue veined cheese by each of five participating countries.

Laboratory number	[a]MEAN YEAST POPULATIONS (LOG_{10} cfu/g)					
	RECOVERY MEDIA					
	RBCA	DRBC	DG18	MES	MEB	Significance level
1	5.0423[b]	5.0435[b]	5.0981[b]	5.0536[b]	4.5773[a]	***
2	4.3633	4.4773	4.7168	4.5440	4.5958	NS
3	7.0827	7.0523	7.0976	7.1158	6.9278	NS
4	4.2233[b]	3.4275[a]	3.9798[ab]	3.6587[a]	3.9818[ab]	**
5	4.5921[b]	4.3913[b]	4.5441[b]	4.5162[b]	3.8499[a]	***

RBCA - Rose-Bengal chloramphenicol agar;

DRBC - Dichloran Rose-Bengal chloramphenicol agar;

DG18 - Dichloran 18% glycerol agar;

MES - Malt extract agar supplemented with NaCl and oxytetracycline;

MEB - Malt extract agar with biphenyl.

Means with different superscripts within the same row differed significantly.

NS - Not significant.

** - P < 0.01.

*** - P < 0.001.

a - means of values obtained from three similar cheese samples, each sampled in duplicate (3 x 2).

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Chapter 3

Development of yeast populations during the processing and maturation of blue-mould cheese

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ABSTRACT

Varieties of blue-mould cheeses were analysed regularly during different stages of manufacturing and maturation to determine the origin of contaminating yeasts present, their population diversity and development until end of storage. Yeast diversity and development in the inner and outer core of the cheeses during maturation were also compared.

Air samples revealed few if any yeasts whereas contact samples from equipment and the surroundings revealed high numbers of yeasts, implicating it as the possible main source of post-pasteurisation contamination, as very few yeasts were isolated from the milk and cheese making process itself. Samples from the inner and outer core of the maturing cheeses had typical survival curves. The number of yeasts on the outer core was about 100 fold more than those in the inner core.

The most abundant yeasts isolated from the environment and maturation cheeses were identified as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida versatilis* and *Cryptococcus albidus*, while the yeasts *Candida antillancae* and *Dekkera anomala* were additionally isolated from the environment. Yeasts were present in high numbers, making their occurrence in blue-mould cheeses significant.

Keywords: Blue cheese, yeasts, lactic acid bacteria, identification

1. INTRODUCTION

The occurrence of yeasts in dairy products like cheese is not unexpected as these products have various properties encouraging the proliferation of yeasts such as high acidity, storage at low temperature, low moisture content and elevated salt concentrations (Fleet, 1990; Viljoen, 2001). Their presence is of major importance as they can be beneficial or detrimental, being a major component of the microflora that contribute to the maturation and flavour development (Fleet, 1990; Beresford *et al.*, 2001) as well as causing spoilage in cheese (Fleet and Mian, 1987; Jakobsen and Narvhus, 1996).

Blue cheese varieties have further unique physical and chemical properties, which select for the growth and prevalence of specific yeast species (Roostita and Fleet, 1996; van den Tempel and Jakobsen, 1998; Addis *et al.*, 2001) such as high fat and protein concentrations, low pH levels, residual unfermented lactose, high concentrations of lactic acid, elevated concentrations of salt, the presence of citric and acetic acids and storage at low temperatures.

The yeasts most frequently isolated from blue cheeses include, *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus, Kluyveromyces lactis* and *Candida* spp. (de Boer and Kuik, 1987; Besançon *et al.*, 1992; Roostita and Fleet, 1996; van den Tempel and Jakobsen, 1998). These yeasts form an integral part of the microflora of blue cheese where they contribute to the maturation process by flavour formation through lipolytic and proteolytic activity (Suzzi *et al.*, 2001), production of aroma compounds, gas production which assists the development of *Penicillium roqueforti* and increase of the pH on the surface of the cheese resulting in the development of secondary microflora, like *Brevibacterium linens* (Devoyod, 1990; Wyder and Puhan, 1999).

Traditionally raw milk was used for the manufacture of blue cheese varieties,

which is still the case for some varieties (de Boer and Kuik, 1987). Currently, however, cheese processors prefer pasteurized milk to minimise the microbial diversity (Fleet, 1990). This resulted in several advantages like the improvement in the bacteriological quality and consequently also the quality of the cheese, with less defects incurred by the poor quality of the raw milk (de Boer and Kuik, 1987). Also, pasteurized milk resulted in a less diverse population of yeasts, usually considered as undesirable and a sign of poor hygiene (Fleet, 1990; Jakobsen and Narvhus, 1996). As yeasts are not able to survive pasteurization temperatures any yeasts present in the cheese will be due to post pasteurization contamination (Devoyod, 1990; Fleet, 1990).

Sources of contamination within the dairy environment responsible for the contamination of the milk and cheese during processing are primarily factory surfaces such as the floors, walls, equipment and brine (Seiler and Busse, 1990; Welthagen and Viljoen, 1999; Leclercq-Perlat *et al.*, 2000). The contribution of these surfaces to contamination, however, varies from factory to factory, depending on the sanitizing practices of the factory (Laubscher, 1999). Brine contains yeast populations (Seiler and Busse, 1990) ranging from 10^4 cfu/ml up to 10^6 cfu/ml (van den Tempel and Jakobsen, 1998). Because of its high salt content, the brine selects for a specific microflora, and consequently halotolerant yeasts like *D. hansenii* and *Y. lipolytica* are frequently encountered (van den Tempel and Jakobsen, 1998; Leclercq-Perlat *et al.*, 2000). Secondary sources include equipment, the air and the worker's hands and aprons (Viljoen and Greyling, 1995; Welthagen and Viljoen, 1999).

After yeast contamination in the cheese, due to the immediate environment, the development of the yeasts during the processing and maturation is governed by properties prevailing in the cheese and its vicinity. Due to the production of lactic acid, by lactic acid starter bacteria, during the manufacturing process, the pH of the cheese decreases (Devoyod, 1990) and as a consequence it offers a selective environment for yeast growth, which is

on the other hand unfavorable for most contaminating bacteria (Devoyod, 1990; Beresford *et al.*, 2001). Similarly the high salt concentration in the brine, storage at low temperatures and lower water-activity render yeasts a competitive advantage (Viljoen, 2001). Spoilage in most dairy products becomes evident when the yeast population reaches $10^5 - 10^6$ cells.g^{-1} (Fleet, 1990), but counts as high as 10^{-8} cells.g^{-1} were detected in cheeses without any visible defects.

The size and composition of the yeast populations during the maturation process on the exterior and interior of the cheese differ dramatically within blue cheese (Devoyod, 1990). A 10-fold difference in yeast population size was encountered between the exterior and the interior, with the exterior exhibiting higher yeast populations (Devoyod, 1990). The interior yeast population showed a less diverse group compared to the exterior and composes only a few species like *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Candida versatilis* (Devoyod, 1990). The yeast population representative of the exterior comprised a wider diversity and in addition included strains of *Candida sake, Candida intermedia* and *Yarrowia lipolytica* (Devoyod, 1990). These populations remained viable until retail although minor differences may be expected (Choisy *et al.*, 1987).

Although several publications indicated the occurrence and numbers of yeasts in blue cheeses, no studies were performed to investigate the growth and development of yeasts populations during manufacturing and maturation. Consequently in this study we endeavoured to determine the origin of contaminating yeasts in a blue cheese plant as well as their development during processing and maturation.

2. MATERIALS AND METHODS

2.1. Blue-mould cheese manufacturing

Blue type cheeses (Gorgonzola-style and Danish-style) were manufactured on four occasions at a commercial cheese factory in the Western Cape region of South Africa. The basic cheese making procedure for Danish- and Gorgonzola-style was the same and carried out as described by Mistry and Kosikowski (1997).

The main differences between the two types of cheese are the type of mould spores and starter culture used (spores of Danish-style more proteolitic and of Gorgonzola-style more lipolytic), fat content (Gorgonzola, 65%; Danish-style, 45%), formation of the mould during maturation and time of maturation.

2.2. Sampling methods and selection of isolates

On all four occasions, several surfaces (Table 3.1) were sampled in duplicate by means of RODAC contact plates (Favero *et al.*, 1968) using DeMann Rugosa and Sharpe agar (MRS) (Merck, C86 - pH 6.5, Darmstadt, Germany) for the analyses of lactic acid bacteria, and Dichloran Rose Bengal Chloramphenicol (DRBC) (Oxoid, Basingstoke, UK) agar for yeasts. Air was sampled by using standard settle plates (the same media in 90 mm Petri dishes) with an exposure time of 5 min.

Samples were also taken during the manufacturing of the blue cheeses at selected points as indicated in Table 3.2; the basic process for Danish- and Gorgonzola-style was the same. Liquid samples were taken by aseptically scooping with a sterile McCartney bottle in the cheese vat, and solid samples by aseptically cutting and transferring into sterile bags.
For the solid samples, 10 g portions were weighed into 90ml sterile peptone water and homogenized in a Colworth 400 stomacher (London, UK) for 2 min.

Further decimal dilutions of the suspensions and liquid samples were performed in 9 ml sterile peptone water. Aliquots (0,1 ml) of the dilutions were spread inoculated over the surface of plates containing the media. DRBC plates were aerobically incubated at 25°C for 4 days, and MRS plates at 25°C for 48 h.

2.3. Sampling during maturation

Gorgonzola-style and Danish-style blue cheeses from the same batch were kept under controlled conditions at 4°C at the site of cheese production and sampled directly after processing at consecutive intervals on a weekly basis during maturation over a 13-week period for Gorgonzola-style and 26 weeks for Danish-style. Duplicate cheese samples were prepared for microbiological analysis by aseptically cutting a cheese wedge from the cheese. A 10 g cheese sample was respectively taken from the surface and the center of the cheese wedge and further microbiologically analyzed as described earlier.

2.4. Enumeration and isolation

All plates containing between 25 and 250 colony-forming units (cfu) (or the highest dilution if below 25), were enumerated and the mean values determined from duplicate samples. Results were recorded as the mean value of two trials, from duplicate plate samples originating from duplicate cheese samples from the same batch (2X2X2).

Yeast colonies were isolated from the highest dilution plates onto DRRBC plates. The yeast isolates were sub-cultured on Malt Extract agar (MEA) (Merck, C10 - pH 5.4), incubated for 48 h at 25°C and checked for purity by colony morphology and microscopy. Pure cultures were stored on Yeast Malt Extract (YM) agar slants at 4°C (Wickerham, 1951) during the period of investigation, until characterisation.

2.5. Yeast Identification

Individual yeast isolates were identified by conducting physiological, sporulation and morphological tests as described by Kurtzman and Fell, (1998). Data were interpreted using the keys of Kurtzman and Fell, (1998) and the computer program of Barnett *et al.* (1987). Each isolate was inoculated into 6 sugar fermentation media and 32 carbon source assimilation media (van der Walt and Yarrow, 1984). Additional tests performed included growth at 37°C and in 50% D-glucose medium, urea hydrolysis and assimilation of nitrogen compounds, performed by means of auxanographic method (Lodder and Kreger-van Rij, 1952) was also included.

Ascospore formation was examined on McClary's acetate agar, potato glucose agar, Gorodkowa agar, corn meal agar and MEA (Kreger-van Rij, 1984). The inoculated media were incubated at 18°C for 4 weeks and examined at 4-day intervals. Cell morphology and mode of reproduction were examined on MEA (Biolab, Merck, Darmstadt) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudo- and true mycelium was examined on corn meal agar according to the Dalmau plate technique (Wickerham, 1951).

3. RESULTS AND DISCUSSION

3.1. Yeast development during processing

Air samples, in the vicinity of processing equipment, revealed no yeasts from the production room, and low numbers from the brine and cheese maturation rooms (Table 3.1). In contrast to the low yeast counts observed from air samples, higher counts of bacteria were present in the air, especially in the brine room. The low number of yeasts corresponds to earlier data (Viljoen and Greyling, 1995; Welthagen and Viljoen, 1998) indicating that the air contributes very little to yeast contamination in the dairy industry.

Contact samples taken within the factory environment associated with the equipment revealed limited presence of yeasts and bacteria (<10^3 cfu/25cm^2), mainly attributed to a successful cleaning program. The stainless steel equipment, if properly cleaned, usually harbors low numbers of yeasts and bacteria, which consequently contribute little to post pasteurization contamination (Welthagen and Viljoen, 1998). However, these microbial numbers increased during processing and successive batches (Welthagen and Viljoen, 1999). Building surfaces, including the floors, walls and doors as well as the shelves in the second maturation room exhibited high numbers of yeasts whereas low numbers were detected on the surfaces in the processing room (Table 3.1). The higher yeast counts observed in the second maturation room is attributed to the usage of wooden shelves, which maintain higher loads of microbial contamination due to difficulty for proper cleaning. Movement between the rooms, as indicated by the high yeast counts on the doors leading to the maturation rooms (>2000 cfu/25cm^2) also played an important role in the spreading of yeasts within the plant. Surfaces like walls and floors, therefore, make an important contribution to yeast contamination (Welthagen and Viljoen, 1998). Similar results were obtained during the making of Gouda and Cheddar and other cheeses (Fleet and Mian, 1987; Welthagen and Viljoen, 1998).

The brine has been shown to be a major source of yeast contamination (10^4 to 10^5 cfu/ml) (Table 3.1) as also indicated by Seiler and Busse (1990). Some of these yeasts, like *Debaryomyces hansenii,* which showed to have an enhanced resistance to such adverse conditions like high salt concentrations are likely to increase in numbers at later stages of the production or during the maturation period (Welthagen and Viljoen, 1998). Despite the frequent occurrences of yeasts in the brine, it must be kept in mind that the numbers and diversity may vary between dairy plants and even between consecutive days in the same plant, due to variation of salt concentrations and age of the brine (Seiler and Busse, 1990; Welthagen and Viljoen, 1998).

Yeasts isolated from the dairy environment are listed in Table 3.3. The occurrence of *D. hansenii* on all occasions during environmental samplings was expected, as the species has been isolated from the dairy environment by numerous researchers (Fleet and Mian, 1987; Welthagen and Viljoen, 1998). *Torulaspora delbrueckii* and *Cryptococcus albidus* were found predominantly on the equipment, whereas, the former was found infrequently from the air. Both species have been isolated from the dairy environment at limited numbers (Welthagen and Viljoen, 1998). Despite the presence of *Dekkera anomala* and *Dekkera bruxellensis* on the walls and equipment, these species have not previously been isolated from the dairy environment, and were not recovered during processing or maturation. Therefore, their presence might be regarded as accidental (Deàk and Beuchat, 1996). *Candida* spp. usually account for a small percentage of yeasts isolated from the dairy environment, which corresponds to our data.

Very low counts of yeasts were recorded during the manufacturing process (Table 3.2). Counts of yeasts generally tended to slightly increase towards the end. The highest count recorded was 70 cfu/g at the end of the process, during moulding. The only yeast species isolated during the processing stage was a representative of *Torulaspora delbrueckii*.

3.2. Yeast development during the maturation period

Survival of yeasts in the interior and exterior of Danish-and Gorgonzola-style blue cheese during maturation are summarised in Fig. 3.1 and 3.2 respectively. The yeast count includes the total number of yeasts present.

3.2.1. Danish-style blue cheese

Initially, the yeast count, directly after processing, on the surface and in the interior of the cheese revealed no yeasts, but within four weeks numbers rapidly increased on the exterior in excess of 10^7 cfu/g ($2{,}39 \times 10^7$ cfu/g).

Yeasts on the interior, however remained absent for the initial four weeks, following a substantial increase in yeast numbers reaching counts as high as 10^5 cfu/g within the next two weeks (Fig. 3.1). These excessive increases in yeast numbers during the initial stages may be attributed to favourable conditions for the yeasts such as the temperature (8-10°C) during the first maturation period (Fleet, 1990), which suppresses the growth of lactic acid bacteria (Choisy *et al.*, 1987) and the high acidity of the curd, caused by fermentation of lactose by lactic acid bacteria (Fleet, 1990), while yeasts originating as contaminants from the brine and the environment are osmotolerant (Seiler and Busse, 1990).

The development of yeasts during the whole maturation period exhibited similar patterns between the exterior and the interior, although a substantial difference of more than one log unit in the number of yeast populations on the exterior compared to the interior of the cheese (Fig. 3.1) was evident. After the excessive increase in yeast numbers at the initial stages of the maturation, the numbers remained more or less stable for the next 84 days. For the next period until end of maturation yeast numbers representative of the interior, gradually increased to $>10^6$ cfu/g, whereas the numbers on the surface decreased to $>10^5$ cfu/g.

The high incidence of yeasts on the exterior, originating from direct contact with the environment, (Devoyod, 1990) are furthermore exposed to aerobic conditions, whereas, the yeasts in the interior have limited access to oxygen (Fleet, 1990; Deàk and Beuchat, 1996) and consequently, the former has a competitive advantage reaching higher numbers (Devoyod, 1990). According to literature, the proportion between the yeast numbers on the exterior and in the interior during maturation is about 100 to 1 (Lenoir, 1984; Choisy *et al.*, 1987).

Table 3.4 shows the composition and proportionate representation of yeasts during the maturation period on the surface and the interior of the cheese. A

total of 30 species were isolated from the interior whereas 34 species were isolated from the surface. The most predominant species on both was *D. hansenii* representative of >50% of the isolates on the surface and interior. All other species accounted for less than 20% on all occasions. *D. hansenii* was the most frequently occurring species occurring in blue cheese in other studies (de Boer and Kuik, 1987; Besançon *et al.*, 1992; Roostita and Fleet, 1996). The overall predominance of *D. hansenii* can be attributed to the species' salt resistance (Fleet, 1990), growth at low temperatures (Welthagen and Viljoen, 1999), poteolytic and lipolytic activity (Welthagen and Viljoen, 1998), the ability to utilise lactic and citric acid (Roostita and Fleet, 1996) and the frequent association with environmental samples (Table 3.3).

Torulaspora delbrueckii was found to be the second most abundant yeast species on the exterior and in the interior of the Danish Blue-style cheese being significantly higher than *Saccharomyces cerevisiae* (Table 3.4). This species has been typically found in the raw milk of several dairies and according to van den Tempel and Jakobsen (1998) the possibility might exist that it is not killed through pasteurization. Despite the absence of yeasts in the pasteurized milk during our survey, the species has been isolated from the equipment (Table 3.3) and therefore it is possible that the species became established through post pasteurization contamination. Welthagen and Viljoen (1998) found that *Torulaspora delbrueckii* was in 40% of hard- to soft-cheese varieties surveyed. *Candida versatilis* was present on the exterior and in the interior of the Danish Blue-style cheese, originating from the brine (Table 3.3). *Candida versatilis* has been isolated from pickling brines (Barnett *et al.*, 1990) and to a lesser extent in cheese samples (Devoyod, 1990).

Cryptococcus albidus was only found in the interior of the blue cheeses. The most likely source of contamination was the processing equipment (Table 3.3), which corresponds with data as indicated by Welthagen and Viljoen (1999). The species has been recovered from different dairy products such

as ice cream, butter and cheese (Fleet and Mián, 1987; Welthagen and Viljoen, 1998) as well as blue cheese-varieties where it predominated (Roostita and Fleet, 1996). Its ability to grow at low temperatures and lipolytic activity mainly contribute to its presence (Welthagen and Viljoen, 1998).

Saccharomyces cerevisiae strains were present in the interior and on the surface of the cheeses despite their absence in environmental samples during this survey. According to Roostita and Fleet (1996) *S. cerevisiae* is sensitive to high salt concentrations, but capable of growth in dairy products with reduced salt values utilising cheese components as growth substrates. The occurrence of this yeast species in blue cheese is probably based on the utilization of protein and fat breakdown products from other species (Roostita and Fleet, 1996) but its origin is unknown.

3.2.2. Gorgonzola-style blue cheese

Yeasts associated with Gorgonzola-style blue cheese (Fig. 3.2) showed similar growth patterns as exhibited by the Danish blue-style cheese (Fig. 3.1). Yeasts present on the surface of Gorgonzola-style blue cheese rapidly increased directly after processing reaching a maximum of 1.34×10^7 cfu/g after 49 days of maturation (Fig. 3.2). In the interior, yeast numbers remained limited for the initial seven days after processing followed by a substantial increase to reach a maximum of 2.99×10^5 cfu/g after 49 days. Low storage temperatures (8-10°C) and high acidity of the curd selected the yeast contaminants originated from the environment. Similar to the Danish style blue cheese, yeasts on the surface were significantly higher compared to the interior.

After 49 days of maturation, yeast numbers on the exterior decreased gradually until the end of maturation at day 91 prior to packaging to 2.4×10^4 cfu/g (Fig. 3.2). A similar decreasing profile was followed by the yeasts present in the interior except for a slight increase at the end of the maturation

period resulting in a final count of 7.1×10^4 cfu/g.

The most frequently occurring yeast species on the exterior of Gorgonzola-style blue cheese were representatives of *D. hansenii*, *Candida versatilis*, *Trichosporon beigelii* and *Torulaspora delbrueckii*. In the interior, an enhanced diversity in the yeast population was obtained (Table 3.4). Again, *D. hansenii* clearly predominated on the exterior and interior of the cheese represented by more than 30% of the population (Table 3.4). *Candida versatilis* was significantly the second most abundant yeast species on the exterior and in the interior of the cheese, whereas, *Torulaspora delbrueckii* strains were also frequently encountered. *Saccharomyces cerevisiae, Rhodotorula glutinis* and *Cryptococcus albidus* were only found in the interior of the Gorgonzola-style blue cheese. Species recovered from Gorgonzola- and not from Danish-style, were *Trichosporon beigelii, Rhodotorula glutinis* and *Candida zeylanoides* (Table 3.4). *Trichosporon* species associated with dairy products have been frequently recovered from cheeses (Kaminarides and Anifantakis, 1998; Welthagen and Viljoen, 1998), raw milk and pasteurized milk (Roostita and Fleet, 1996), and the brine (Seiler and Busse, 1990) originating from the floors and walls, and equipment (Table 3.4). *Rhodotorula glutinis* and *Candida zeylanoides* have been isolated from cheeses (Viljoen and Greyling, 1995) originated from the air, equipment surfaces (Welthagen and Viljoen, 1998) and brine (Seiler and Busse, 1990). The ability of *Rhodotorula glutinis* to grow at low temperatures and its strong ability to hydrolise fat contributes to its presence in cheese (Fleet and Mian, 1987).

Results obtained, clearly indicated that the development of yeasts within the blue cheeses originated as yeast contaminants being present in the environment. With the exception of *Saccharomyces cerevisiae,* all the other yeast species were isolated from the immediate environment. Similar species were obtained in Australian and European blue cheese varieties (Roostita and Fleet, 1996), which can be contributed to the generalized usage of

pasteurized milk for manufacturing of the cheeses (van den Tempel and Jakobsen, 1998). At the end of the maturation period, yeasts were present in both cheese varieties at high numbers, making their occurrence in South African blue cheese varieties meaningful. Those species that proved to be dominant at all times, were naturally selected based on environmental influences or competition between species for survival.

Table 3.1. Enumeration of yeasts and bacteria from environmental samples in cheese plant (results are the means of duplicate samples).

SAMPLE	YEASTS	BACTERIA
Air (cfu per 90 mm Petri dish)		
Production Room	0	2.0
Brine Room	3.0	22.5
1st Maturation room	4.0	11.0
2nd Maturation room	2.5	5.0
Equipment surfaces (cfu per 25 cm^2)		
Cheese vat	0	0
Net	75.0	0
Machinery	0	25.0
Table	126.0	0
Cheese Moulds	0	980.0
Humidifier outlet	0	212.0
Building surfaces (cfu per 25 cm^2)		
Floor – Production room	0	0
Floor – Brine	52.0	1300.0
Floor – 1st Maturation	259.0	0
Wall – Production	0	0
Wall – Brine	0	0
Wall – 1st Maturation	0	0
Wall – 2nd Maturation	27.5	0
Door 1	1900.0	1070.0
Door 2	2105.5	2700.0
Shelf – 1st Maturation	0	880.5
Shelf – 2nd Maturation	375.0	1200.0
Brine (cfu per ml)		
Foam	2.8×10^5	1.8×10^5
Collective outlet	6.6×10^4	1.9×10^4
Collective tank	1.3×10^5	2.3×10^4

Table 3.2. Enumeration of lactic acid bacteria and yeasts during the basic manufacturing process of Blue type cheeses (results are the means of duplicate samples).

Elapsed time (hr)	Procedure	Lactic acid bacteria (cfu/g)	Yeasts (cfu/g)
00:00	Milk in vat	2500	0
01:00	Mould and starter Culture added	1.09×10^5	0
02:40	Curd cutting	3.60×10^8	0
02:45	Stir starts	1.50×10^7	0
03:45		7.30×10^7	10
04:00		1.00×10^8	0
04:30		1.10×10^8	0
05:00	Draining	6.10×10^7	0
05:15	Dry salting	9.10×10^7	10
06:00	Scooping/Moulding	1.39×10^8	0
06:30	Turning	6.60×10^7	0
07:00		1.12×10^8	0
07:30		9.40×10^7	60
07:45	End of turning	6.18×10^8	70

Table 3.3. Yeasts associated with blue cheese manufacture and their sources.

Isolates	Air	Floors/Walls	Equipment	Brine
Candida versatilis				+
Candida zeylanoides				+
Crytococcus albidus			+	
Dekkera anomala		+	+	
Debaryomyces hansenii	+	+	+	+
Dekkera bruxellensis		+		
Rhodotorula glutinis	+			
Torulaspora delbrueckii	+		+	
Trichosporon beigelii		+	+	

Table 3.4. Distribution of yeast populations in interior and exterior of Danish-style and Gorgonzola-style blue cheeses.

Yeast	**INTERIOR** Population (%)	**EXTERIOR** Population (%)
	DANISH-BLUE STYLE	
Debaryomyces hansenii	53	50
Saccharomyces cerevisiae	10	18
Torulaspora delbrueckii	13	20
Candida versatilis	13	12
Cryptococcus albidus	10	ND
	GORGONZOLA STYLE	
Debaryomyces hansenii	30	35
Trichosporon beigelii	ND	15
Torulaspora delbrueckii	14	25
Candida versatilis	19	25
Saccharomyces cerevisiae	13	ND
Candida valdiviana	4	ND
Cryptococcus albidus	11	ND
Rhodotorula glutinis	7	ND

ND = Not detected

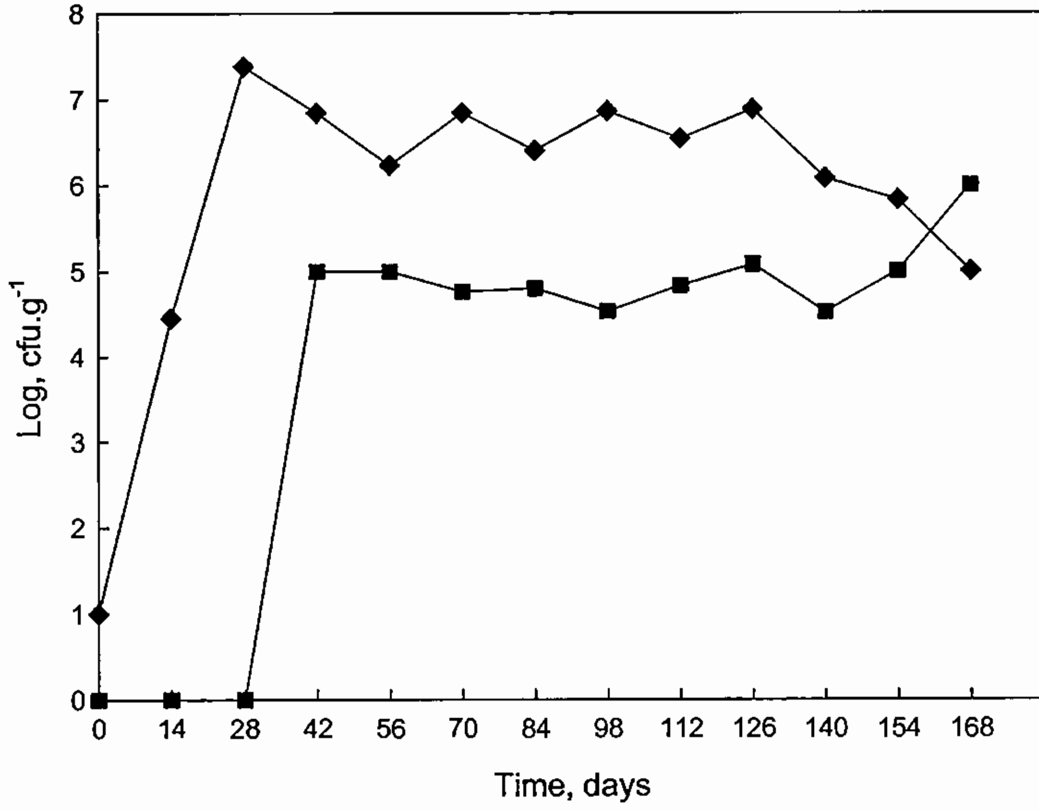

Fig. 3.1. Growth and survival of total yeasts on the interior (■) and exterior (◆) of Danish-style blue cheese during maturation.

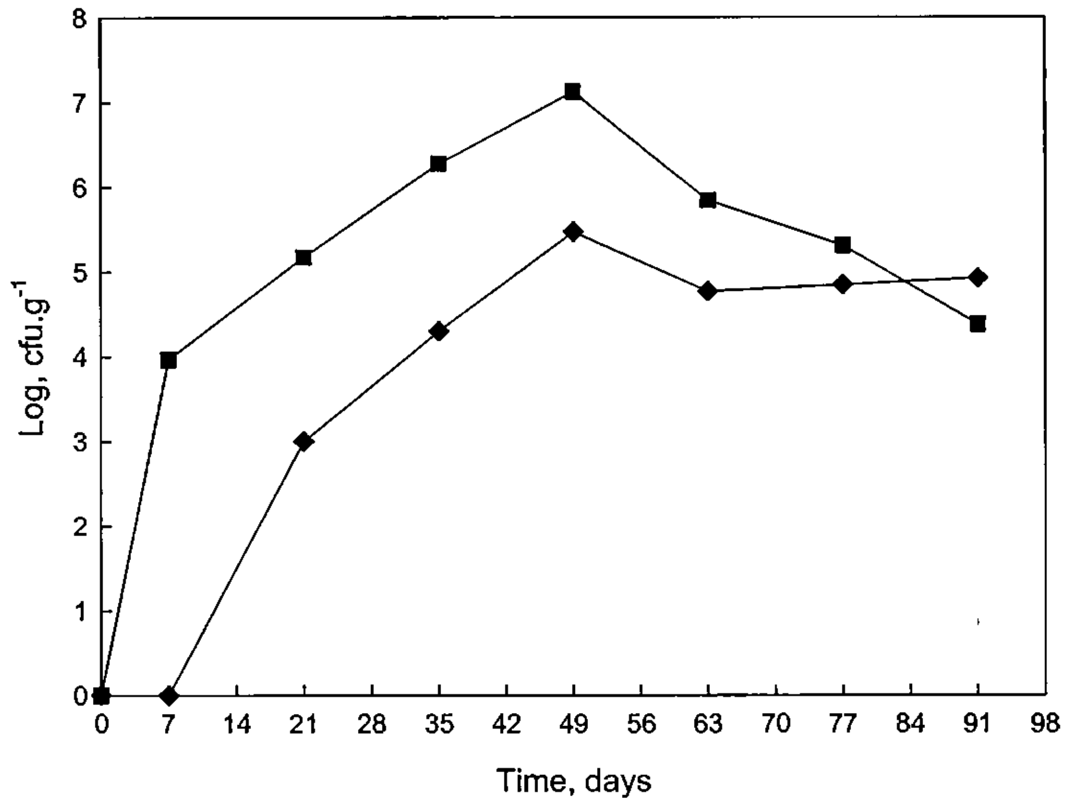

Fig. 3.2. Growth and survival of total yeasts on the interior (■) and exterior (◆) of Gorgonzola-style blue cheese during maturation.

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Chapter 4

Interactive microbial development during the processing and maturation of South African blue-mould cheese

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ABSTRACT

Samples of blue-mould cheeses, manufactured at a commercial cheese factory in the Western Cape region of South Africa, were analysed during processing and maturation. Air and contact samples were taken throughout the cheese factory, while samples from the various stages of processing, as well as regular sampling during maturation were carried out. These samples were microbiologically analysed for lactic acid bacteria, coliforms, *Brevibacterium linens*, yeasts and total counts. In addition, the changes in the pH values, the organic acids and the sugars were determined during the processing and maturation period.

Since no yeasts were isolated from the pasteurised milk it was evident that they derived as contaminants from the environment, mainly from the brine. High numbers of yeasts and lactic acid bacteria were isolated from the brine. The simultaneous presence of yeasts and lactic acid bacteria in the cheese suggested an interactive development whereby the lactic acid bacteria utilised the organic acids present as carbon source increasing the pH while the yeasts in return produced vitamins and growth factors that encouraged the growth of the lactic acid bacteria. The increase in the pH also promoted the growth of *B. linens*. The relationship between pH, organic acid and sugars showed similar profiles to those reported in literature for Cheddar cheese and other mould-ripened cheese.

Keywords: Blue-mould cheese, lactic acid bacteria, yeasts, *Brevibacterium linens* and maturation.

1. INTRODUCTION

Surface ripened cheeses are characterised by complex bacterial flora present on the surface. This smear, often reddish in colour, consists predominantly of yeasts and bacteria (Kammerlehner, 1995). These aerobic microorganisms originating as contaminants have a direct impact on the flavour development, texture and appearance of these cheeses. They however may also contribute positively towards the maturation of the cheese, by reducing the maturation time from 4 - 6 months to only two months as reported by Bikash *et al.* (2000) for Cheddar cheese.

Numerous studies have been conducted on the acceleration of cheese maturation, urged by the economic advantages of a rapid development of stronger cheese flavour in a shorter time (Law, 1984). Lactic acid bacteria applied as starter cultures contain the desired enzymes required for cheese production and maturation 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 and 1992). Fleet and Mian (1987) and van den Tempel and Jakobsen (1998) have shown the presence of yeasts in blue mould cheeses. Yeasts numbers as high as 10^6 cfu/g have been detected in Danish-blue mould ripened cheeses. Yeasts contribute both positively and negatively to the fermentation and maturation process. Controlled yeast growth can be beneficial by inhibiting undesired microorganisms (Kaminarides and Laskos, 1992) and supporting the function of the starter culture (Kalle *et al.*, 1976). In addition, yeasts have proteolytic and lipolytic activity (Coghill, 1979; Wyder and Puhan, 1999a and b), produce gas that leads to curd openness (Jakobsen and Narvhus, 1996), excrete growth factors (Jakobsen and Narvhus, 1996) and by metabolising lactic acid leading to an increase in pH (Fleet, 1990). All these

factors promote a favourable environment for non-starter microorganisms to proliferate.

The dominant yeast species found during cheese maturation are *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* and species of *Candida* (Lenoir, 1984; Nooitgedagt and Hartog, 1988; Devoyod, 1990; Fleet, 1990; Roostita and Fleet, 1996), whereas the yeast species most frequently isolated from blue cheeses include, *D. hansenii, Y. lipolytica, K. marxianus, K. lactis, Y. lipolytica* and *Candida* spp. (de Boer and Kuik, 1987; Besançon *et al.*, 1992; Roostita and Fleet, 1996; Barth and Gaillardin, 1997; van den Temple and Jakobsen, 1998). For more details also see Chapter 3, Development of yeast population during the processing and maturation of blue-mould cheese. These yeast species form an integral part of the microbiota of blue cheeses and positively contribute towards maturation by the production of aroma compounds (Hanssen *et al.*, 1984; Martin *et al.*, 1999), flavour formation through lipolytic and proteolytic activity (Coghill, 1979; Wyder and Puhan, 1999 a and b), the excretion of growth factors (Jakobsen and Narvhus, 1996), the production of gas which leads to curd openness (Jakobsen and Narvhus, 1996), and assists with *Penicillium roqueforti* development. In addition, yeasts cause an increase in the surface pH, which facilitates secondary microflora development such as *Brevibacterium linens* (Karahadian *et al.*, 1985; Addis *et al.*, 2001). Furthermore, Martin *et al.* (1999) found that the changes in sensory profiles indicated that the yeasts contributed to the development of specific fruity odours, especially when associated with bacteria.

Although some yeasts can survive the pasteurisation process to a limited extent (Fleet, 1990), the primary sources of yeast contamination during cheese processing derived from the brine and factory surfaces, including the floors, walls and equipment (Seiler and Busse, 1990; Welthagen and Viljoen, 1999). To a lesser extent, post-pasteurisation contamination sources include the air, the

workers hands and aprons (Viljoen and Greyling, 1995). The ability of yeasts to grow and survive in dairy products is primarily due to their 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).

As reported in Chapter 2, retail samples of blue-mould cheeses purchased in various countries had high occurrences of yeasts. Fleet (1992) noted a unique relationship between the different microorganisms associated with mould-ripened cheeses. Thus, the main objective of this study was a survey of the evolution of the microflora present during the processing and maturation of blue-mould cheeses. In addition, the main sources of contamination were identified and the chemical changes incurred by these microorganisms during processing and maturation were analysed.

2. MATERIALS AND METHODS

2.1. Blue-mould cheese production

Blue-mould type cheese was manufactured on two different occasions at a commercial cheese factory in the Western Cape region of South Africa. The basic cheese making procedure was carried out as described by Kosikowski (1997).

For the production of the Blaaukrantz-type blue-mould cheese, *Penicillium roqueforti* spores (Visbyvac DIP Dosis 5, Wisby, GmbH and Co. KG, Germany) were prepared according to the manufactures instructions and added to the pasteurised milk prior to the addition of the starter culture. The lactic acid bacteria starter culture (DL-mix, M FZ 3-22, 50 u, Wisby, Danisco Cultor Niebüll,

GmbH, Germany) was prepared according to the manufactures instructions and added at the appropriate time to the pasteurised milk.

2.2. Sampling methods and media

All media were prepared in accordance with the manufacturer's instructions and autoclaved at 121°C for 15 min. Aliquots of between 15 and 20 ml sterile media were dispensed into 90 mm Petri dishes and allowed to dry overnight at room temperature. The experimental media included: Plate count agar (PCA) (Biolab diagnostics, Merck) for the enumeration of the total counts was aerobically incubated at 30°C for 48 h, Rose-bengal chloramphenicol agar (RBCA) (Merck, C107, Darmstadt, Germany – pH 7.2) for the enumeration of yeasts was aerobically incubated at 25°C for 4 days, DeMann, Rugosa and Sharpe (MRS) agar (Merck, C86 - pH 6.5, Darmstadt, Germany) which is elective for lactic acid to lactococci bacteria was incubated under aerobic conditions at 25°C for 48 h, MacConkey agar (Merck, C2 – pH 7.1, Darmstadt, Germany) for the enumeration of coliform bacteria was aerobically incubated at 37°C for 24 h, and in accordance with Toolens and Koning-Theune (1970), Lab Lemco agar, with 0.5% glucose, 0.5% $CaCo_3$ 5.5% NaCl and 0.02% pimafucin (LCGS) was used for the isolation and enumeration of *B. linens*. LCGS was incubated aerobically at 25°C for 5 to 7 days.

Samples from the equipment and the surrounding vicinity were taken during the manufacture of the blue-mould cheeses using RODAC contact plates (Favero *et al.*, 1968) containing the above media at selected points as indicated in Table 4.1 and 4.2. Surfaces areas that could not be sampled by RODAC contact plates due to inaccessibility were sampled by swabbing adjacent surface areas of *ca.* 25 cm^2 per swab for 30 sec each using five replicate swabs moistened in Bacto Peptone Water (Difco, Laboratories, Detroit, MI).

Liquid samples were taken by aseptically scooping with a sterile McCartney bottle in the cheese vat and solid samples by aseptically cutting pieces of the curd and cheese and transferring into sterile plastic bags (Whirl Pak, Nasco, U.S.A.). For solid samples, 10 g portions were diluted into 90 ml sterile peptone water in sterile plastic bags (Whirl Pak, Nasco, U.S.A.) and homogenized with the aid of a Colworth 400 stomacher (London, UK) for 2 min. Further decimal dilutions of the suspensions and liquid samples were performed in 9 ml sterile peptone water. Aliquots (0.1 ml) of the dilutions were spread inoculated over the surface of plates containing the relevant selective media.

Air was sampled by using standard settle plates (the same selective media as above in 90 mm Petri dishes) with an exposure time of 5 min (Welthagen and Viljoen, 1999). Samples were taken as indicated in Table 4.1.

Brine samples were taken on a weekly basis over a six-week period by sampling directly from the brine vat using sterile McCartney bottles (Fig. 4.1 a and b and Table 4.4). The appropriate serial dilutions were performed and spread plated on the respective media.

2.3. Sampling during maturation

Blaaukrantz-type blue-mould cheeses (4 randomly selected cheeses were collected at different time slots) from two separate batches were kept under controlled conditions at 4°C at the site of cheese production and sampled directly after processing and at consecutive intervals on a weekly basis during maturation up to week 7 and thereafter at week 9 and 14 (Fig. 4.2 and 4.3). Cheese samples were prepared for microbiological analysis by aseptically cutting a cheese wedge from the cheese. Two 10 g cheese samples were taken from the surface and the centre of the cheese wedge respectively and microbiologically analysed as described earlier.

2.4. Enumeration and isolation

All plates containing between 25 and 250 colony-forming units (cfu) (or the highest dilution if below 25) were enumerated and the mean values determined from duplicate samples. Results were recorded as the mean value of two trials, from duplicate plate samples originating from duplicate cheese samples from the same batch (2 x 2 x 2).

2.5. Physical and chemical analysis

The pH, sugars and organic acid content of the cheese samples were determined at selected intervals during the production and manufacture of the cheese over a 30-week period (Fig. 4.4, 4.5 and 4.6). As described for the microbial analysis, an additional 10 g of cheese was 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 pH determination. The pH of the homogenised cheese samples was measured at room temperature using a Cyberscan 500-pH meter (Eutech instruments, Singapore, serial number 35268) according to the method described by Kosikowski (1970).

The presence of the sugars (lactose, galactose, glucose) and the organic acids (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 microbial 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.). A volume of 2.5 ml of the slurry was transferred to Eppendorf tubes and three drops of 30% H_2SO_4 added 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 - 5°C until determination (Roy *et al.*, 1997). The

organic acids were determined using a HPLC system equipped with a variable wavelength detector set at 220nm and a Biorad-aminex 87H column with a 0.01 N H_2SO_4 eluent set at 0.6ml/min (Molnar-Perl and Morvai, 1992). The sugar content was measured using a Waters HPLC system with a Biorad-aminex C42 column and refractive index detector (Molnar-Perl and Morvai, 1992).

3. RESULTS AND DISCUSSION

3.1. Microbial interactions

A large variety of cheeses and most notably the mould-ripened cheese are characterised by the surface development of specific microflora, including yeasts, moulds and bacteria. These are responsible for creating the unique properties that characterise these cheeses (Lenior, 1984). Since only *Penicillium* and lactic acid bacteria are directly added to the cheese during production, a substantial group of microorganisms originated as predominantly post-pasteurisation contaminants.

3.1.1. Air and surfaces

Enumeration of samples from the dairy environment associated with blue-mould cheese manufacture (Tables 4.1, 4.2, 4.3 and 4.4) showed similar results to those obtained during the making of Gouda, Cheddar and other cheeses (Fleet and Mian, 1987; Welthagen and Viljoen, 1998). An overall profile of the microbial populations considered as contaminants throughout the cheese manufacturing plant was constructed. Table 4.1 details the results obtained by means of air sampling whereas the results of microbial loads associated with the equipment are tabulated in Table 4.2. The results indicated that the hygiene of the air regarding the presence of coliforms was of a good standard since no coliforms were detected. The viable aerobic total bacterial counts, however reached

counts of 62 cfu per 90 mm Petri dish in the brine room and 30 cfu per 90 mm Petri dish in the cold room next to the pallets where the cheeses were matured. As expected, the lactic acid bacterial counts were high in the preparation (begin) room (92 cfu per 90 mm Petri dish) and slowly declined as the cheese progresses through the factory to counts of less than 10 cfu per 90 mm Petri dish in the end processing room. Despite low numbers of yeasts detected in the cold and packaging rooms (<10 cfu per 90 mm Petri dish), very high numbers (>300 cfu per 90 mm Petri dish) were observed in the maturation rooms, presumably leading to contamination of the cheeses. Similarly, high loads of *B. linens* (90 cfu per 90 mm Petri dish) were also detected in the maturation room. The high numbers of airborne microbial species in the maturation room is a clear indication of the major source of contamination enhancing contamination.

As indicated by the results in Table 4.2, the lactic acid bacteria and the coliforms were very low or absent on the surfaces of the equipment sampled. The yeast counts obtained from the cheese vat (31 cfu per 25 cm^2), the processing room walls (<10 cfu per 90 mm Petri dish), brine vat (52 cfu per 25 cm^2) and the maturation room walls (15 cfu per 25 cm^2) remained low. This can mainly be attributed to a successful cleaning program. The stainless steel equipment, if properly cleaned, usually harbors low numbers of yeasts and lactic acid bacteria which consequently contribute little to post pasteurisation contamination (Welthagen and Viljoen, 1998). In contrast, high numbers of yeasts (>300 cfu per 25 cm^2) were obtained from the surfaces of the wooden pallets in the cold room. Similarly the total counts obtained from the wooden pallets were very high (>300 cfu per 25 cm^2). These wooden pallets maintain higher loads of microbial contamination due to the increased difficulty of proper cleaning and acted as a major source of post pasteurisation contamination. The *B. linens* counts remained relatively constant, with the exception of the increased counts being recorded on the pallets in the cold room (16 cfu per 25 cm^2) and the cheese vat

(24 cfu per 25 cm^2). It was interesting to note that *B. linens* was present on many surfaces located throughout the cheese factory (Table 4.2).

A survey was also conducted to determine the spread of the blue-mould spores throughout the cheese factory (Table 4.3). From the results obtained, it is clear that the hairnets, yellow jackets and shoes worn by the workers were the main contaminating sources responsible for counts exceeding 300 cfu per 25 cm^2. Despite mechanisms in place for the cleaning of shoes upon exiting and entering the blue-mould factory, the high counts (>300, >300 and 30 cfu per 25 cm^2 for the total bacterial counts, yeasts and *Penicillium* respectively) on the shoes were obtained from workers on the premises outside the blue-veined cheese plant and may therefore act as a major source of cross contamination. The blue jackets worn over the yellow jackets, in an attempt to prevent the spread of the *Penicillium* spores to the remainder of the factory had limited success. Spore counts as high as 33 cfu per 25 cm^2 were obtained on the blue jackets. The possible use of ozone to remove any reaming spores may reduce the cross contamination of moulds. Serra *et al.* (2003) obtained successful results using ozone to reduce the moulds in the cheese maturation rooms. Movement within the cheese factory plays an important role in the spreading of yeasts within the plant. Welthagen and Viljoen (1998) reported that surfaces, like walls and floors, contained a high number of yeasts and therefore make an important contribution to yeast contamination.

As previously stated, yeasts are not traditionally associated with cheese, but have been isolated from cheese, with both beneficial and negative effects (see Chapter 3). One of the main contaminating sources, in terms of yeasts, was the brine (Table 4.4 and Fig. 4.1). The brine has been reported to contain the highest number of yeasts (Viljoen and Greyling, 1995), while specific yeasts such as *Debaryomyces hansenii* have been repeatedly isolated from the brine (Mrak and Bonnar, 1939; Seiler and Busse, 1990; Fleet 1992). Some of these yeasts,

like *D. hansenii,* showed a higher resistance to adverse conditions like high salt concentrations and are likely to increase in numbers at later stages of the production or during the maturation period (Welthagen and Viljoen, 1998). Despite the frequent occurrences of yeasts in the brine, it must be kept in mind that the numbers and diversity may vary between dairy plants and even between consecutive days in the same plant, due to variation of salt concentrations and age of the brine (Seiler and Busse, 1990; Welthagen and Viljoen, 1998). Results obtained (Table 4.4) showed that despite high bacterial counts that usually out-compete the growth of yeasts, the yeast counts remained dominant. The yeasts in the brine initially increased to a highest count of 5.4 log units/ml obtained at week 3 (Fig. 4.1b), followed by a slight decrease. The brine, a heavy suspension of water and salt, usually has whey and many small pieces of curd suspended in it. Since lactic acid bacteria are not considered tolerant towards high salt concentrations (Fox *et al.*, 2000) the presence of the curd and whey may attribute to the enhanced lactic acid bacterial count (Fig. 4.1). The coliforms remained very low and/or absent in the brine, probably due to the unfavourable environment (Fig. 4.1a). In contrast, the levels of *B. linens* increased throughout the six-week period to values of almost 8.0 log units/ml obtained in the final week of microbial analysis (Fig. 4.1b). This rapid proliferation of *B. linens* may, in part, be attributed to the presence of the yeasts, which produce vitamins and other growth factors required by *B. linens* (Valdés-Stauber *et al.*, 1997; Busse, 1989; Lenoir, 1984; Purko *et al.*, 1951). However, the ability of this bacterium to withstand high salt concentrations (up to 15%) is well documented (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000). In addition, despite an optimum growth temperature of 20 - 30°C, *B. linens* species is very capable of growth at 10°C, (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000), suggesting that the brine creates a favourable environment to encourage its growth.

Based on these data it is evident that the brine was one of the main sources of contamination of both yeasts and *B. linens*. Mrak and Bonar (1939), reported yeasts survival at NaCl concentrations of 4.0 - 5.0% (w/w), whereas Welthagen and Viljoen (1998) indicated growth of yeasts in brine with salt concentrations as high as 15%. The pasteurisation of the brine on a weekly basis (Fig 4.1a and b) could significantly reduce contamination of the cheeses and the cheese factory environment.

3.1.2. Processing and Maturation

The microbial analysis performed on the cheese during production and maturation comprised the enumeration of the lactic acid bacteria, yeasts, coliforms, *B. linens* as well as the determination of the viable total bacterial counts.

Lactic acid bacteria were absent in the pasteurised milk, but after addition as starter cultures, rapidly increased to counts as high as 9.5 log units/g for both the inner core and outer surface of the cheese samples during processing (Fig. 4.2 and 4.3). Thereafter the counts remained relatively stable in both the interior and surfaces samples throughout the maturation processes. A slight decline in the lactic acid bacteria counts was observed towards the latter part of the maturation process. This reduction is mainly attributed to a reduction in moisture content (results not shown) and the depletion of lactose (Fig. 4.6) after six weeks of maturation.

The yeasts counts remained low during processing (< 1 log unit) but increased substantially within the first week after the maturation process commenced to reach maximum counts of 5 log units in the inner core after three weeks of maturation and exceeding 7 log units on the surface after seven weeks (Fig. 4.2 and 4.3). This enhancement in yeast numbers directly after processing

corresponds with the high numbers of yeasts in the brine the cheeses were subjected to at the end of processing. The low numbers of yeasts during the initial stages of processing, may be attributed to pasteurisation, whereas during the later stages the yeasts were out-competed by the rapid growth of the lactic acid bacteria when favourable conditions prevailed encouraging the growth of the bacteria, the lack of the majority of yeasts to utilise lactose as a carbon source and the low numbers of contaminating yeasts at the initial stages of processing (Table 4.1 and 4.2) serving as yeast inoculum. The surrounding environment, including the air, walls, floors, pallets and shelves all contributed as sources of yeast contamination during the maturation process (Table 4.2). This corresponds with the data reported by Welthagen and Viljoen (1998) as found during the making of Cheddar and Gouda cheeses.

The increase in the yeast numbers during maturation could be attributed to the utilisation of available organic acids produced by the lactic acid bacteria as well as galactose being initially present for a short period at the beginning of maturation. Yeast numbers in the inner core of the cheese declined after three to four weeks due to lower oxygen levels, but continued to proliferate on the surface well after seven weeks. At these later stages of maturation, the yeasts have been shown to rely on the lactic acid bacteria to provide organic acids for growth (Roostita and Fleet, 1996) although lipolytic and proteolytic activity by the yeasts also played a supported role. In return, the increased yeasts numbers aid in the production of additional growth factors and vitamins (Purko *et al.*, 1951; Lenoir, 1984; Busse, 1989; Valdés-Stauber *et al.*, 1997), which not only help to supported the growth of the lactic acid bacteria but the growth of other microorganisms like *B. linens* (Fig. 4.2 and 4.3) (Purko *et al.*, 1951; Devoyod, 1969; Devoyod and Sponem, 1970; Leclercq-Perlat *et al.*, 2000). For both the inner core and the surface samples the growth of *B. linens* followed similar growth profiles to that of the yeasts (Fig. 4.2 and 4.3). This apparent stimulation of growth of *B linens*, further contributed to an enhancement in pigmentation

causing an orange-reddish colouration on the cheese surface during maturation (Valdés-Stauber *et al.*, 1997).

Since *B. linens* is aerobic (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000), numbers were significantly higher ($p < 0.05$) on the cheese surface (a highest count of 7.9 log units/g obtained after week 7) compared to the inner core (a highest count of 3.6 log units/g obtained after week 5) (Fig. 4.2 and 4.3). The inner core of the cheese showed a significant decline in the numbers of *B. linens* present at the end of maturation due to limited oxygen whereas the surface samples remained high in *B. linens* numbers with only a slight decrease. It may be speculated that the slight decrease in the numbers of *B. linens* on the surface was a result of a lower pH (Fig. 4.4) after seven weeks or the reduction in yeast numbers at the time resulting in less stimulating growth factors.

The coliform counts were low or absent (Fig. 4.2 and 4.3) during the production process. During the initial stages of maturation, however these numbers increased to the highest value of 3.4 log units/g recorded after week 7 for the outer surface and 2.2 log unit/g after week 5 for the inner core. The outer surface samples showed notably higher counts (ranging from < 1 to 3.4 log units/g) compared to the inner core samples (< 1 to 2.2 log units/g).

Of importance to note is the fact that the microbial counts can vary from cheese batch to batch as well as from day to day within a specific dairy plant, due to variations in temperature (Davenport, 1980), NaCl concentrations (Seiler and Busse, 1990), the efficiency of pasteurization and/or the standards of hygiene during the making of a specific batch of cheese (Fleet and Mian, 1987).

3.2. Physical and chemical analysis

3.2.1. pH analysis

The initial pH of the raw milk was 6.58 (Fig. 4.4). It began to decrease during renneting, and further decreased to 6.35 at the end of the cutting process (Fig. 4.4). The decrease in pH has been shown to be primarily due to starter cultures producing organic acids (Welthagen and Viljoen, 1999). Over the remainder of the production process active acidification caused the pH to decreased to a value of 4.99 at the end of scooping (Fig. 4.4) and slightly during the first 24 h of maturation to a value of 4.97. After an initial increase in pH at the beginning of the maturation process from pH 4.97 to pH 6.01 (Fig. 4.4) the pH continued to show a very slightly increased despite high numbers of lactic acid bacteria, and therefore this increase may probably be attributed to the excessive growth of the yeasts at the time (Fig. 4.2 and 4.3) utilising the lactic acid as carbon source. From week 15, the pH slowly increased corresponding with a decrease in the lactic acid values and reduction in viable lactic acid bacterial numbers (Fig. 4.6).

3.2.2. Sugars and organic acid analysis

Mould-ripened cheeses have unique chemical properties (Roostita and Feet, 1996). In addition to the high concentrations of fat and protein, residual unfermented lactose, high concentrations of lactic acid, low concentrations of citric and acetic acids and notable amounts of NaCl are characteristic of mould-ripened cheese (Choisy *et al.*, 1987).

According to literature the lactose content of bovine milk is 4.6%, on average ranging between 3.8 – 5.3% (Tamime and Robinson, 1985; Fox and McSweeney, 1998; Walstra *et al.*, 1999). In correspondence with literature the lactose content of the milk before processing was 4.3% (Fig. 4.6). Immediately

after the addition of the starter cultures, the lactose decreased due to metabolism by the lactic acid bacteria and was depleted after seven weeks of maturation. Laubscher and Viljoen (1999a) reported depletion of lactose after 2 days of maturation during the making of Gouda cheese whereas Welthagen and Viljoen (1999) reported a decrease in the lactose content of Cheddar cheese to a value of 0.26% after 51 days of maturation.

Since lactose is a disaccharide comprising glucose and galactose, it must be hydrolysed before it can enter the catabolic pathway for hexoses (Schlegel, 1993). All the lactose was hydrolysed to glucose and galactose (Fig. 4.6). The glucose was immediately utilised by the microorganisms (Schlegel, 1993) and there was accumulation of galactose (Fig. 4.6). The galactose content increased to a maximum value of 0.85% at the beginning of the maturation process but was depleted within five weeks of maturation. As the lactic acid bacteria utilised the lactose converting it to lactic acid, an increase in the lactic acid content from 0.54% after stirring to a value of 1.79% after the second addition of water was observed (Fig. 4.6). (Schlegel, 1993). The lactic acid content of the cheese decreased slightly during the initial stages of maturation followed by an increase and only showed a decrease after the 14th week (Fig. 4.6).

Certain yeasts have the ability to assimilate acetic and citric acid (see Chapter 1, Table 1.3). As the yeast began to increase in numbers (Fig. 4.2 and 4.3) the acetic acid in particular showed a decrease (Fig. 4.5), suggesting assimilation by the yeast species present. Citric acid remained relatively constant throughout the production and the maturation process.

Sufficient and/or additional citrate causes an increases the buffering capacity of the milk, resulting in a lower water retention and decrease in acidification. This causes an increase in pH and thus promoting the growth of secondary microflora such as *B. linens*. In addition, citrate can cause the withdrawal of Ca^{2+} ions from

the cheese, inhibiting the normal process of casein coagulation and thereby aids in preventing the eye formation characteristic often noted in selected cheeses (Hugenholtz and de Felipe, 2002).

In conclusion, environmental sampling again showed (see Chapter 3) that yeasts were most prevalent in the maturation rooms and that the number of the yeasts increased during maturation. Since the brine was noted to be one of the major contamination sources, the possibility of pasteurising the brine on a regular basis should be investigated. The simultaneous presence of yeasts, lactic acid bacteria and *B. linens* suggested an interactive development between the pH values, organic acid profiles as well as the growth and proliferation of these microbiota.

Table 4.1. Air samples taken throughout the blue-mould cheese factory. Results indicate the number of colonies obtained per 90 mm Petri dish.

Location	Lactic acid bacteria	Total counts	Yeasts	*B. linens*	Coliforms
Begin room	92	4	6	5	0
Brine room	2	62	<10	<10	0
Maturation room (back)	9	8	>300	90	0
Maturation room (front)	8	4	>300	5	0
Cold room (next to pallet)	<10	30	<10	13	0
Cold room (in pallet)	<10	2	<10	10	0
Packaging room	2	5	<10	<10	0
End room	<10	24	<10	<10	0

Table 4.2. Samples taken from various equipment surfaces located throughout the cheese factory. Results indicate the number of colonies obtained per 25 cm^2.

Sample	Lactic acid bacteria	Total counts	Yeasts	*B. linens*	Coliforms
Cheese vat, begin process	0	31	6	24	0
Walls, begin process	0	6	1	12	0
Brine vat surface	0	52	2	6	0
Pallet, cold room	0	>300	>300	16	3
Maturation room walls	0	15	8	11	1
Packaging room walls	0	440	4	10	0

Table 4.3. The results of contact samples taken from various surfaces in order to determine the spread of the blue-mould spores through out the cheese manufacturing plant. Results indicate the number of colonies obtained 25 cm^2.

Sample location	Total counts	Yeasts	*Penicilliun*
Change room	120	4	3
[a]Yellow jacket (lower end)	>300	285	>300
[a]Yellow jacket (middle)	>300	>300	>300
In ladies locker	28	2	5
Canteen air (outside)	64	11	15
Hair nets	>300	>300	300
Shoes	>300	>300	210
[b]Blue jacket (lower end)	36	12	6
[b]Blue jacket (sleeve)	11	3	0
[b]Blue jacket (at canteen)	72	33	11

[a] Yellow jackets worn inside blue-mould factory.

[b] Blue jackets worn when leaving the blue-mould factory over the yellow jackets.

Table 4.4. Yeasts and aerobic bacterial counts associated with the brine.

Brine-Sample	Yeasts ($cfu.ml^{-1}$)	Aerobic Bacteria ($cfu.ml^{-1}$)
Foam	278×10^3	180×10^3
Collective outlet	66×10^3	19×10^3
Collective tank	134×10^3	23×10^3

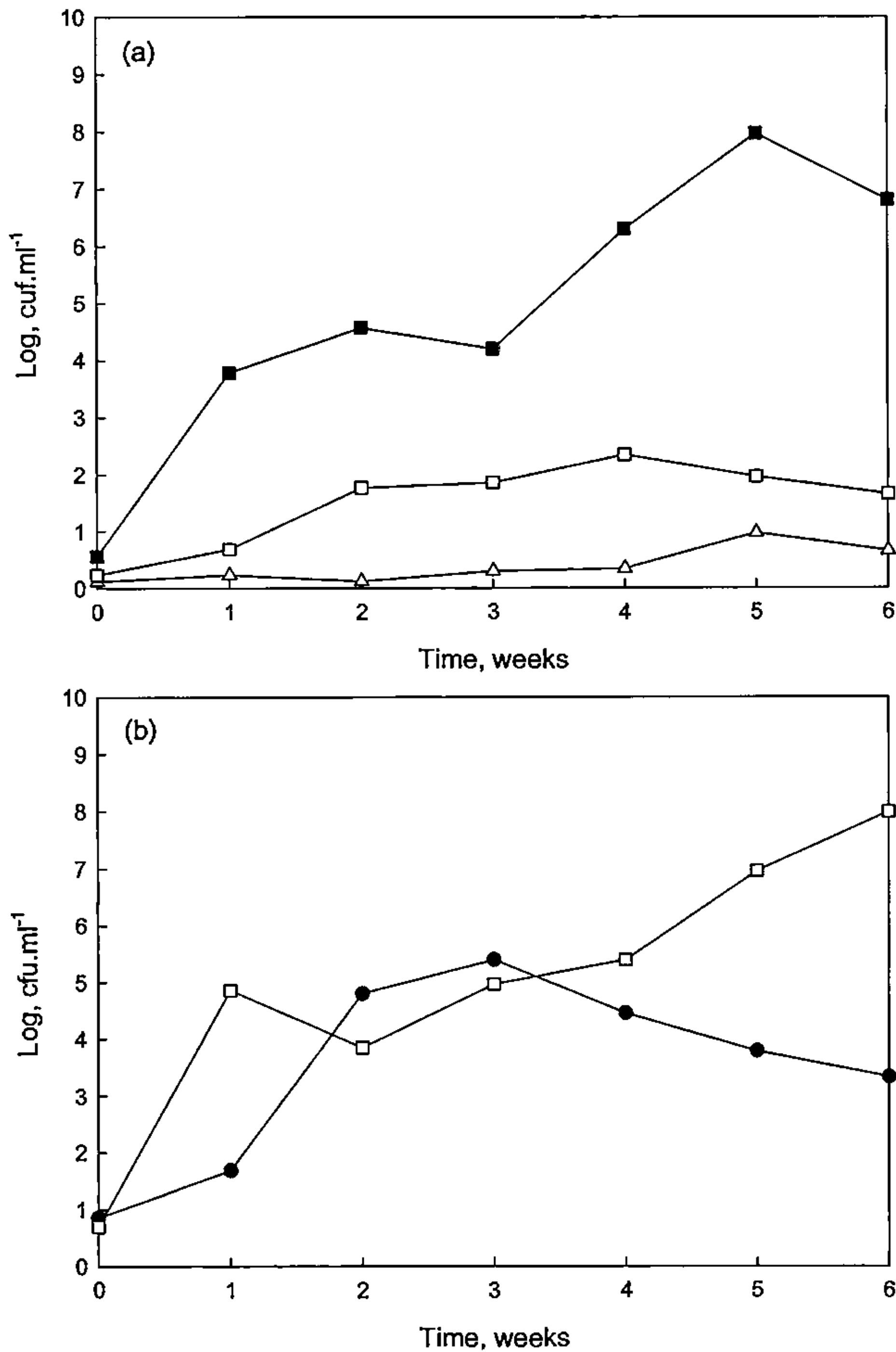

Fig. 4.1. The development of (a) the viable total bacterial counts (□) lactic acid bacteria (■), coliforms (△) and (b) *Brevibacterium linens* (□) and yeasts (●) in the brine of blue-mould cheese, taken over a six-week period. Note: the brine was replaced every 4 to 6 weeks.

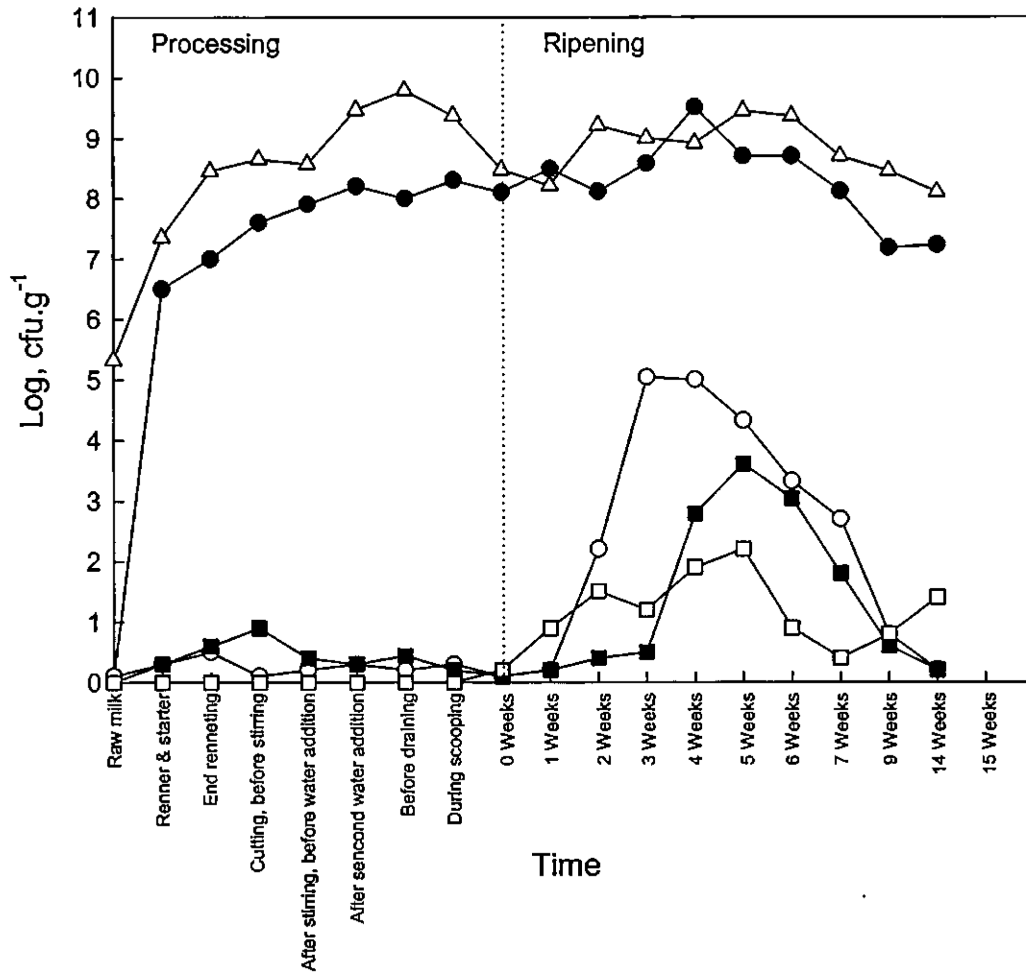

Fig. 4.2. The changes in the microbial counts obtained from the inner core of blue-mould cheese during processing and maturation. Symbols: Yeasts (○); coli (□); lactic acid bacteria (●); total counts (▲); *Brevibacterium linens* (■).

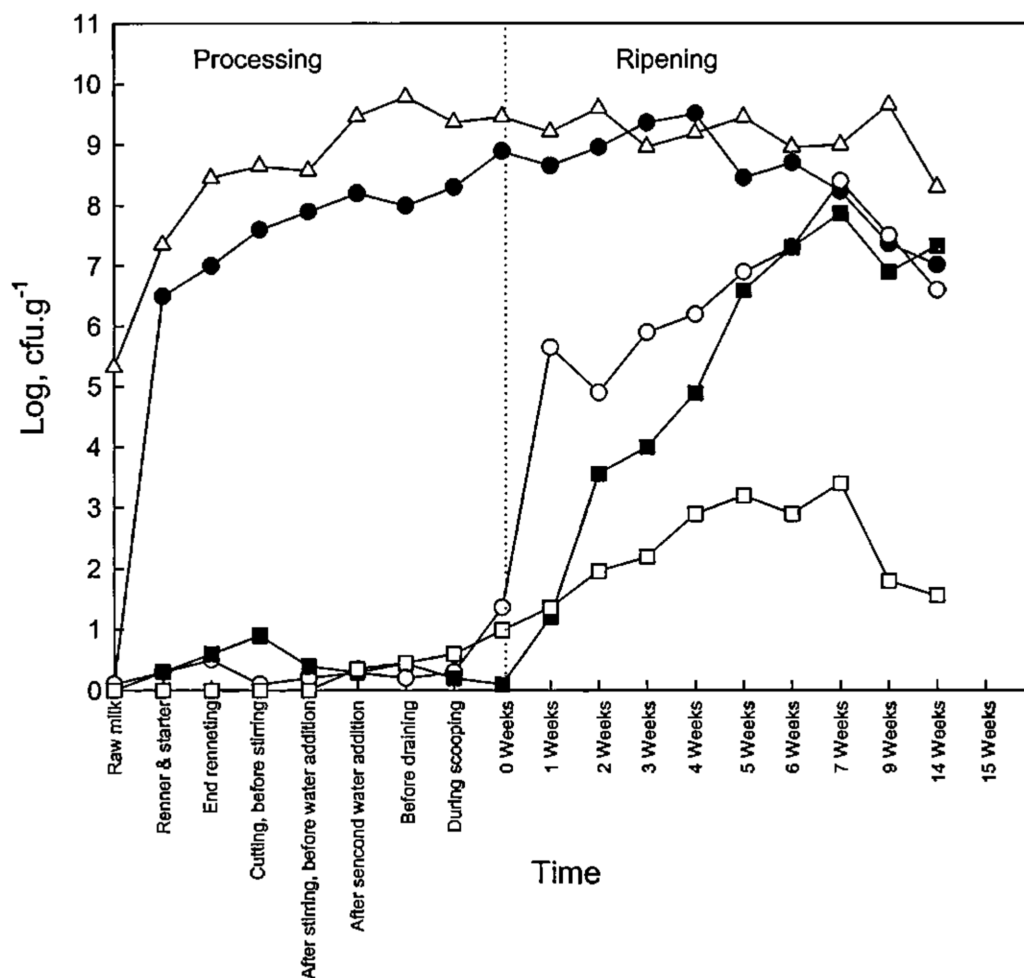

Fig. 4.3. Changes in microbial counts (log units per g^{-1}) obtained from the surface samples of blue-mould cheese during processing and maturation. Symbols: Yeasts (○); coli (□); lactic acid bacteria (●); total counts (▲); *Brevibacterium linens* (■).

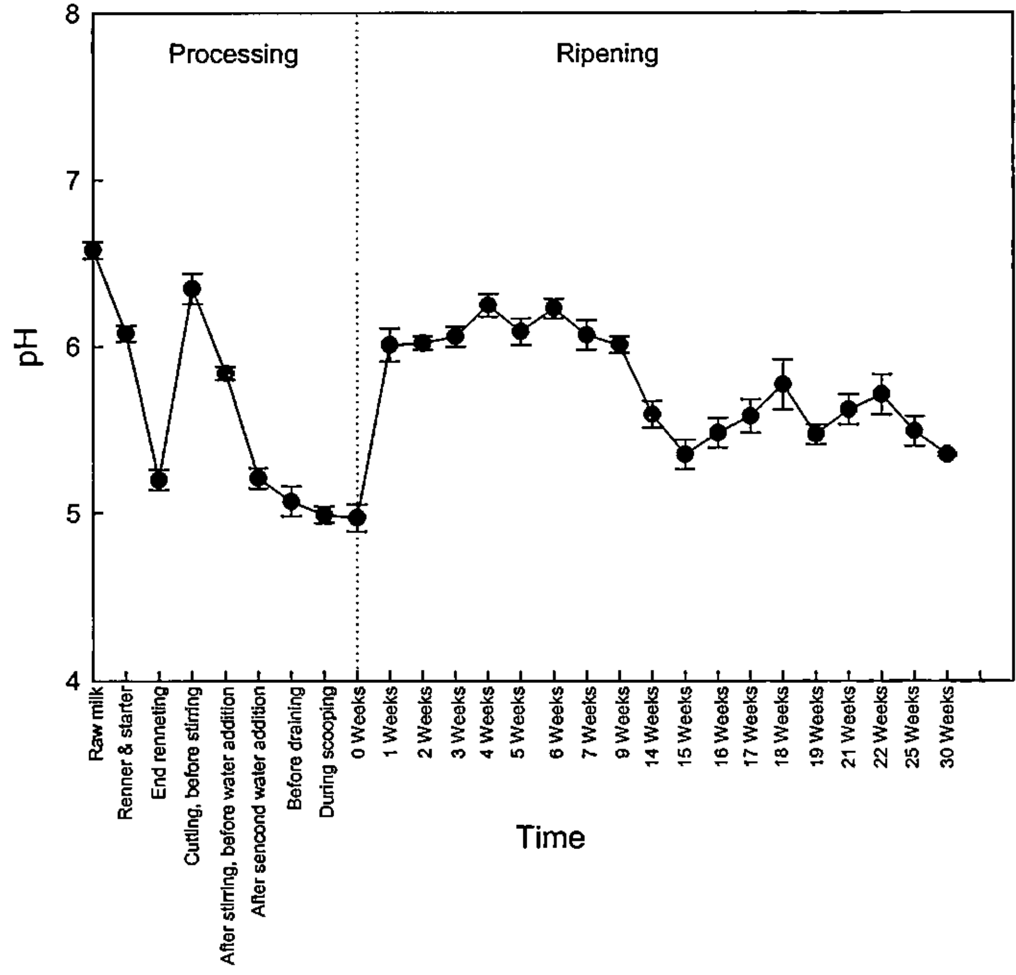

Fig. 4.4. The pH profile of blue-mould cheese during the production and the maturation process.

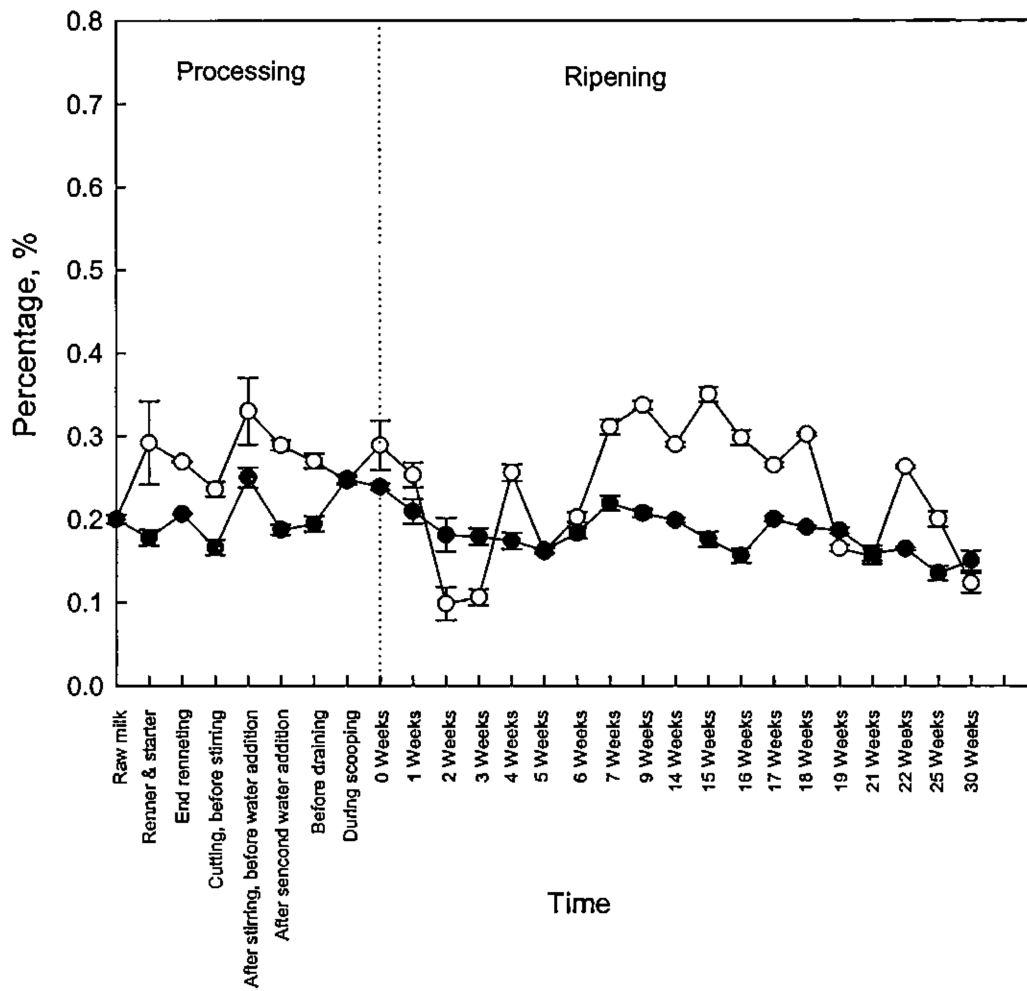

Fig. 4.5. The changes in the acetic and citric acid concentrations of blue-mould cheese during the production and the maturation process. Symbols: acetic acid (○) and citric acid (●).

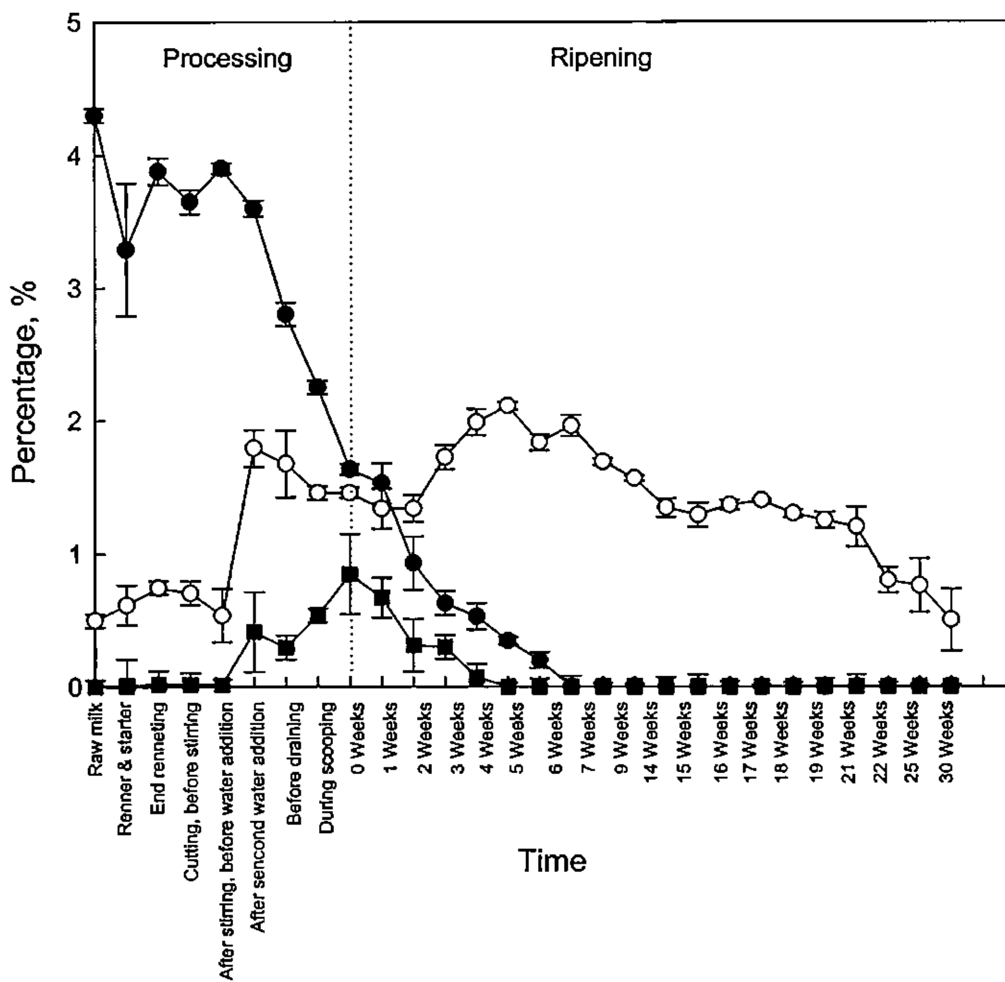

Fig. 4.6. The changes in the lactic acid, galactose and lactose concentrations present in blue-mould cheese during the production and the maturation process. Symbols: lactic acid (○), lactose (●) and galactose (■).

5. References

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a synergistic effect between lactic acid bacteria and *D. hansenii* with a resulting prolonged survival of the lactic acid bacteria.

Several other possibilities regarding the inhibition of *B. linens* were also investigated including the use of Radical water™, as well as the use of other non-toxic chemicals or methods. Radical water™ (trade name) is prepared through a process known as Electrolytic Activation of Water (EAW) (Food review, 2002). This process involves the activation of water using the basis principles of electro-chemistry and a flow-through electrolytic module (FEM) reactor. This reactor designed by Dr Vitold Bakhir and Yuri G. Zadorozhny of the All-Russian Institute for Medical Engineering, was adapted for production in South Africa by Dr Gilbert Hinze (Radical Waters, Pty, Ltd., South Africa). During the preparation of Radical water™, normal portal water and a small quantity of salt (approximately 0.2%) are passed through an electrolytic cell where it is exposed to a high-density electrical field. Using the salt to enhance conductivity, the water is separated into two active streams of water. The positively charged anolyte stream is now a strong biocide and disinfectant solution (Food review, 2002). Conversely, the negatively charged catholyte stream has strong surfactant properties allowing these streams to be customised according to the requirements of the consumer (Radical Waters, Pty, Ltd., South Africa). Radical water™, is user friendly and cost effective, has multiple applications, including the treatment of biofilms. Being water, it is compatible with any food system and leaves no residues. Kirkpatrick, (2000) reported significant reductions in the total bacterial numbers obtained from the skin surfaces of chickens when treated for 9 days with Electro-Activated Water (EAW) Anolyte solution. Radical water™ was thus applied in the production and maturation of blue mould cheese with the aim of reducing or eliminating *B. linens* growth and surface discolouration.

2. MATERIALS AND METHODS

All experiments were conducted at a commercial blue-mould cheese factory in the Western Cape region of South Africa.

2.1. Enumeration media

All media were prepared in accordance with the manufacturer's instructions and autoclaved at 121°C for 15 min. An aliquot of between 15 and 20 ml sterile media were dispensed into 90 mm Petri dishes and allowed to dry overnight at room temperature. The experimental media included: Plate count agar (PCA) (Biolab diagnostics, Merck) for the enumeration of the total counts, Rose-bengal chloramphenicol agar (RBCA) (Merck, C107, Darmstadt, Germany – pH 7.2) for the enumeration of yeasts, DeMann, Rugosa and Sharpe (MRS) agar (Merck, C86 - pH 6.5, Darmstadt, Germany) which is elective for lactic acid bacteria, MacConkey agar (Merck, C2 – pH 7.1, Darmstadt, Germany) for the enumeration of coliform bacteria and in accordance with Toolens and Koning-Theune (1970), Lab Lemco agar, with 0.5% glucose, 0.5% $CaCo_3$ 5.5% NaCl and 0.02% pimafucin (LCGS) was used for the isolation and enumeration of *B. linens.*

2.2. Radical water™ treatments

Wooden pallets used for the storage and maturation of blue-mould cheeses were sprayed with Radical water™, obtained from Q-radical (South Africa), for varying time intervals (Table 5.1 and 5.2) making sure the entire surface was wet. Triplicate microbial samples were subsequently taken for analysis. Further experiments involved submerging the wooden pallets in Radical water™ over different time periods. Similarly, the blue-mould test cheeses were submerged or sprayed with Radical water™ over various time intervals (Fig. 5.2, 5.3, 5.4 and 5.5). Samples were analysed in duplicate. All controls were kept under the same conditions as the tests but were not treated with Radical water™.

2.3. Microbial sampling

Microbial sampling was performed by aseptically removing wood shavings or cuttings from the wooden pallets and placing them in sterile plastic bags (Whirl Pak, Nasco, U.S.A.). Similarly, cheese samples from both the inner core and outer surfaces were obtained by aseptically cutting pieces of the cheese and transferring into sterile plastic bags (Whirl Pak, Nasco, U.S.A.). The inner core samples were taken from the cheese core, or a minimum depth of 50 mm inside the surface of a wedge that was cut from the large block of cheese. Surface samples were taken no deeper than 10 mm from the rind of the cheese. A 10 g sample of either the wood or the cheese was weighed into 90 ml sterile peptone water and homogenized in a Colworth 400 stomacher (London, UK) for 2 min. Further decimal dilutions of the suspensions were performed in 9 ml sterile peptone water. Aliquots (0,1 ml) of the dilutions were spread inoculated over the surface of plates containing the relevant selective media. RBCA, MRS and PCA plates were aerobically incubated at 30°C for 4 days and 48 h respectively. MC plates were incubated at 37°C for 24 h, while the LCGS plates were incubated for 5 to 7 days at 30°C. On exposure to light the intensity of the orange/yellow colonies increased (Bergey's Manual of Determinative Bacteriology [7th Ed]).

2.4. Enumeration and isolation

All plates containing between 25 and 250 colony-forming units (cfu) or the highest dilution if below 25 were enumerated and the mean values determined from duplicate samples. For the microbial sampling of the cheese, results were recorded as the mean value of two trials (i.e. two batches), from duplicate plate samples originating from duplicate cheese samples (2X2X2). For the microbial sampling of the wooden pallets, results were recorded as the mean of triplicate analyses.

2.5. Preparation of yeast culture

Debaryomyces hansenii (CHR Hansen, France) was prepared, according to the manufacture's instructions, as a liquid culture that was hand sprayed onto the test surfaces of the cheese (5 cheeses) ensuring the entire surface was wet. Microbial samples were subsequently taken from both the outer surface and the inner core (Fig. 5.6, 5.7, 5.8 and 5.9). Further experiments involved dipping/submerging the cheeses (5) in the Radical water™ over different time intervals (Fig. 5.6, 5.7, 5.8 and 5.9). Five control cheeses from the same batch were kept under the same conditions as the test cheeses and sampled as indicated for the hand sprayed and dipped cheeses (Fig. 5.6, 5.7, 5.8 and 5.9).

2.6. Vacuum packaging of cheese

Four production size whole cheeses selected at random from two different batches, were vacuum packed in industrial strength plastic bags after the completion of the brining process and after piercing. Piercing the whole cheese aids the growth of the *Penicillium* throughout the whole cheese by allowing oxygen to penetrate the cheese. Two cheeses were kept as controls and were only packed in an industrial strength plastic bag after being matured at 9°C for 4 – 5 weeks, prior to being moved to the second maturation room. For the remaining two test cheeses the plastic layer of the bags remained in full contact with the cheese at all times, ensuring no or little contact with the air. For the control cheese only the larger surface area (i.e. the top of the cheese) remained in contact with the plastic (Fig. 5.10). Both the test and control cheeses were matured for 4 – 5 weeks at 9°C, followed by a further 6 - 10 weeks at 4°C.

2.7. Cheese manufacture and maturation

The procedure for cheese manufacture was carried out according to Kosikowski (1997). *Penicillium roqueforti* spores (Visbyvac DIP Dosis 5, Wisby, GmbH and Co. KG, Germany) were prepared according to the manufactures instructions and added to 4950 l of pasteurised milk prior to the addition of the starter culture. The lactic acid bacteria starter culture (DL-mix, M FZ 3-22, 50 u, Wisby, Danisco Cultor Niebüll, GmbH, Germany) containing, *Lactococcus lactis* subsp. *lactic, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactic biovar. diacetylacis* and *Leuconostoc mesenteroides* subsp. *cremoris*, was prepared according to the manufactures instructions and added at the appropriate time to the pasteurised milk.

All cheeses were matured at 9°C for 4 - 5 weeks and then washed and packed in plastic bags before being moved to the second maturation room (4°C) for 4 - 10 weeks.

2.8. Sensory analysis of matured cheeses

The sensory quality of the control, dipped and hand sprayed cheeses were evaluated by a panel of experts in the field of cheese evaluation, based on a standard protocol including openness, texture, taste, flavour, and maturity at various intervals during the maturation period. The cheeses were evaluated after 5 weeks, 10 weeks and 13 weeks and the results compared.

3. RESULTS AND DISCUSSION

Brevibacterium linens and similar coryneform bacteria have been isolated from the surface of smear cheeses at levels as high as 10^9 - 10^{11} cfu/g (Neve and Teuber, 1989; Piton and Fontainer, 1990; Grand *et al.*, 1992; Piton-Malleret and Gorrieri, 1992; Eliskases-Lechner and Ginzinger, 1995; Galli *et al.*, 1996; Schubert *et al.*, 1996; Bockelmann *et al.*, 1997; Valdés-Stauber *et*

al., 1997; Bikash *et al.*, 2000; Leclercq-Perlat, 2000). In Chapter 4 we reported a highest count of 7.9 log units/g on the surface of the cheese, obtained after 7 weeks of maturation and a highest count of 3.6 log units/g in the inner core of the cheese, obtained after 5 weeks of maturation.

3.1. Treatment of pallets for the inhibition of *B. linens*

3.3.1. Radical water™

An initial survey conducted to establish a profile of the microbial populations on the pallets revealed that the populations of yeasts, lactic acid bacteria and coliforms remained relatively constant regardless of use and/or location of the wooden pallets (Table 5.1). In contrast, the populations of *B. linens* varied depending on the location and use of the wooden pallets. Pallet 2 (packaging room, recently used) showed values of 1.47 x10^7 cfu/g, which was between 10^2 and 10^4 cfu/g lower than values reported in literature for cheese factory environments and equipment (Neve and Teuber, 1989; Piton and Fontainer, 1990; Grand *et al.*, 1992; Piton-Malleret and Gorrieri, 1992; Eliskases-Lechner and Ginzinger, 1995; Galli *et al.*, 1996; Schubert *et al.*, 1996; Bockelmann *et al.*, 1997; Valdés-Stauber *et al.*, 1997; Bikash *et al.*, 2000; Leclercq-Perlat, 2000). On the contrary, pallet 1 (packaging room, unused at time of sampling) and pallet 3 (cold store room) yielded values of 1.8 x 10^4 and 7.5 x 10^3 cfu/g respectively (Table 5.1). Since, *B. linens* has an optimum growth temperature of 20 - 37°C (varies from species to species) (Bikash *et al.*, 2000; Rattray and Fox, 1999; Toolens and Koning-Theune, 1970) the low counts obtained in the cold store room could be expected. The packaging room, which is at room temperature and varies between 20°C - 30°C depending on the time/season of the year yielded the highest counts (1.47 x10^7 cfu/g for pallet 2, packaging room, recently used) since this environment was more conducive towards the proliferation of *B. linens*. In Chapter 4, it was speculated that the growth of yeasts aids the growth and proliferation of *B. linens* and since yeasts are dominant in and on the cheeses, which are stored on the wooden pallets

during maturation (Chapter 3), this could explain the higher counts of *B. linens* obtained on the pallets in used at the time of sampling as the yeasts may have contaminated the pallets. Further investigations were therefore focused on the pallets in use at the time of sampling.

Table 5.2 details the results obtained for the first trial involving the pallets treated with the Radical water™. These results clearly showed that there were no significant changes in the counts of yeasts, coliform, *B. linens* and consequently in the total counts. Further investigation revealed that the Radical water™ used for this trial was less active than freshly prepared Radical water.

Ensuring that the Radical water™ was active a second trial was initiated. As before, the wooden pallets were sprayed with the Radical water™ ensuring the entire surface was wet and microbial samples were subsequently taken over varying time intervals as indicated in Fig. 5.1. Initial *B. linens* counts for the untreated wooden pallets averaged 9.3 x 10^4 cfu/g (Fig. 5.1c), again significantly lower than the 10^9 - 10^{11} cfu/g reported in literature for cheese factory environments and equipments (Neve and Teuber, 1989; Piton and Fontainer, 1990; Grand *et al.*, 1992; Piton-Malleret and Gorrieri, 1992; Eliskases-Lechner and Ginzinger, 1995; Galli *et al.*, 1996; Schubert *et al.*, 1996; Bockelmann *et al.*, 1997; Valdés-Stauber *et al.*, 1997; Bikash *et al.*, 2000; Leclercq-Perlat, 2000). After 60 min of treatment with the Radical water™ the *B. lines* counts were reduced to 3.6 x 10^1 cfu/g, an average decrease of three log cycles in the overall counts. The counts were slightly further reduced after 120 min to 3.2 x 10^1 cfu/g, but increased to a value of 5.9 x 10^3 cfu/g after 240 min, followed again by a small decrease to 3.1 x 10^2 cfu/g after 360 min. This sudden increase observed after 240 min may have been due to the short half-life of the Radical water™. From Fig. 5.1c it is evident that 90 min was the most suitable time observed for effective treatment as this was calculated to be the average time between the first significant decrease and the next increase. As the *B. linens* numbers decreased, the total bacterial

counts also declined to a value of 2.4 x 10^4 cfu/g after 45 min of treatment with the Radical water™ (Fig 5.1d). Despite an increase in the total bacterial counts the final enumeration value was ten fold lower than the initial count. Similarly, the lactic acid bacteria, yeasts and coliforms numbers decreased over the tested time (Fig. 5.1a, b and e). The lactic acid counts showed the greatest decrease after 45 min of treatment with the Radical water™, with the counts being reduced from 4.5 x 10^5 cfu/g initially to 1.9 x 10^3 cfu/g (Fig. 5.1a). As with the *B. linens* and the total bacterial counts the LAB numbers again increased slightly, with the final enumeration value being ten fold lower than the initial count (Fig. 5.1a, c and d). Similarly, the coliform counts showed a maximal decrease after 45 min from an initial count of 3.8 x 10^4 cfu/g to 6.7 x 10^2 cfu/g (Fig. 5.1e). The yeast counts (Fig. 5.1b) showed minimal variation over the treatment period in comparison to the lactic acid bacteria, *B. linens*, total bacterial counts and coliform counts (Fig. 5.1a, c, d and e). In contrast to the other counts the yeast counts showed less than a ten fold decrease after 360 min of treatment with the Radical water™ with the counts being reduced from 6.7 x 10^3 cfu/g initially to 2.2 x 10^3 cfu/g (Fig. 5.1b).

For a product or process to be considered as successful in terms of complete elimination or inhibition a minimum of a four log cycle decrease is required (Royer *et al.*, 2001). Since we only obtained a three log cycle decrease in the overall *B. linens* counts, it was evident that the application of Radical water was effective at reducing the numbers *B. linens* but did not completely inhibit or eliminate this bacterium. Despite the lack of complete inhibition, these results were the most promising obtained thus far and hence the used of Radical water™ in the treatment of the wooden pallets has been implemented in the cheese factory.

3.2. Cheese treatments

3.2.1. Radical water™

Based on the success obtained in treating the pallets with Radical water™ a trial was conducted on 15 blue veined cheeses selected from the same batch. Five cheeses were selected as controls. An additional five cheeses were sprayed with the Radical water™ ensuring the entire surfaces were wet. The remaining five cheeses were submerged in the Radical water™ for 90 min, since this was the most suitable time determined in the previous experiments carried out on the wooden pallets (Fig. 5.1). All cheeses were manufactured and matured under the same controlled conditions as specified in the materials and methods above. Microbial samples were taken at selected intervals from both the outer surface and inner core as depicted in Fig. 5.2, 5.3, 5.4 and 5.5.

From Fig. 5.2 and 5.3 it is evident that the Radical water spray technique was not very effective since the *B. linens* count continue to increase during the maturation time in both the outer surface and inner core samples up to week eight. Thereafter the counts began to decrease to a final value of 1.25×10^2 and 0.6×10^1 cfu/g for the outer surface and inner core samples respectively (Fig. 5.2 and 5.3). As reported in Chapter 4 the decrease in the *B. linens* can in part, be attributed to the slight decrease in the pH of the cheese at the time, the depletion of available carbon compounds and to the decrease in the yeast counts after seven to eight weeks, resulting in a reduction in the production of vitamins and additional growth factors. The inefficiency of the Radical water™ to inhibit the proliferation of *B. linens* numbers may be attributed to the evaporation of the Radical water™ from the surface of the cheese during the maturation period. Despite the low temperatures (4 – 9°C) in the maturation rooms and the cheeses being bagged in plastic films, surface evaporation could still take place. The lack of inhibition of *B. linens* as obtained in the inner core may be due to limited penetration of the Radical water™ since only

the surfaces of the cheeses were sprayed with the Radical water™, as well as the anoxic environment prevailing within the inner core of the cheese. It has previously been stated that *B. linens* is aerobic (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000).

The cheese dipped in the Radical water™ showed notable differences in the *B. linens* counts (Fig. 5.4 and 5.5). In contrast to the cheeses sprayed with the Radical water™ showing an increase in the *B. linens* counts from week 1, the cheese dipped in the Radical water™ only showed an increase in the *B. linens* count from week 3. A comparison of the numbers of *B. linens* present on the surface samples between the sprayed and the dipped cheeses at week 5 (Fig. 5.2 and 5.4) showed values of 8.9×10^3 and 3.1×10^2 cfu/g respectively. Despite these initial differences, the enumeration values after 14 weeks for both the sprayed and the dipped cheese exhibited similar values of 10×10^1 and 12.6×10^1 cfu/g respectively (Fig. 5.2 and 5.4). In addition, the surface sample values for the sprayed and dipped cheeses ranged from 0.13×10^1 cfu/g to 2.8×10^4 cfu/g and from 0.13×10^1 cfu/g to 1.6×10^5 cfu/g respectively.

The inner core samples for the dipped cheese yielded average *B. linens* values of 100 fold lower than the values obtained for the spray treated cheese (Fig. 5.3 and 5.5). This improvement in the inhibition can be attributed to the longer exposure time to the Radical water™ facilitating absorption and penetration into the inner core. Despite limited success of treating the whole cheeses by means of dipping in Radical water™ for 90 min, it proved to be very time consuming and thus not an economically viable option for incorporation on an industrial scale.

3.2.2. Incorporation of yeasts during maturation

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). The possibility of using *D. hansenii,* included as an adjunct starter culture, has been investigated (Yamauchi *et al.*, 1976; Ferreira and Viljoen, 2003) with varying degrees of success. *D. hansenii* is known to enhance the maturation of cheese since it can stimulate maturation by means of lipolytic and proteolytic activities (Devoyod, 1990; Fleet, 1990) and control the pH of the cheese by means of the utilisation of the lactic and acetic acid present (Devoyod, 1990; Fleet, 1990). However, the use of this yeast as a bio-control agent for *B. linens* growth on blue-mould cheeses has not previously, to our knowledge, been investigated.

Fifteen cheeses from the same batch were selected divided into three groups of five and treated as follows: five were dipped and five were hand sprayed with *D. hansenii* in solution, while the remaining five were kept as controls. All cheeses were kept under the same controlled conditions as specified earlier. Sampling for microbial analysis from both the outer surface and the inner core was performed for lactic acid bacteria, yeasts, *B. linens* and total bacterial counts over selected time intervals as depicted in Figs. 5.6, 5.7, 5.8 and 5.9.

The lactic acid bacterial counts for the dipped, hand sprayed and control cheeses remained similar for both the inner core and outer surface samples (Figs. 5.7 and 5.9). This indicated that the application of *D. hansenii,* did not substantially influence the growth and proliferation of the lactic acid bacteria during the maturation of the blue-mould cheeses. Despite being treated with the *D. hansenii* suspension, the overall yeast counts for the hand sprayed cheeses were much lower than those of the control, most notably for the inner core samples (Figs. 5.7 and 5.9). Due to the aerobic nature of most yeast, significantly ($p < 0.05$) higher yeast counts were observed on the surface compared to the inner core. The reduced surface and inner core yeast counts, compared to the yeast counts associated with the dipped cheeses suggested that hand spraying did not allow sufficient time for the yeasts to penetrate the cheese. Alternatively the reduced count may have resulted due

to settling of the yeast solution during the application process or ineffective application. In contrast, the dipped cheeses showed higher overall counts of yeasts for both the inner core and the outer surface (Figs. 5.7 and 5.9).

Neither the dipped nor the spray treated cheeses showed reduced *B. linens* counts when compared with the control cheeses (Figs. 5.6 and 5.8). In fact both the dipped and sprayed cheeses inner core exhibited higher *B. linens* counts than the control. The higher *B. linens* counts associated with the presence of high numbers of yeasts may be indicative of the stimulating effect of yeasts on the growth of *B. linens* by means of an increase in the pH, via lactic acid utilisation and the production of growth vitamins (Devoyod, 1990; Fleet, 1990).

Treatment of the cheeses with the yeast solution resulted in enhanced numbers of *B. linens* for both application techniques. However, despite the lack of reducing the *B. linens,* the addition of *D. hansenii* had a positive effect on the maturation and development of the cheese.

3.3. Sensory analysis

All fifteen of the cheeses (5 controls, 5 dipped and 5 sprayed) were subjected to a sensory evaluation by a panel of experts in this field. The results were summarised per group of five cheeses and are detailed in Table 5.3. The hand sprayed and dipped cheeses both yielded similar results in terms of flavour and appearance. Both these trial cheeses reached maturation at the packing stage, as opposed to the control cheese, which was only ready for consumption at the end of the cold chain processing. This clearly indicated that the presence of *D. hansenii* enhanced the maturation process. The appearance of the sprayed and dipped cheeses remained similar at all three grading stages. Interestingly, *B. linens* was highly visible on the control cheese at the final grading, similar in appearance to that of the sprayed and dipped cheeses (Table 5.3). Since *B. linens* favours a neutral to alkaline pH

environment, the excessive growth on the trial cheeses can be explained by the slight increase in pH due to the utilisation of the lactic and acetic acid present by *D. hansenii* (Devoyod, 1990; Fleet, 1990). Despite a reduction in maturation time as well as an improvement in the overall texture, the presence of *B. linens* was not reduced and therefore the cheeses had a strong flavour, which although favoured by the European market, was not suitable for the South African market.

3.3.1. Other techniques

It is well known that *B. linens* is an aerobic bacterium (Bikash *et al.*, 2000; Leclercq-Perlat, 2000; Rattray and Fox, 1999; Toolens and Koning-Theune, 1970). Thus the possibility of limiting the oxygen availability to the surface of the cheese during maturation was investigated. However, it is important to kept in mind that *Penicillium roqueforti,* an integral part of blue cheese making, used to create the traditional aroma, appearance and texture of the blue-mould cheese, is a strict aerobe and complete removal of oxygen would have a detrimental effect on the final flavour, texture and aroma of the cheese. With this in mind the cheeses were packed in industrial strength plastic bags thereby limiting the oxygen supply to the surface of the cheese. The plastic was applied with surface-to-surface contact on upper side of the cheese, while the remainder of the cheese was allowed free flow of the oxygen available in the bag and the minimal amount that could permeate the plastic. As indicated in Fig. 5.10, the areas of direct contact with the plastic (a), i.e. the anaerobic or reduced oxygen areas, showed a definite reduction in *B. linens* growth (evident by the reduced orange-reddish colouring of the cheese), when compared with the non-contact areas or areas allowing for the free flow of air (b). This technique allows the continued proliferation of the *Penicillium,* since the cheeses were pierced prior to maturation and this facilitated the movement of air throughout the cheese, while inhibiting *B. linens* growth on a large portion of the surface area, thus saving costs in terms of wastage.

Despite this technique being effective, it remains a time consuming and expensive means of controlling or reducing the *B. linens* growth.

Table 5.1. Enumeration results obtained from wood shavings taken from various wood pallets in the blue-mould cheese factory. The results shown are the average of duplicate samples.

Location	Lactic acid bacteria	Total colony counts	Total yeasts counts	*Brevibacterium linens*	Coliforms
	MRS	PCA	RBCA	LCGS	M[c] Conkey
Pallet 1	4.0×10^5	1.1×10^7	3.8×10^2	1.8×10^4	4.6×10^2
Pallet 2	8.8×10^5	2.5×10^7	3.0×10^2	1.47×10^7	9.8×10^2
Pallet 3	8.0×10^3	1.76×10^7	4.0×10^1 ESPC	7.5×10^3	5.2×10^3

Pallet 1	Packaging room, unused at time of sampling.
Pallet 2	Packaging room, recently used.
Pallet 3	Cold store room

Table 5.2. Enumeration results obtained from wood shavings taken from various wood pallets after treatment with Radical water™ in the blue-mould cheese factory. The results shown are the average of duplicate samples.

Sample pallet[a]	Total colony counts	Total yeasts counts	*Brevibacterium linens*	Coliforms
	PCA	RBCA	LCGS	M[c] Conkey
Before treatment	3.1×10^7	3.8×10^2	2.1×10^7	9.7×10^2
After 15 min	2.8×10^7	3.0×10^2	1.95×10^7	8.8×10^2
After 30 min	2.75×10^7	6.8×10^1	2.0×10^7	5.2×10^3
After 45 min	2.4×10^7	3.6×10^2	1.95×10^7	4.6×10^3
After 60 min	2.65×10^7	3.25×10^2	1.85×10^7	9.9×10^2
After 90 min	2.95×10^7	3.4×10^2	2.15×10^7	9.4×10^2
After 120 min	2.55×10^7	4.1×10^2	2.19×10^7	9.2×10^2

[a] Pallet — Packing room, recently used.

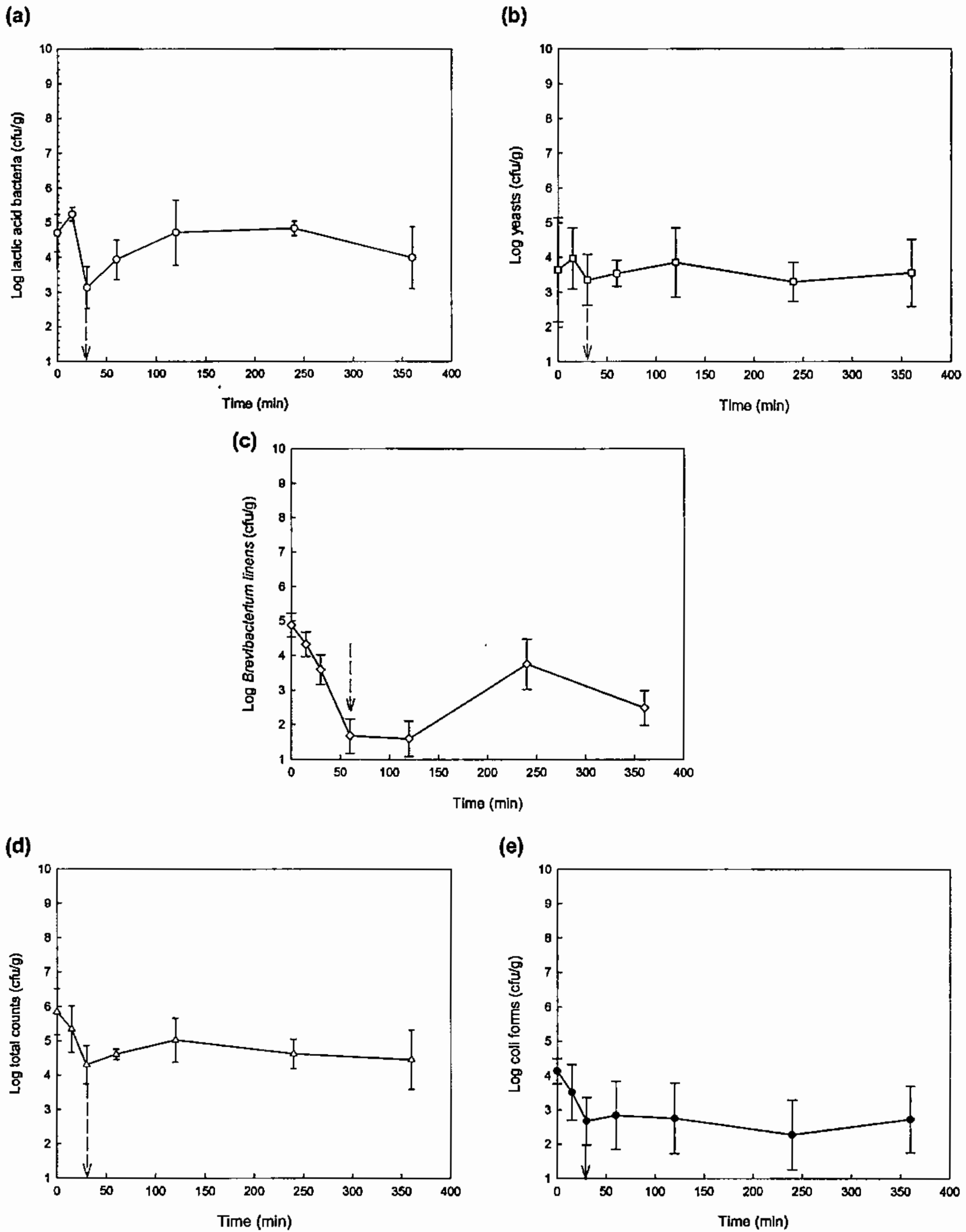

Figure 5.1. The graphical representation of the microbial loads obtained after treatment of the wooden pallets with Radical water™. The arrows indicate the most suitable time observed for effective treatment. N = 3.

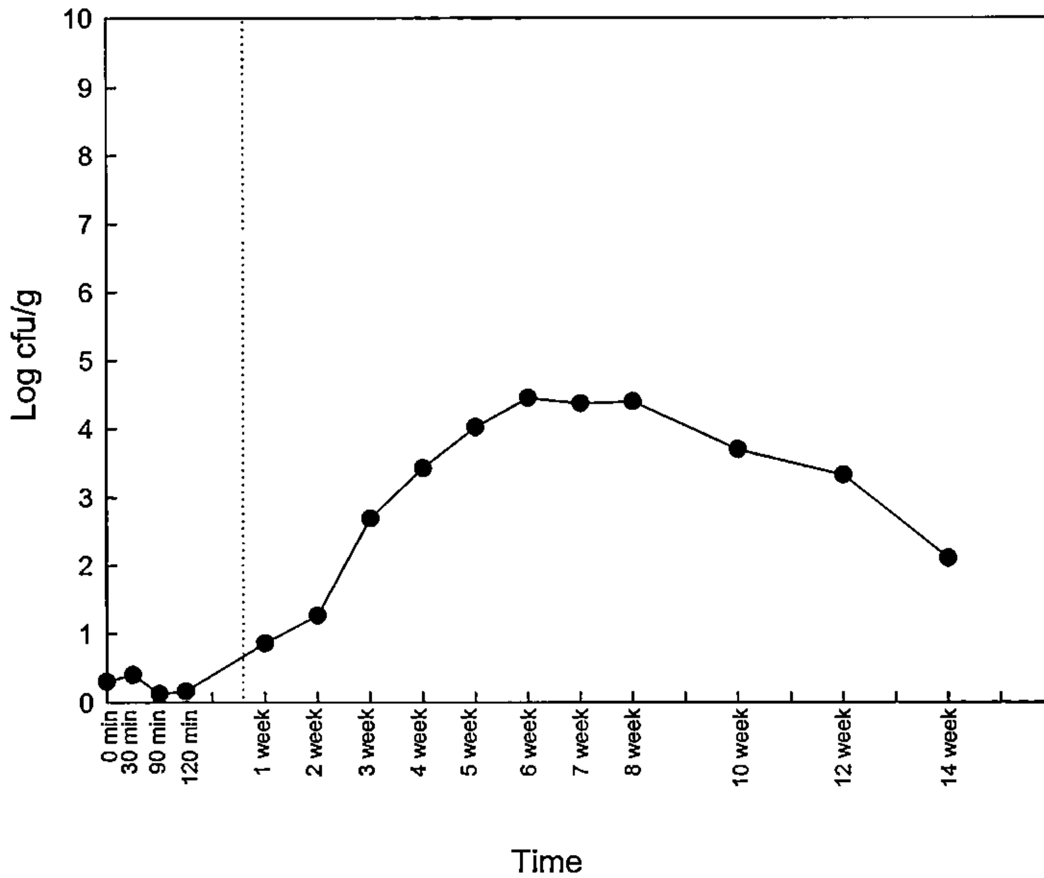

Fig. 5.2. The changes in the *Brevibacterium linens* counts sampled from the outer surface of blue-mould cheese during the maturation process after the cheeses were sprayed with Radical water™.

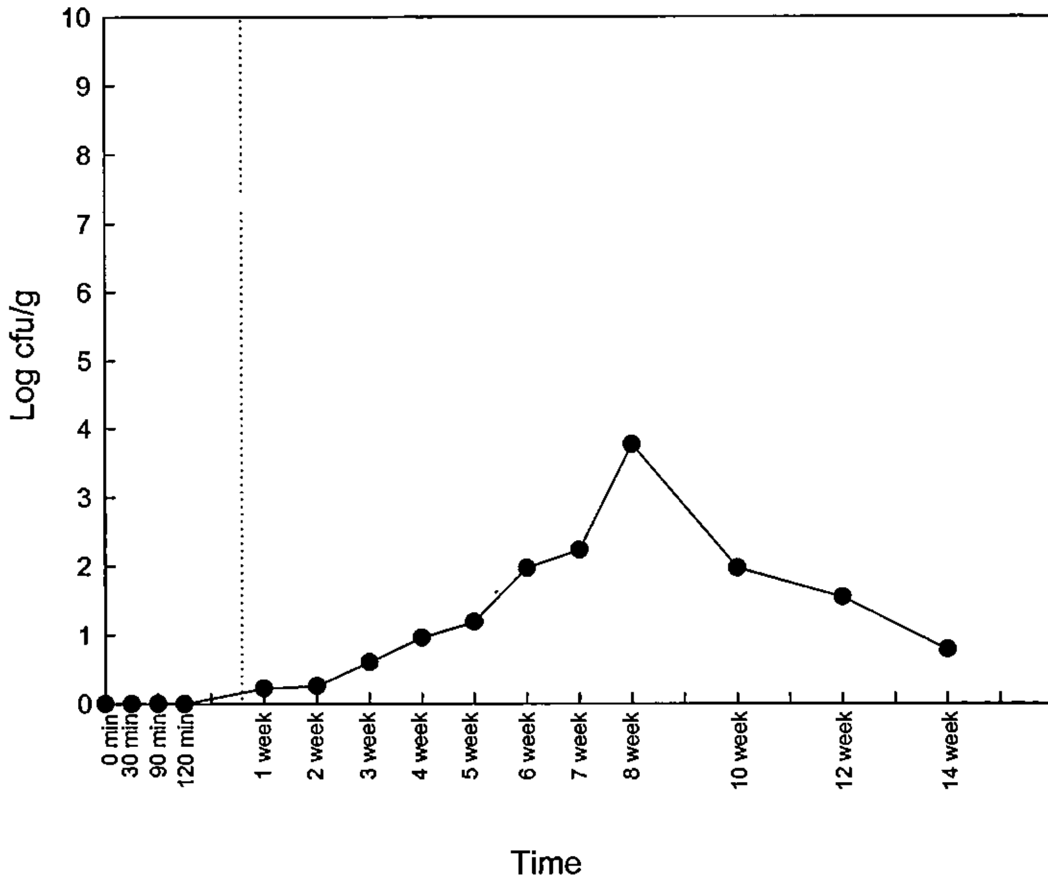

Fig. 5.3. The changes in the *Brevibacterium linens* counts sampled from the inner core of blue-mould cheese during the maturation process after the cheeses were sprayed with Radical water™.

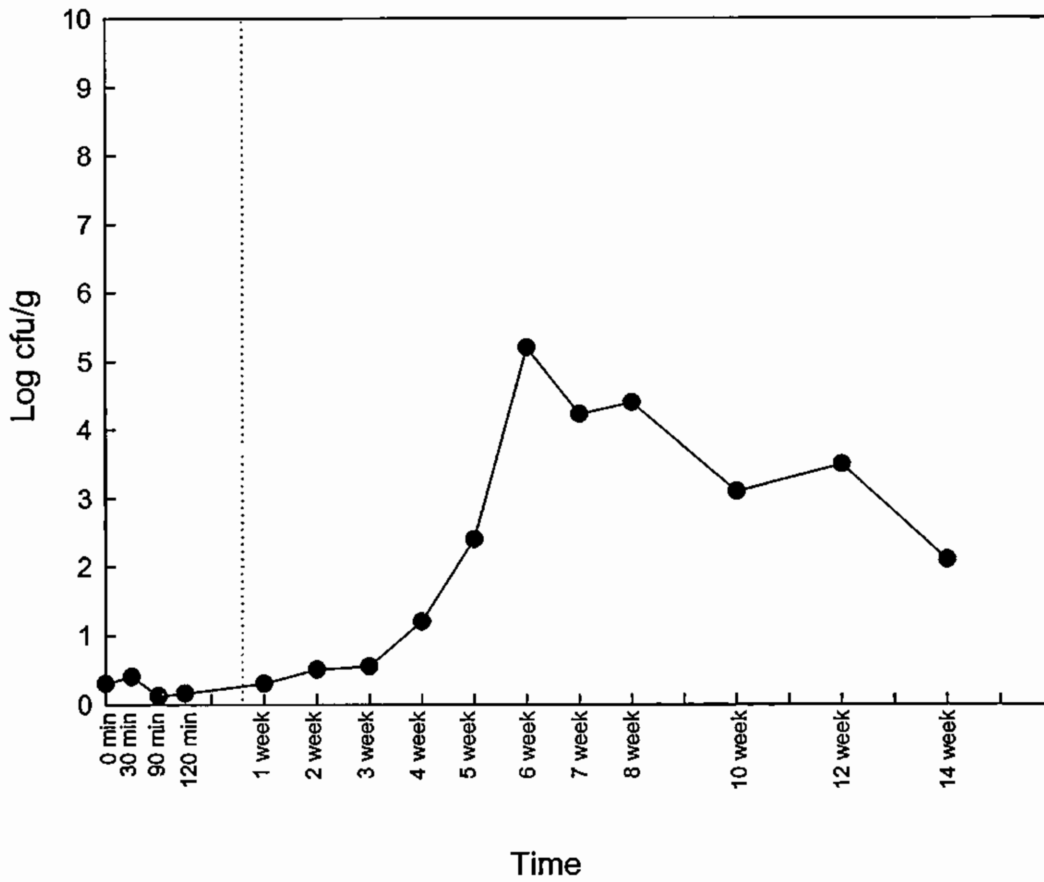

Fig. 5.4. The changes in the *Brevibacterium linens* counts sampled from the outer surface of blue-mould cheese during the maturation process after the cheeses were dipped in Radical water™ for 90 min.

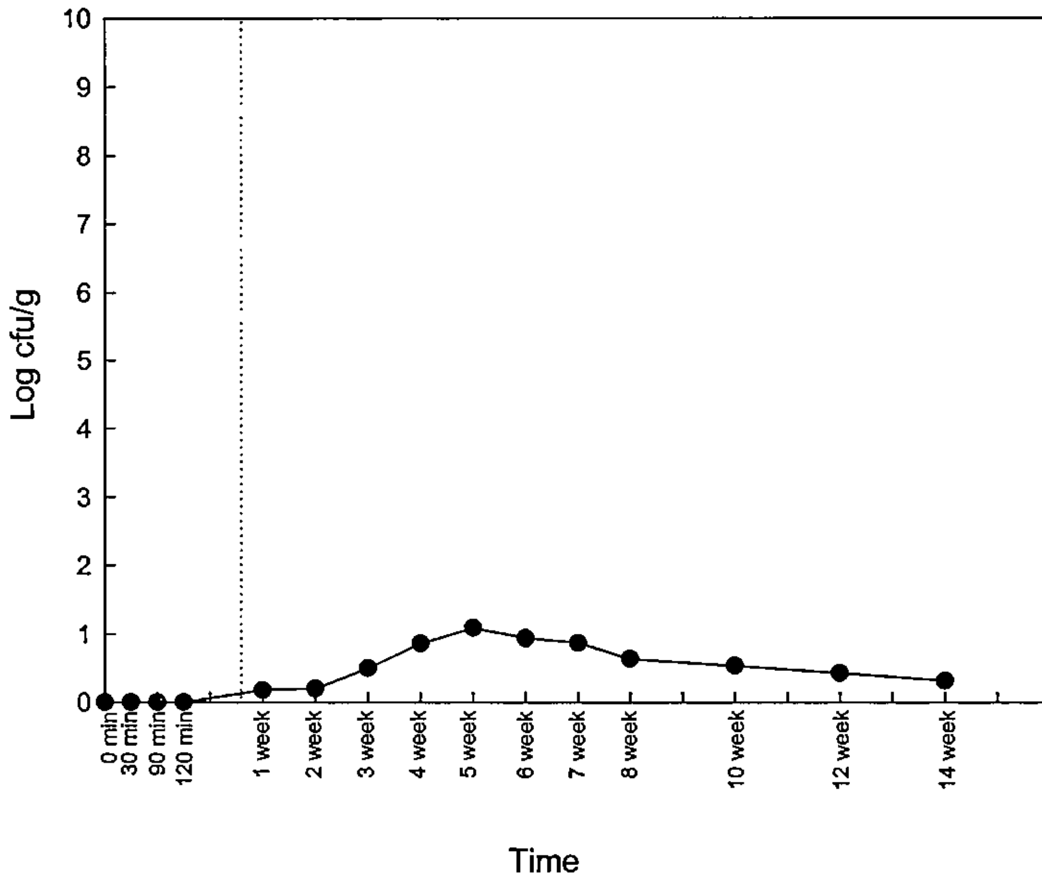

Fig. 5.5. The changes in the *Brevibacterium linens* counts sampled from the inner core of blue-mould cheese during the maturation process after the cheeses were dipped in Radical water™ for 90 min.

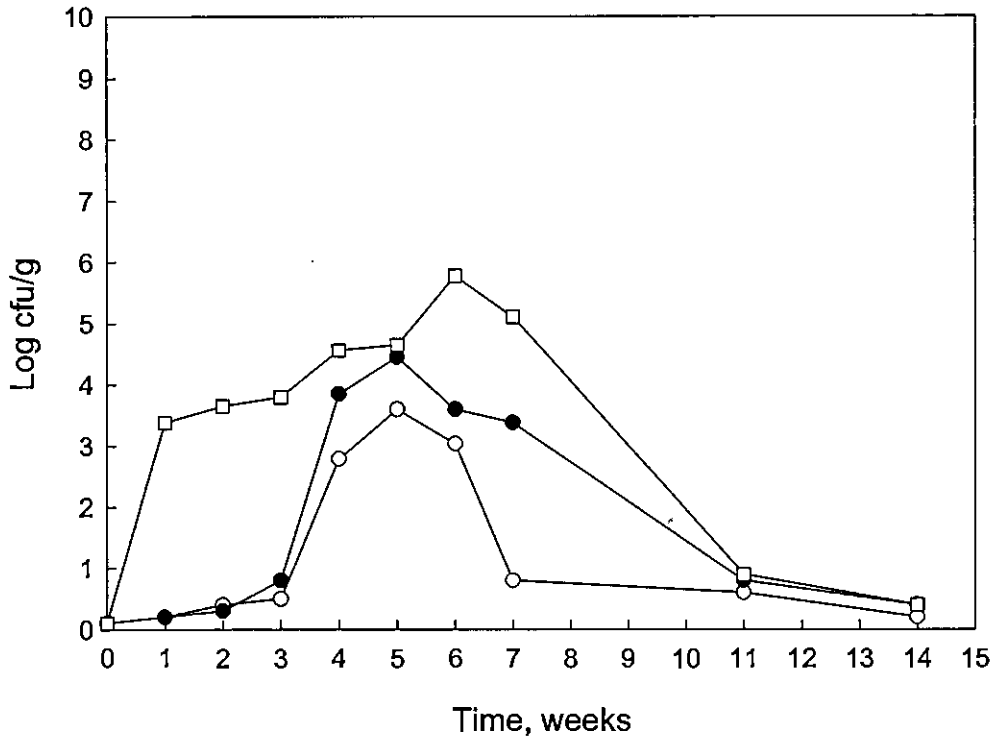

Fig. 5.6. The profiles of *Brevibacterium linens* sampled over 14 week period from the inner core of Blaaukrantz PCS 32263 blue-mould cheese. The cheeses were either keep as a control (O), dipped (□) or hand sprayed (●) with the *Debaryomyces hansenii* solution.

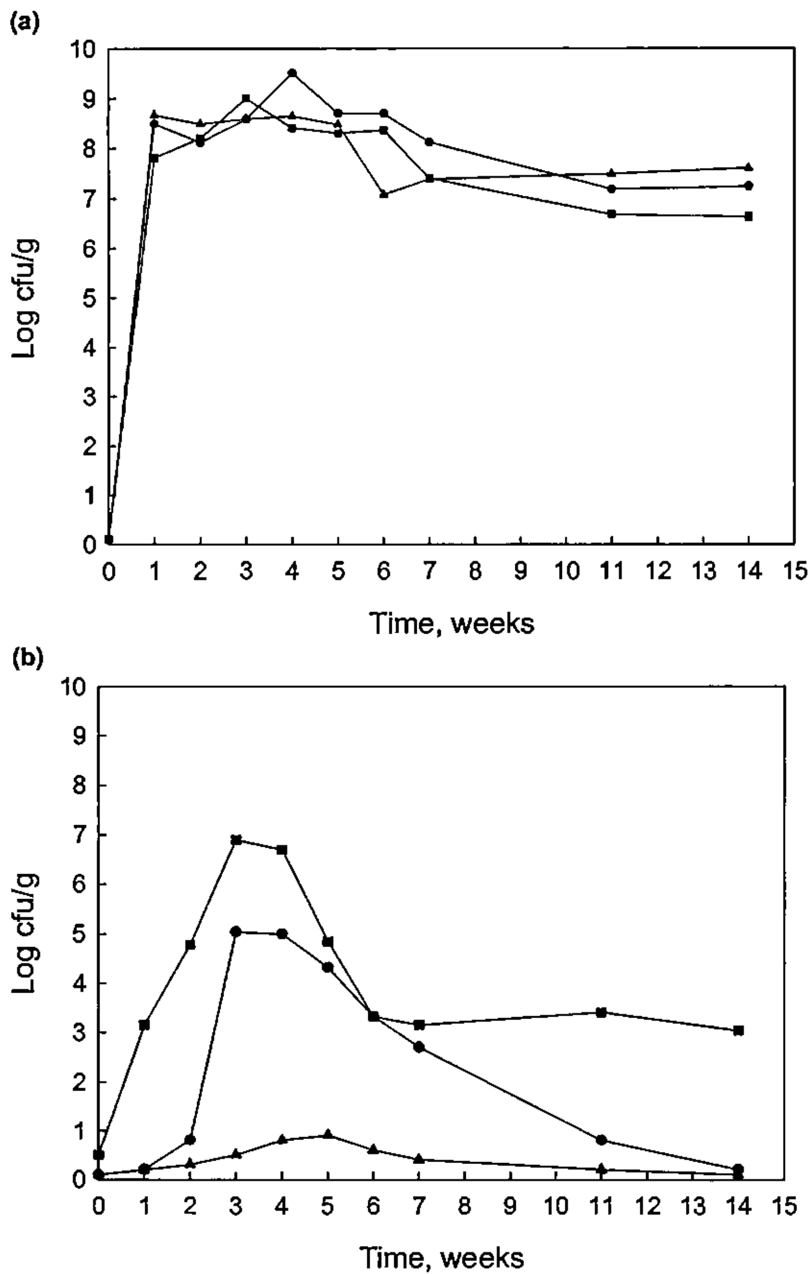

Fig. 5.7. The profiles of lactic acid bacteria (a) and yeasts (b) sampled over a 14 week period from the inner core of Blaaukrantz PCS 32263 blue-mould cheese. The cheeses were either keep as a control (●), dipped (■) or hand sprayed (▲) with the *Debaryomyces hansenii* solution.

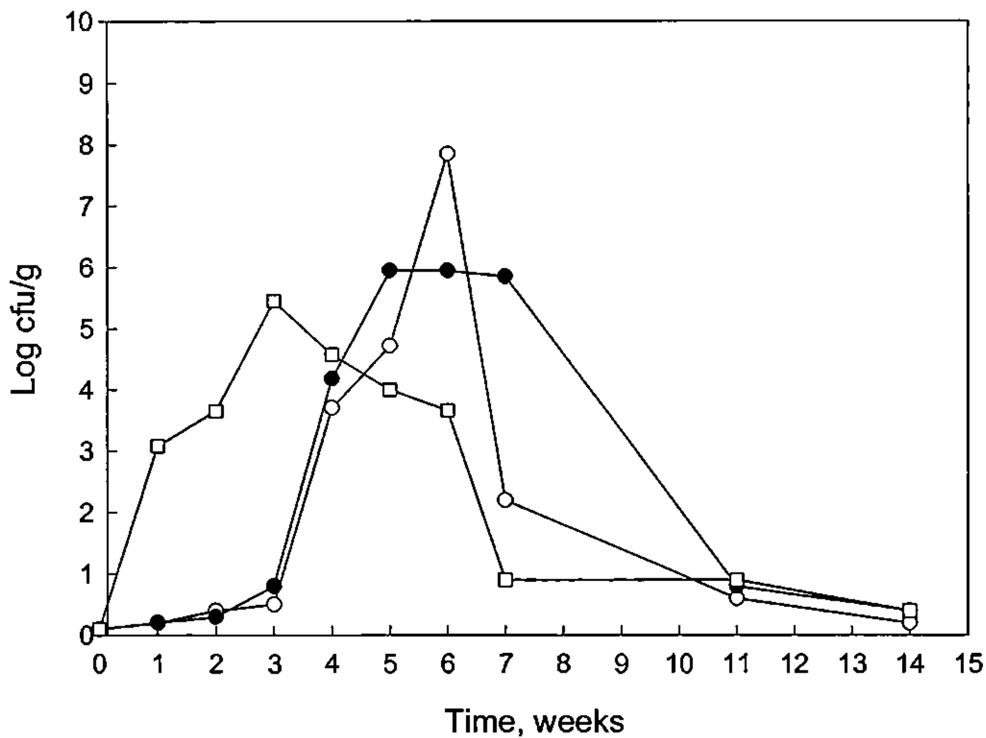

Fig. 5.8. The changes in the *Brevibacterium linens* profiles sampled over a 14 week period from the outer surface of the Blaaukrantz PCS 32263 blue-mould cheese. The cheese were either kept as a control (O), dipped (■) or hand sprayed (●) with the *Debaryomyces hansenii* solution.

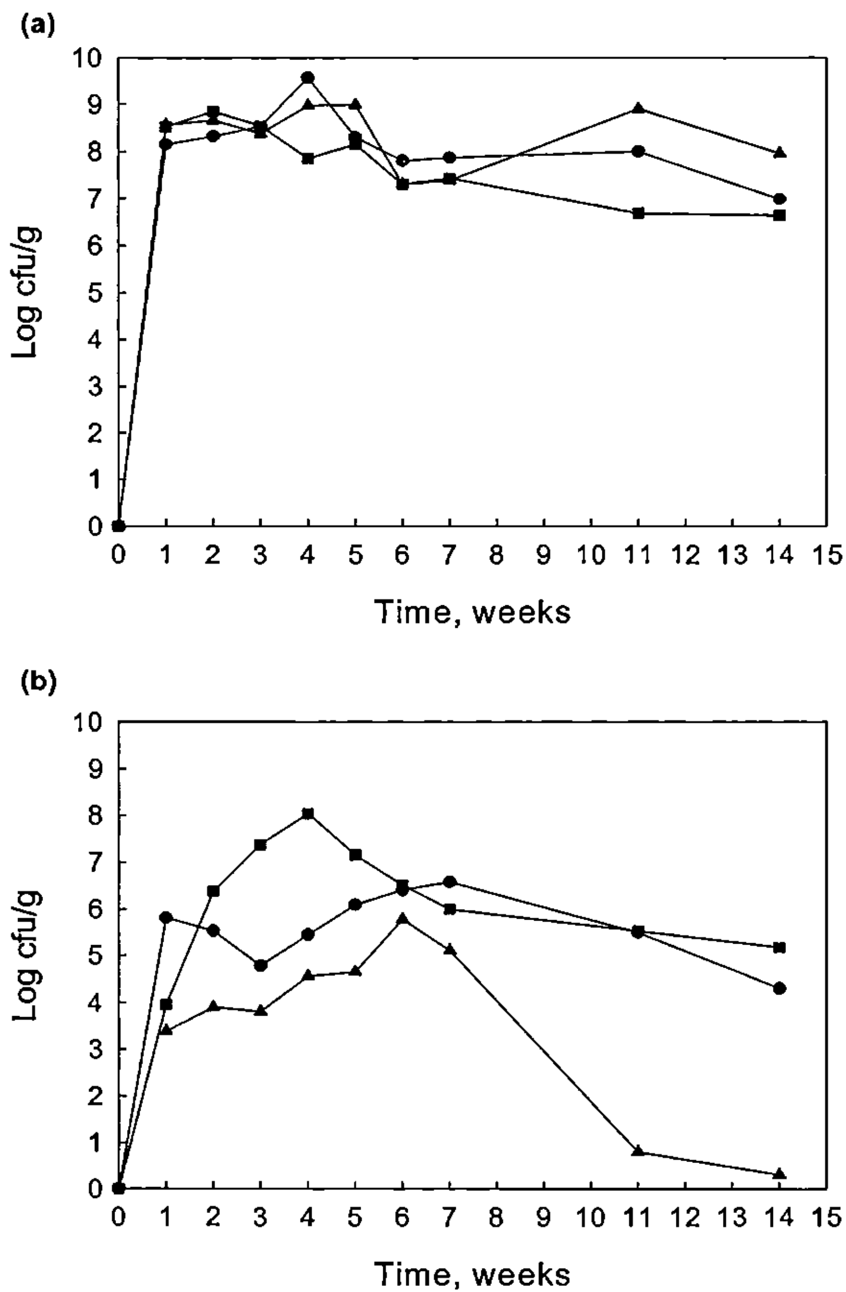

Fig. 5.9. The changes in lactic acid bacteria (a) and the yeasts (b) profiles sampled over a 14 week period from the outer surface of the Blaaukrantz PCS 32263 blue-mould cheese. The cheese were either kept as a control (●), dipped (■) or hand sprayed (▲) with the *Debaryomyces hansenii* solution.

Table 5.3. Sensory analysis of the cheese treated with the yeasts.

CONTROL CHEESE	SPRAYED CHEESE	DIPPED CHEESE
1ST GRADING Cheese moved from first maturation room to second maturation room		
Appearance		
Clean and dry on outside	A bit yeasty and slimy on the outside	Yeasty and slimy on the outside, but not as much as sprayed cheese
Texture		
Slightly drier than normal	Softer texture than control	Softer texture, but slightly rubbery
Flavour		
Still bland	Flavour well developed, much more than control	Flavour more developed than control
Overall		
Acceptable for moving to second maturation room		
2ND GRADING Ready for weighing and packaging		
Appearance		
Slightly dry on the outside, but more or less normal for this stage	High yeast growth visible. Yeasts need to be removed before packaging	Yeast growth visible, but not too much more than normal
Texture		
Bordering on the dry side, but acceptable for packaging	Good texture	Good texture
Flavour		
Distinctive	Well developed	Flavour well developed, but not as much as sprayed cheese

Table 5.3 (Continued). Sensory analysis of the cheese treated with the yeasts.

CONTROL CHEESE	SPRAYED CHEESE	DIPPED CHEESE
Overall		
Good for packaging, should however still ripen during the cold chain to reach optimum flavour	Bordering on optimum flavour profile	Good packaging profile
3RD GRADING End of shelf life		
Appearance		
B. linens highly visible on the outside of cheese	Yeasts and *B. linens* clearly visible on outer layers	Yeasts and *B. linens* clearly visible on outer layers
Texture		
Soft, but slightly rubbery	Reasonably soft, can still be sliced	Good soft texture
Flavour		
B. linens well developed, flavour off	Strong flavour with definite *B. linens* note	Strong flavour with definite *B. linens* note
Overall		
Still acceptable at this stage, but may become to strong	Too strong for the average South African consumer	Nearly to strong for optimal consumption

Data obtained from the sensory analysis panel at Simonsberg Cheese factory, South Africa.

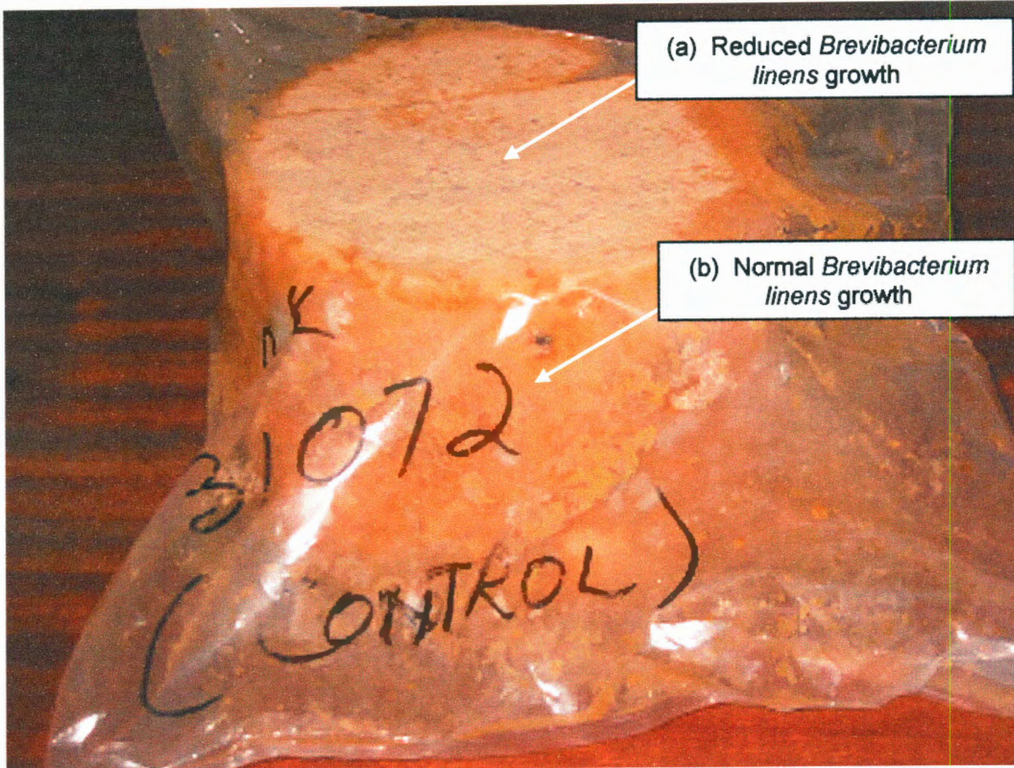

Fig. 5.10. The control cheese, cover with the layer of plastic, clearly indicating a reduction in the *Brevibacterium linens* growth at the areas of contact (a).

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Chapter 5

Inhibition of *Brevibacterium linens* during the production and maturation of South African blue-mould cheese

ABSTRACT

Brevibacterium linens, a natural dairy associated bacterial species, renders a unique taste and aroma, as well as an orange-reddish colour to the surface of various blue-mould cheeses. Although European cheese producers favour the presence of *B. linens,* the South African consumers demand a product void of the species. Accordingly, various natural methods regarding the inhibition of *B. linens* have been investigated. Initial surveys conducted throughout a South African cheese-making factory revealed that the *B. linens* was present on the wooden pallets used for the storage of the cheese during maturation. Treatment of the pallets with Radical water™ indicated that a 90 min washing period was the optimum time required for effective reduction of *B. linens.* Treatment of the whole cheese with Radical water™ indicated that application by submerging the whole cheese in Radical water™ for 90 min significantly reduced the *B. linens* counts during the first six week of maturation when compared with the hand sprayed technique. Further investigation showed that treatment of the whole cheeses with a solution of *Debaryomyces hansenii* either by hand spraying or dipping did not reduce the *B. linens* despite a reduction in maturation time, an improvement in the overall texture and the development of a stringent flavour. Wrapping of the cheese in plastic films to create an anoxic environment showed limited success.

Keywords: *Brevibacterium linens*, blue-mould cheese, Radical water™, *Debaryomyces hansenii* and inhibition.

1. INTRODUCTION

Numerous European smear cheeses are ripened by a complex bacterial flora present on the surfaces of these cheeses. Well-known examples of these European smear cheeses include Tilsit, Romadour, Limburger, Harzer, Münster and Weinkäse (Valdés-Stauber *et al.,* 1997). This smear, often reddish in colour, consists primarily of yeasts and bacteria (Kammerlehner, 1995). These aerobic microorganisms have a direct impact on the flavour, texture, appearance and development of these cheeses. *Brevibacterium linens,* one of the main microorganisms present in the surface flora, contributes significantly to the final surface flavour, colour and aroma due to its strong proteolytic activity as well as the production of methanethiol (Bikash *et al.*, 2000).

Although the presence of *B. linens* in cheese is not harmful to humans, the South African consumer in general demands cheese devoid of the taste/aroma and/or the off colour caused by *B. linens* (see Chapter 1, Section 11.1). This bacterium is strictly aerobic, non-motile and non-spore forming (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000). The classification of this bacterium has altered numerous times over the last two decades (Fiedler *et al.*, 1981). Breed first described the genus *Brevibacterium*, with its type species *B. linens* in 1953. The role of *B. linens* in the flavour and aroma formation during the production and maturation of cheeses has been investigated by a number of authors (Valdés-Stauber *et al.*, 1997; Adamitsch and Hampel, 2000; Leclercq-Perlat, *et al.*, 2000; Ummadi and Weimer, 2001; Bockelmann, 2002). Most authors indicated how this bacterium contributes positively towards the cheese production by accelerating the maturation process. The orange-reddish colour in conjunction with the aroma, primarily ammonium, is essential to the production of many European smear-ripened cheeses, including Limburger, Münster, Brick, Tilsiter and Appenzeller (Rattray and Fox, 1999).

Numerous authors speculated about the enhanced growth of *B. linens* in blue-mould cheeses. Yeasts, for instance have been reported to stimulate the growth of *B. linens* (Purko *et al.*, 1951; Devoyod, 1969; Devoyod and Sponem, 1970; Leclercq-Perlat *et al.*, 2000) by metabolising the lactic acid, produced by the lactic acid bacteria at the beginning of the ripening process. This leads to an increase in pH (surface pH varies from 7.0-7.4), and with the additional growth factors, such as vitamins, produced by the yeasts, salt tolerant bacteria including *Micrococcus* spp., *Brevibacterium* spp. and *Arthrobacter* spp. proliferate (Purko *et al.*, 1951; Lenoir, 1984; Busse, 1989; Valdés-Stauber *et al.*, 1997). The application of yeasts as bio-control agents however, also proved to be successful (Kalle et al., 1976; Fleet, 1990; Kaminarides and Laskos, 1993) in inhibiting undesired microorganisms. Little research however has been done on the interactions, either positive or negative, between yeasts and *B. linens* in blue-mould cheeses. Hence, in this study we endeavoured to select for a possible yeast species, *D. hansenii*, capable of inhibiting the *B. linens* growth during the production and maturation of South African blue-mould cheese.

The possibility of using *D. hansenii* as adjunct starter cultures for cheese production has previously been investigated 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, 1999). 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, 1999). *D. hansenii* also inhibits the germination of undesired microorganisms like *Clostridium butyricum* and *C. tyrobutyricum* in cheese brines (Fatichenti *et al.*, 1983). Furthermore, Yamauchi *et al.* (1976) reported

ABSTRACT

Brevibacterium linens is an important dairy associated species rendering a specific taste and aroma to numerous smear ripened and blue-mould cheeses due primarily to proteolysis. Despite the beneficial effects, the presence of the species in South African blue-mould cheeses is undesired and consumers demand a product void of the species. Accordingly, numerous methods including microbial inhibition studies using yeasts and probiotic cultures with possible inhibitory effects were applied in an attempt to inhibit the species. None of the yeasts, however proved to be successful, whereas *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, two typical probiotic species applied in dairy products, showed inhibitory effects against *B. linens* when tested using the spot-on-lawn assay. In addition, wrapping the blue-mould cheese in Parafilm®, plastic and foil, in order to create an anoxic environment, proved effective in reducing *B. linens* surface growth.

Keywords: Inhibition, yeasts, *Brevibacterium linens*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis.*

1. INTRODUCTION

Breed first described the genus *Brevibacterium*, with its type species *B. linens* in 1953. The role of *B. linens* in the flavour and aroma formation during the production and maturation of cheeses has been investigated by a number of authors (Valdés-Stauber *et al.*, 1997; Adamitsch and Hampel, 2000; Leclercq-Perlat, *et al.*, 2000a; Ummadi and Weimer, 2001; Bockelmann, 2002). Most state how this bacterium contributes positively towards the cheese production by accelerating the ripening process. The orange-reddish colour in conjunction with the aroma, primarily ammonia, are essential to the production of many European smear-ripened cheeses, including Limburger, Münster, Brick, Tilsiter and Appenzeller (Rattray and Fox, 1999). *B. linens* is not harmful if consumed, however, the South African consumer rejects cheese with the taste and orange-reddish colour created by *B. linens* growth.

Yeasts have been reported to stimulate the growth of lactic acid bacteria, *B. linens* and moulds in cheeses (Purko *et al.*, 1951; Devoyod, 1969; Devoyod and Sponem, 1970; Leclercq-Perlat *et al.*, 2000a) due to the metabolism of the lactic acid, produced by the lactic acid bacteria, at the beginning of the ripening process. This increase in pH and additional growth factors, produced by the yeasts allow *Brevibacterium linens* to proliferate (Valdés-Stauber *et al.*, 1997; Busse, 1989; Lenoir, 1984; Purko *et al.*, 1951). The colour intensity of the *B. linens* on the surface of the cheese is directly related to the growth of yeasts, especially *Debaryomyces hansenii* and *Kluyveromyces marxianus*, since they de-acidify the surrounding environment via lactic acid utilisation (Leclercq-Perlat *et al.*, 2004).

Lactic acid bacteria have been utilised in the preservation of food and other food areas for centuries. They are recognised as safe for human consumption since they are present on the surface of the human body and in the gastrointestinal tract (Wright and Saxelin, 1996). The word 'Probiotic' is a Greek word meaning 'for life' and is presently defined as 'viable microorganisms' that exert beneficial effects on the host after ingestion

through the improvement of the intestinal indigenous microflora balance in addition to general nutrition (Gomes and Malcata, 1999, Ouwehand *et al.*, 2002; Maus and Ingham, 2003). Two of the most well known probiotics are *Lactobacillus rhamnosus* and *Bifidobacterium lactis*. *Lactobacillus* is a gram-postive, non-spore forming, non-motile rod or coccobacillus. This microaerophillic probiotic microorganism shows optimum growth at 35-40°C and a pH range of 5.5-6.0 (Gomes and Malcata, 1999; Narayanan *et al.*, 2004;). *Bifidobacterium*, first described in 1899-1900 by Tissier (Norris *et al.*, 1950), is a gram-positive, non-spore forming, non-motile, catalase-negative anaerobe, which varies in shape from short curved rods to club-shaped rod and even bifurcated Y-shaped rods (Gomes and Malcata, 1999). The optimum growth occurs at a pH of 6-7, with a temperature range of 36-41°C under anaerobic conditions (Martin and Chou, 1992; Gomes and Malcata, 1999). *Lactobacillus rhamnosus* and *Bifidobacterium lactis* are capable of producing bacteriocins, which are known to have activity towards a wide range of gram-positive species (deVuyst and Vandamme, 1994).

Some of the most favourable advantages of ingestion of probiotics include: alleviation with lactose intolerance (Martini *et al.*, 1991; Jiang *et al.*, 1996; Curry and Crow, 2003); inhibition of pathogenic bacteria (Anand *et al.*, 1985; Curry and Crow, 2003); enhancement of the immune system (Fuller, 1998; Curry and Crow, 2003); anitcholesterolemic effects (Gilliland, 1990; Bukowska *et al.*, 1998); enhanced mineral absorption (Gibson and Roberforid, 1995); alleviation of constipation (Fuller, 1989); maintenance of normal intestinal microflora (Fuller, 1989; Curry and Crow, 2003).

Since probiotics are beneficial in terms of human health when consumed and may have an inhibitory effect via bacteriocin activity towards the gram-positive *B. linens* this possibility was further investigated. In addition, since the application of yeasts as bio-control agents proved to be successful (Kalle et al., 1976; Fleet, 1990; Kaminarides and Laskos, 1993) attempts to investigate these organisms as natural inhibitors of *B. linens* seemed logical. Accordingly, in this study we endeavoured to find a natural means of inhibiting

the growth of *B. linens* using selected dairy associated yeast and lactic acid bacteria with probiotic activity. These organisms were selected because, if positive, will encourage further investigative applications.

2. MATERIALS AND METHODS

2.1. Media

All media were prepared in accordance with the manufacturer's instructions and autoclaved at 121°C for 15 min. Aliquots of between 15 and 20 ml sterile media were dispensed into 90 mm Petri dishes and allowed to dry overnight at room temperature. The experimental media included: Plate count agar (PCA) (Biolab diagnostics, Merck) for the enumeration of the total viable bacterial counts and De Mann Rugosa and Sharpe agar (MRS) (Biolab diagnostics, Merck) for the lactic acid bacteria. In accordance with Toolens and Koning-Theune (1970), Lab Lemco agar, with 0.5 % glucose, 0.5 %, $CaCo_3$ 5.5 % NaCl and 0.02 % pimafucin (LCGS) was used for the isolation and enumeration of *B. linens*. LCGS was incubated at 25°C for 5 to 7 days.

2.2. Interactive studies

2.2.1. Microbial interactions

The spot-on-lawn assay (Piddock, 1990; Hoover and Harlander, 1993) was used to examine interactions between microorganisms and *B. linens* cultures. The basal medium used was either Tryptone Soy Agar (TSA) or PCA agar for bacteria and Malt Extract Agar for yeasts (MEA) (Oxoid). The respective basal medium was prepared and kept in a molten state at 50°C. Freshly grown (24 - 48h) cultures were seeded at 10^5 - 10^6 cfu/ml into the molten medium and poured into Petri dishes. After cooling and solidification, the medium was spot-inoculated on the surface with freshly grown (24 - 48h) inoculum species. The controls included the seeded molten basal medium without the inoculum species. In addition, all inoculum species were tested to

ensure growth on the basal mediums used. All plates were incubated at 25°C and observed after 1, 3, 5, 7 and 9 days. Inhibition of the seeded organism by the spot-inoculated isolate was evident as a clear zone surrounding the growing spot culture.

All yeast cultures were selected (Table 6.1) based on previous studies, which indicated the predominant species associated with dairy products (Viljoen, 2001). These cultures were obtained from the yeast culture collection at the University of the Free State, Bloemfontein, South Africa and maintained on YM agar slants at 4°C. Prior to use the yeast cultures were streaked out on fresh Malt Extract agar (MEA) (Merck, C10 - pH 5,4) for 48 h at 25°C and checked for purity.

The probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* (Wisby, Danisco Cultor Niebüll GmbH, Germany) were randomly chosen and reactivated from the freeze-dried state by incubating in 10 ml of sterile UHT low fat milk for 6 h at 43°C. The cultures were subsequently streaked out on MRS agar and further incubated at 43°C under anaerobic conditions for 24 to 48 h.

Orange-reddish coloured cultures were isolated from blue-mould cheeses produced locally, using LCGS media as described by Toolens and Koning-Theune (1970) and identified as representatives of *Brevibacterium linens* according to Bergey's Manual of Determinative Bacteriology (7th Ed). *B. linens* (NCDO 1002) obtained from National Collection of Dairy Organisms was used in addition to the strains isolated. The cultures were streaked out on PCA agar and incubated at 25°C for 24 - 48 h until sufficient growth was observed.

2.3. Other inhibitive methods

The spot-on-lawn assay (Piddock, 1990; Hoover and Harlander, 1993) or a modified version thereof was used to examine interactions between

microorganisms or sample materials and *B. linens* cultures. The basal medium used was Tryptone Soy Agar (TSA) or PCA agar for bacteria. The basal medium was prepared and kept in a molten state at 50°C. Freshly grown (24 - 48h) cultures of *B. linens* were seeded at 10^5 - 10^6 cfu/ml into the molten medium and poured into Petri dishes. After cooling and solidification, the medium was spot-inoculated on the surface with freshly grown (24 - 48h) inoculum species. Alternatively the sterile sample materials were aseptically place on the surface of the solidified medium. Sample materials included foil, Prarafilm® and industrial strength plastic and were cut into squares 1 cm x 1 cm prior to sterilisation. Sterile olive oil, 0.1 ml, was also applied to the surface of the solidified medium. As before, all plates were incubated at 25°C and observed after 1, 3, 5, 7 and 9 days. Inhibition of the *B. linens* by the spot-inoculated isolate or sample material was evident as a clear zone surrounding the growing spot culture.

3. RESULTS AND DISCUSSION

Yeasts stimulate the growth of lactic acid bacteria, *B. linens* and moulds in cheeses (Purko *et al.*, 1951; Devoyod, 1969; Devoyod and Sponem, 1970; Leclercq-Perlat *et al.*, 2000a), by increasing the pH due to lactic acid metabolism and by producing additional growth factors, such as vitamins, (Valdés-Stauber *et al.*, 1997; Busse, 1989; Lenoir, 1984; Purko *et al.*, 1951). This stimulation of growth of *B. linens* in blue-mould cheeses, further contributes to an enhancement in pigmentation causing an orange-reddish colouration in the cheese during the maturation period (Valdés-Stauber *et al.*, 1997). On the other hand, yeasts have also been indicated as acting as antagonistic organisms and applied as natural bio-control organisms (Kaminarides and Laskos, 1993; Fleet, 1990; Kalle *et al.*, 1976) against fruit rotting and the inhibition of *Clostridium* species in dairy products (Deiana *et al.*, 1984). As the discolouration of the blue-mould cheeses is unacceptable, attempts have been made to inhibit *B. linens* by using yeasts and probiotic organisms as natural inhibitors. Thus 21 strains of yeasts were examined as either a possible inhibitor or sensitive counterpart (Table 6.1).

As determined by the spot-on-lawn assay (Piddock, 1990; Hoover and Harlander, 1993), none of the yeasts inhibited or reduced the growth of *B. linens* (Table 6.1). Interesting to note, however was that *Zygosaccharomyces rouxii, Z. mellis* and *Debaryomyces anomala* were almost all completely inhibited by *B. linens*. *Trichosporon beigelii* and *Yarrowia lipolytica* remained unaffected by *B. linens*. Other species including *D. hansenii*, were unaffected by *B. linens* which corresponded with literature, since these yeasts are known to be associated with cheese (Mrak and Bonnar, 1939; Seiler and Busse, 1990).

B. linens colonisation of cheese predominates in blue-mould cheese with the occurrence thereof being very limited or absent in white-mould cheese. Even though white-mould *Penicillium* species could not be incorporated in the blue-mould cheeses, the possibility of finding a *Penicillium* species that could

inhibit *B. linens* was investigated with the aim of identifying the inhibitory substance (i.e. a mycotoxin) and possibly utilising this for inhibition or reduction purposes. Strains of three species of *Penicillium* (Table 6.2) were isolated from locally produced white and blue-mould cheeses and checked for purity. These were again screened using the 'spot-on-lawn' assay (Piddock, 1990; Hoover and Harlander, 1993). However, none of the *Penicillium* species screened inhibited the growth of *B. linens* (Table 6.2).

Alternatively, the probiotics *Lactobacillus rhamnosus and Bifidobacterium lactis,* which have known to have activity towards a wide range of gram-positive species (De Vuyst and Vandamme, 1994), were screened against *B. linens* for possible inhibitory action. Both these probiotics showed strong inhibition of *B. linens* (Figs. 6.1, 6.2 and 6.3; Table 6.2). However, the *B. linens* appeared to show reduced sensitivity to *L. rhamnosus* after approximately one week of incubation. In contrast, *B. lactis* continued to inhibit *B. linens* well after three weeks of incubation (Fig. 6.3). The initial inhibition may be attribute to the acid production by these probiotic strains. The diffusion of the acid into the agar on prolonged incubation could then allow the growth of *B. linens*. Alternatively the inhibition may be attributed to bacteriocin activity.

The addition of *L. rhamnosus* and/or *B. lactis* to the cheese is a strong possibility. As Fig. 6.1 and Fig. 6.2 indicate, these microorganisms significantly inhibited the growth of *B. linens* on agar plates, with *B. lactis* showing extended inhibition thereof. Despite these being preliminary trials, this opens doors for a number of possible applications. The fact that *L. rhamnosus* and *B. lactis* are probiotics with added health benefits is a bonus and allows acceptability of incorporation into foods (Ouwehand *et al.*, 2002; Saarela *et al.*, 2000; Fooks *et al.*, 1999; Salminen *et al.*, 1998).

Further investigation will need to focus on the application of the *Lactobacillus rhamnosus* and/or *Bifidobacterium lactis* at plant scale level into the blue-mould cheese and subsequent analysis thereof. In addition, the effect of

these probiotics on the microbial and chemical interactions with in the cheese as well as the effect on the final flavour, texture and aroma of the cheese will need to be evaluated. Gardiner *et al.* (1998) and Stanton *et al.* (1998) reported no adverse effects on sensory criteria of Cheddar cheese when probiotic bacteria like *Lactobacillus paracasei* were detected. Similarly Dinakar and Mistry (1994) reported no adverse effects in terms of the sensory criteria when *Bifidobacterium bifidum* was detected in Cheddar cheese, while Broome *et al.* (1990), McSweeney *et al.* (1994), Lynch *et al.* (1996) and Gardiner *et al.* (1999) all reported an improvment in the flavour of Cheddar cheese when probiotics were included as adjunct starter cultures. However, Gomes *et al.* (1995) reported that despite a similar texture and appearance to the control cheese, Gouda cheese containing *Bifidobacterium* sp. strain Bo and *L. acidophilus* strain Ki developed a bitter taste after 50 days. Bitter peptides usually develop in dairy products due to the formation of biogenic amines formed through the decarboxylation of the carboxyl group of an amino acid and the liberation of the corresponding amine (Joosten, 1988). In addition, the higher levels of lactic acid, due primarily to the high numbers of *L. acidophilus* could have contribute to the over acidification 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 but still possessing the ability to reduce or inhibit *B. linens* either by means of bacteriocin production or another antagonistic effect.

From Table 6.2, it is evident that by reducing the oxygen transfer, by means of plastic, foil etc, *B. linens* growth was inhibited. This correlates with literature, since *B. linens* is known to be an aerobic microorganism (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000). Despite this positive result it is not always practically possible for the industry to wrap the cheeses being time consuming and often costly. In addition, some wrappings, such as foil have the tendency to curl up and not make complete surface contact allowing *B. linens* to proliferate. Thus an alternative method needs to be found.

Table 6.1. The mean of results obtained from triplicate experiments carried out to determine the efficiency of the selected yeast species inhibition of *Brevibacterium linens* growth.

Inhibition by *B. linens*	**Rating per day**				**Test species**	**Inhibition of *B. linens***	**Rating per day**			
Positive or Negative	***3***	***5***	***7***	***9***		Positive or Negative	**3**	**5**	**7**	**9**
-	2	2	2	2	*D. hansenii*	-	1	1	1	1
Slight	3	3	3	2	*S. cerevisiae*	-	1	1	1	1
Slight	3	3	2	2	*T. delbrueckii*	-	1	1	1	1
Slight	3	3	2	2	*C. sake*	-	1	1	1	1
-	3	2	2	2	*C. intermedia*	-	1	1	1	1
-	2	2	1	2	*C. rugosa*	-	1	1	1	1
-	4	2	2	2	*C. zeylanoides*	-	1	1	1	1
-	3	2	1	1	*C. albidus*	-	1	1	1	1
-	1	1	1	1	*T. beigelii**	-	1	1	1	1
+/-	3	2	2	2	*R. glutinis*	-	1	1	1	1
Slight	3	3	3	3	*R. minuta*	-	1	1	1	1
Slight	3	2	2	2	*R. mucilaginosa*	-	1	1	1	1
+	4	4	4	4	*D. anomala*	-	1	1	1	1
+	4	4	3	3	*D. bruxellensis*	-	1	1	1	1
-	1	1	1	1	*Y. lipolytica*	-	1	1	1	1
Slight	5	3	3	3	*K. marxianus*	-	1	1	1	1
Slight	3	3	3	3	*K. lactis*	-	1	1	1	1
Slight	2	3	3	3	*Z. florentinus*	-	1	1	1	1
-	4	5	5	4	*Z. mellis*	-	1	1	1	1
+	5	5	5	5	*Z. rouxii*	-	1	1	1	1
Slight	3	3	3	3	*P. haplophila*	-	1	1	1	1

**Trichosporon cutaneum* var. *cutaneum*

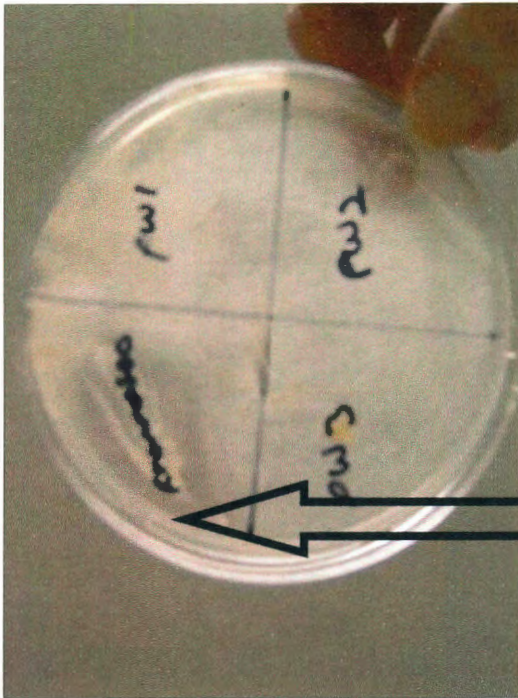
5 – Complete inhibition; 4 – Notable inhibition; 3 – Average inhibition;

2 – Slight inhibition; 1 – No inhibition.

Table 6.2. The mean of results obtained from triplicate experiments carried out to determine the efficiency of the selected species or samples on the inhibition of *Brevibacterium linens* growth.

Test species/ sample	Inhibition of *Brevibacterium linens*	Rating per day					Comment
		1	3	5	7	9	
Foil	+	5	5	4	4	3	*B. linens* began to grow underneath the foil after 5 days
Parafilm®	+	5	5	5	5	5	Very effective in inhibiting *B. linens*. No growth in contact area
Plastic	+	5	5	4	4	3	Effective, plastic began to lift after day 7
Olive oil	-	1	1	1	1	1	Did not inhibit *B. linens* at all
Lactobacillus rhamnosus	+	5	5	4	4	3	Initial complete inhibition
Bifidobacterium lactis	+	5	5	5	5	5	Complete inhibition of *B. linens* for first two weeks
Penicillium candidum	-	1	1	1	1	1	Did not inhibit *B. linens* growth
Penicillium camemberti	-	1	1	1	1	1	Did not inhibit *B. linens* growth
Penicillium caseicolum	-	1	1	1	1	1	Did not inhibit *B. linens* growth

5 – Complete inhibition; 4 – Notable inhibition; 3 – Average inhibition; 2 – Slight inhibition; 1 – No inhibition.



The lighter zone indicates the inhibition of the *Brevibacterium linens* by the *Lactobacillus rhamnosus*.

Fig. 6.1. An agar plate clearly indicating the inhibition of *B. linens* by *L. rhamnosus* after 24 hours. PW 1, 2 and 3 indicates three *Penicillium* strains that tested negative for *B. linens* inhibition.

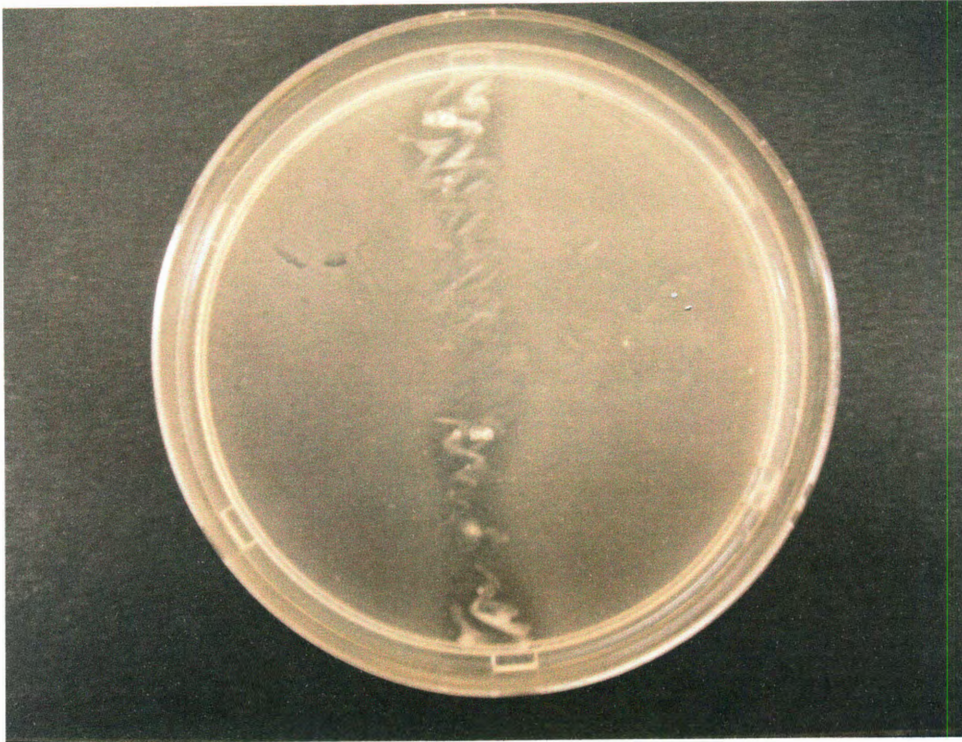


Fig. 6.2. An agar plate indicating the inhibition of *Brevibacterium linens* by *Lactobacillus rhamnosus* after three weeks of growth.

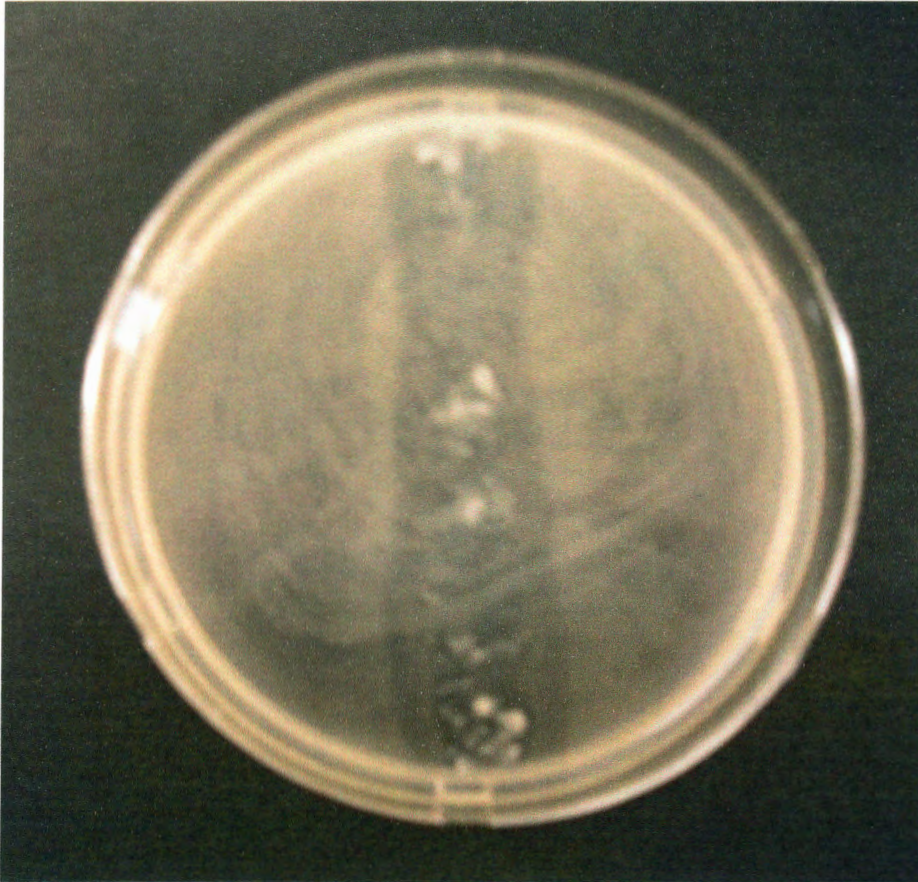


Fig. 6.3. An agar plate clearly indicating the inhibition of *Brevibacterium linens* by *Bifidobacterium lactis* after three weeks of growth.

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Chapter 7

General discussion and conclusions

Mould or surface ripened cheeses are characterised by complex bacterial flora present on the surface of these cheeses. This flora consists primarily of yeasts and bacteria (Kammerlehner, 1995). These aerobic microorganisms have a direct impact on the flavour, texture, and appearance development of these mould-ripened cheeses. Various 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).

Yeasts were previously considered as contaminants in dairy products due to spoilage during the fermentation process and were recognised as a potential problem in cheese (Fleet, 1990; 1992). Several studies reported yeast numbers as high as 10^6 cfu/g in blue-mould cheeses with both positive and negative effects on the final product (Fleet and Mian, 1987; van den Tempel and Jakobsen, 1996). In addition, yeasts have been reported to assist with *Penicillium roqueforti* development as well as facilitating secondary microflora development such as *Brevibacterium linens* (Karahadian *et al.*, 1985; Addis *et al.*, 2001), while influencing the development of specific fruity odours.

B. linens is an important dairy associated species rendering a unique taste and aroma to numerous smear ripened and blue-mould cheeses due, primarily, to proteolysis. The orange-reddish colour in conjunction with the aroma, primarily ammonium, is essential to the production of many European smear-ripened cheeses, including Limburger, Münster, Brick, Tilsiter and Appenzeller (Rattray and Fox, 1999). Despite the beneficial effects, the South African consumer in general demands cheese void of *B. linens*, as they do not prefer the strong taste created or the off colour (see Chapter 1, Section 11.1). Consequently, large portions of the blue-mould cheese surfaces must be cut off prior to packaging to eliminate the surface flora, resulting in huge financial losses for the dairy industry. Hence, we endeavoured to find a natural means of inhibiting the *B. linens* growth both during the production and maturation of South African blue-mould cheese. Accordingly, numerous methods including microbial inhibition studies using yeasts and probiotic cultures with possible inhibitory effects were applied in an attempt to inhibit the species.

1. An inter-laboratory evaluation of selective media for the detection and enumeration of yeasts from blue-mould cheese.

The initial step of this investigation involved the identification of a medium suitable for the selection and enumeration of yeasts in the presence of moulds. This was imperative since various yeasts had been reported not only to stimulate the growth of lactic acid bacteria, *B. linens* and moulds in cheeses (Purko *et al.*, 1951; Devoyod *et al.*, 1969; Devoyod and Sponem, 1970; Leclercq-Perlat *et al.*, 2000a), but also contribute significantly to the final product, as they form an integral part of the microflora of blue-mould cheeses and positively contribute towards ripening through the production of aroma compounds (Hanssen *et al.*, 1984; Martin *et al.*, 1999), flavour formation through lipolytic and proteolytic activity (Coghill, 1979; Wyder and Puhan, 1999a and b), the excretion of growth factors (Jakobsen and Narvhus, 1996) and assist with *P. roqueforti* development.

This inter-laboratory study involved five countries represented as laboratories 1 to 5. Each evaluated eleven different selective media, designed to suppress mould and bacterial growth and support yeasts growth, for the recovery of yeast populations from blue veined cheeses. In addition, qualitative results were also incorporated. The yeast enumeration values were subjected to statistical analysis using analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test (NCSS, 2001).

With the exception of Laboratory 3, none of the other laboratories were successful in recovering yeasts on all the media. No significant differences in quantitative data obtained on Rose-Bengal Chloramphenicol Agar (RBCA), Dichloran Rose-Bengal Chloramphenicol Agar (DRBC), Dichloran 18% Glycerol Agar (DG18), and Molybdate agar with 10% Sodium Propionate (MES) were detected by four of the collaborating laboratories whereas one laboratory found RBCA to be superior for yeast enumeration. DG18 and Malt Extract Agar with Biphenyl (MEB), however, were ranked superior based on qualitative results compared to the other media, attributed to distinctive

individual yeast colonies and mould inhibition. Since RBCA was readily available and reasonably priced this medium was selected for further use in yeast analyses in the presence of moulds.

2. Development of yeast populations during the processing and ripening of blue-mould cheese.

Considering the importance of yeasts in terms of cheese development an overall profile of the yeast population was constructed during the production and maturation processes. Blue-mould cheeses were analysed at regular intervals during manufacturing and ripening to determine the origin of contaminating yeasts present, their population diversity and development until end of storage. Yeast diversity and development in the inner and outer core of the cheeses during maturation were also compared.

Air samples revealed few if any yeasts, whereas contact samples from equipment and the surroundings revealed high numbers of yeasts suggesting that this was the main source of post-pasteurization contamination, as very few yeasts were isolated from the milk and cheese making process itself. This corresponded with results obtained by Welthagen and Viljoen (1998). Samples from the inner and outer core of the maturing cheeses had typical growth curves. The number of yeasts on the outer core was about 100 fold more than those in the inner core.

The most dominant yeasts isolated included *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida versatilis* and *Cryptococcus albidus*, while the yeasts *Candida antillancae* and *Dekkera anomala* were isolated from the environment. Yeasts were present in high numbers, making their occurrence in blue-mould cheeses significant.

3. Microbial interactions during the production and maturation of South African blue-mould cheese.

The next stage of the investigation was to evaluate the interaction between the various microflora present during the production and maturation of the blue-mould cheese. Samples taken were analysed for lactic acid bacteria, coliforms, *B. linens*, yeasts and total counts. In addition, the changes in the pH values, the organic acids and the sugars were determined during the ripening period.

Since no yeasts were enumerated from the pasteurised milk it was evident that these were present as contaminants from the environment. The growth of yeasts and the lactic acid bacteria, not only supported each other, but the yeasts reduced the pH within the cheese via lactic acid utilisation and may produce vitamins and growth factors, all of which promoted the growth of *B. linens*.

Lactic acid bacteria were absent in the pasteurised milk, but after starter addition, rapidly increased and thereafter the counts remained relatively stable for both the interior and surface samples throughout the ripening processes. Interestingly, the lactic acid bacteria counts on the outer surface were higher than in the inner core. The yeasts counts remained low or absent during processing and only increased from the time ripening commenced. The levels of yeasts were significantly higher on the surface than in the inner core, while the high concentrations of NaCl present in the brine had little or no influence on the survival of the yeasts. The increased yeast numbers resulted in the production of additional growth factors and vitamins (Purko *et al.*, 1951; Lenoir, 1984; Busse, 1989; Valdés-Stauber *et al.*, 1997), which not only supported the growth of the lactic acid bacteria but also the growth of *B. linens* (Figs. 4.2 and 4.3) (Purko *et al.*, 1951; Devoyod *et al.*, 1969; Devoyod and Sponem, 1970; Leclercq-Perlat *et al.*, 2000a). This stimulation of growth of *B linens*, further contributed to an enhancement in pigmentation causing an orange-reddish colouration on the cheese surface during ripening

(Valdés-Stauber *et al.*, 1997). The coliform counts were low or absent during the production process indicating sufficient levels of hygiene. The pH, sugars and organic acid profiles complimented and confirmed the growth and interaction profiles obtained for the microflora analysed.

4. Inhibition of *Brevibacterium linens* during the production and maturation of South African blue-mould cheese.

Although European cheese making favours the presence of *B. linens*, the South African consumers demand a product void of the species. Accordingly, various natural methods on the inhibition of *B. linens* were investigated. Initial surveys conducted throughout a South African cheese-making factory revealed that the *B. linens* proliferated on the wooden pallets used for the storage of the cheese during maturation. Treatment of the wooden pallets with the novel Radical water™ suggested that a 90 min period was the optimum time required for effective reduction of *B. linens.* The treatment of the whole cheeses with the Radical water™ yielded limited success, which was in part due to the short half life time of the Radical water™. Based on the results obtained, the usage of Radical water™ has been implemented in the cheese factory for treating the pallets.

Further investigation showed that the addition of *Debaryomyces hansenii* as a possible bio-control agent during the cheese making process did not reduce *B. linens* despite a reduction in maturation time as well as an improvement in the overall texture and the development of a stringent flavour. Since it is well known that *B. linens* is an aerobic bacterium (Toolens and Koning-Theune, 1970; Rattray and Fox, 1999; Bikash *et al.*, 2000; Leclercq-Perlat, 2000b) the possibility of limiting oxygen supply to the surface of the cheese during maturation was investigated. However, *Penicillium roqueforti,* used to create the traditionally aroma and appearance of the blue mould cheese, is a strict aerobe and complete removal of oxygen would have a detrimental effect on the final flavour and appearance of the cheese. The areas of contact with the plastic i.e. the anaerobic or reduced oxygen areas, showed a definite

reduction in *B. linens* growth, when compared with the non-contact areas. Despite this technique being effective it remains a time consuming and expensive means of controlling or reducing the *B. linens* growth.

5. Inhibition of *Brevibacterium linens* during the maturation and ripening of blue-mould cheese by means of bio-control.

Further investigations into reducing or inhibiting *B. linens* involved the usage of yeasts and probiotic cultures. However, none of the yeasts applied proved to be successful. In contrast, *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, two typical probiotic species applied in dairy products, showed strong inhibition of *B. linens* when tested using the 'spot-on-lawn' assay (Piddock, 1990; Hoover and Harlander, 1993). However, the *B. linens* appeared to show reduced sensitivity to *L. rhamnosus* after approximately one week of incubation, while *B. lactis* continued to inhibit *B. linens* well after three weeks of incubation. The initial inhibition may be attribute to the acid production by these probiotic strains. The diffusion of the acid into the agar on prolonged incubation could then allow the growth of *B. linens*. Alternatively the inhibition may be attributed to bacteriocin activity. *L. casei* var. *rhamnosus* is known to produce bacteriocins such as caseicin 80 and caseicin LHS, both heat lable bacteriocins (Rammelsberg and Radler, 1990 and Dicks *et al.*, 1992). Bacteriocins produced by gram-positive bacteria exhibit activity towards a wide range of gram-positive species (de Vuyst and Vandamme, 1994), hence the effect on *B. linens*.

The addition of *L. rhamnosus* and/or *B. lactis* is a strong possibility. The fact that *L. rhamnosus* and *B. lactis* are probiotics with added health benefits is a bonus and allows for ease of incorporation into foods (Salminen *et al.*, 1998; Fooks *et al.*, 1999; Saarela *et al.*, 2000; Ouwehand *et al.*, 2002).

Further investigation will need to focus on the application of the *Lactobacillus rhamnosus* and/or *Bifidobacterium lactis*, at plant scale level, into the blue-mould cheese and subsequent analysis thereof. In addition, the effect of

these probiotics on the microbial and chemical interactions with in the cheese as well as the effect on the final flavour, texture and aroma of the cheese will need to be evaluated. Gardiner *et al.* (1998) and Stanton *et al.* (1998) reported no adverse effects on sensory criteria of Cheddar cheese when probiotic bacteria like *Lactobacillus paracsei* were detected. Similarly Dinakar and Mistry (1994) reported no adverse effects in terms of the sensory criteria when *Bifidobacterium bifidum* was detected in Cheddar cheese, while Broome *et al.* (1990), McSweeney *et al.* (1994), Lynch *et al.* (1996) and Gardiner *et al.* (1999) all reported an improvment in the flavour of Cheddar cheese when probiotics were included as adjunct starter cultures. However, Gomes *et al.* (1995) reported that despite a similar texture and appearance to the control cheese, Gouda cheese containing *Bifidobacterium* sp. strain Bo and *L. acidophilus* strain Ki developed a bitter taste after 50 days. Bitter peptides usually develop in dairy products due to the formation of biogenic amines formed through the decarboxylation of the carboxyl group of an amino acid and the liberation of the corresponding amine (Joosten, 1988). In addition, the higher levels of lactic acid, due primarily to the high numbers of *L. acidophilus* can contributed to the over acidification 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 but still possessing the ability to reduce or inhibit *B. linens* either by means of bacteriocin production or another antagonistic effect.

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Chapter 8

Summary

From the extensive literature review given in Chapter 1, it was evident that blue-mould cheeses are unique in terms of the flavour, aroma, texture and microbial developments that take place during production and maturation. Despite surface mould-ripened cheeses only representing a small portion of the annual world cheese production, their popularity is increasing worldwide making it important to study their microbiological, biochemical and technological properties. Consequently, the aims of this study were to identify a medium suitable for the isolation and enumeration of yeasts in the presence of moulds, elicit the establishment, growth and survival of yeasts during the processing and ripening of blue-mould cheeses, to follow the interaction between the microbiota present and to determine their influence on each other. In addition, since the interactive development of the different microbiota also affects the final cheese product, the technological changes were determined. As *Brevibacterium linens* plays a substantial role on the quality of blue veined cheese, various methods were applied as a means of inhibiting the growth and development of the species.

The enumeration of yeasts in the presence of moulds proved to be difficult since the yeasts are easily overgrown by the rapid spreading of moulds. We therefore aimed to find a medium that not only supported the growth of the yeasts, but also inhibited mould growth to such an extent that the isolation and enumeration of the yeasts could be performed with relative ease. Ten different selective media with anti-mycotic properties were evaluated and compared statistically using analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test, for their ability to suppress the growth and spreading of moulds and to enhance the enumeration and recovery of yeasts from blue-mould cheeses. No significant differences in quantitative data obtained on Rose-Bengal Chloramphenicol Agar (RBCA), Dichloran Rose-Bengal Chloramphenicol Agar (DRBC), Dichloran 18% Glycerol Agar (DG18), and Molybdate agar with 10% Sodium Propionate (MES) were detected by four of the collaborating laboratories whereas one laboratory found RBCA to be superior for yeast enumeration. DG18 and Malt Extract Agar with Biphenyl (MEB), however, were ranked superior based on qualitative results compared

to the other media, attributed to distinctive individual yeast colonies and mould inhibition.

Yeasts are one of the main microbial groups that occur during the ripening of blue veined cheese varieties, originating as post-pasteurisation contaminants. In Chapter 3 the development of yeasts derived mainly from the dairy environment and their establishment during the ripening process was examined. Yeast species isolated and identified corresponds with those obtained by other researchers on similar cheese varieties. A 10 to 100 fold difference in yeast numbers were observed during the ripening period between the exterior and interior. *Debaryomyces hansenii* predominated in the present study. This was due to its ability to produce extracellular proteases and lipases and the species high tolerance towards low temperatures and high salt concentrations.

Although yeasts are one of the major microbial groups present in blue veined cheese other microorganisms like *Penicillium roqueforti* and several different bacterial genera are also frequently encountered. All of these organisms collectively contribute to the final flavour and aroma of the product based on interactive proliferation. Consequently it was necessary to study the interactions between these microorganisms to improve our understanding of the ripening process and the contribution each makes to the process. Studies revealed that various interactions occurred between yeasts and *P. roqueforti*, as well as yeasts and bacteria essential to the final outcome of the cheese product. The majority of yeast-bacterial interactions were neutral, although some interactions were antagonistic and others stimulative. *Debaryomyces hansenii* was the most stimulatory yeast species towards bacterial isolates while *Torulospora delbrueckii* was the most antagonistic. All three microbial groups studied dominated throughout the ripening period and were present in meaningful numbers at the end of the ripening period.

The use of Radical water™ to reduce or inhibit *B. linens* growth yielded limited results. A treatment time of 90 mm with Radical water™ for the wooden

pallets used for storage of the blue-mould cheese during maturation was determined to be optimal for inhibiting or reducing *B. linens* growth on these pallets and thus reducing the spread to the cheese. Despite this being a time consuming process, the treatment of the pallets with Radical water™ has since been implemented in the cheese factory. The addition of the yeast *D. hansenii* did not reduce the presence of *B. linens*, but did however improve the overall texture and flavour of the final cheese product. While covering the whole cheese with a layer of plastic to create an anoxic layer around the cheese, proved to be the most effective means of controlling *B. linens*, time constraints proved it not an economically viable option.

Further investigations based on the inhibition of *B. linens* included the application of yeasts and probiotics as possible bio-control agents. None of the dairy associated yeasts tested proved to be successful, whereas *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, two typical probiotic species applied in dairy products, showed inhibitory effects against *B. linens* when tested using the spot-on-lawn assay.

Key words: Blue-mould cheese, yeast development, interaction, inhibition, *Brevibacterium linens*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis.*