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Yeast diversity in blue mould ripened cheeses.

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by

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Man wonders over the restless sea, the flowing water, the sight of the sky, and forgets that of all wonders man himself is the most wonderful; St. Augustine, (quoted from Hoebel, 1949, 1.).

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CHAPTER 1

Literature review

1.1 INTRODUCTION

The occurrence of yeasts in dairy products, like cheese, is not unexpected as products like these have a high acidity, low moisture content and high salt concentration, all of which contribute to a competitive advantage and proliferation of the species (Fleet, 1990). The role of yeasts in cheese is not well known nor understood and consequently not recognised as a major component of the microflora of cheese nor its contribution to the ripening and flavour development of cheeses (Fleet and Mian, 1987).

The presence of yeasts in dairy products is important, because they can either cause spoilage, or contribute to biochemical changes that are desirable, and to the extreme, have an effect on public health (Fleet and Mian, 1987). The desirability of the presence of yeasts in dairy products like cheese, depends mainly on the type of cheese. The presence of yeasts may be extremely desirable in blue veined cheese or Camembert for example, where the growth of the yeast contributes to the ripening and flavour formation of the cheese, while (Jakobsen and Narvhus, 1996) their presence might however not be as desirable in Cheddar and Swiss cheeses. Excessive growth of yeasts during ripening in these types of cheese is a potential source of spoilage (Beresford et al., 2001).

Blue veined cheese is manufactured in many countries around the world, each having its own specific procedure and ingredients, which add to the diversity of the cheeses. Further adding to the diversity is the fact that milk from different animals are used to produce the cheese resulting in variation in the end product (Gripon, 1987). A very broad definition for the blue veined cheeses would be, a cheese characterised in appearance and flavour by the growth and development of the fungal-species, *Penicillium roqueforti*, either naturally or through inoculation, that has a 4-5 % salt content, possesses a spicy, piquant flavour and a moist texture showing slight stickiness with a tendency to crumble (Kosikowski, 1970). According to the International Dairy Federation (IDF) blue veined cheese varieties are classified under two groups of cheeses, namely semi-hard and semi-soft and soft cheese.

Some of the most famous blue veined cheese varieties include, Roquefort from France, which is made with unpasteurised ewe's milk, Stilton from Great Britain and Gorgonzola from Italy, the latter made with pasteurised cow milk. The most important blue veined cheese varieties in terms of South African blue veined cheeses, are Danablu style and Gorgonzola style. The cheese manufactured in South Africa closest resemble Danablu in many regards, which include the use of pasteurised cows milk, the forming of blue-green mould veins in the interior of the cheese and the lack of rind formation.

The mould associated with the cheese gives it a distinct appearance, compared to other cheeses, and the presence of the mould also leads to a more complex ripening than in other cheeses with a simpler microflora (Gripon, 1987). Moulds are however not the only organisms responsible for the development of flavour during ripening, although they are the most important. The millions of bacteria trapped in the inside of the cheese as well as the bacteria and yeasts on the surface of the cheese, also contribute to the flavour and the ripening of blue veined cheese varieties (Kosikowski, 1970). The contribution of yeasts to the ripening and flavour development of blue veined cheese varieties is still relatively unknown but it is becoming increasingly evident that yeasts play an important role in the ripening of these cheeses.

The main objective of this study was to study the diversity of yeasts associated with the production and maturation of South African produced blue veined cheese. This was done for two reasons. Firstly, not much is known about the diversity of yeasts associated with South African produced blue veined cheese. Secondly, although an appreciable amount of literature exists regarding the diversity of yeasts associated with blue veined cheese varieties, much is still unknown and needs to be studied. In order to study the diversity and the establishment, growth and survival of yeasts during the ripening of blue veined cheese varieties, media optimisation was performed prior to commencement of these studies. The possible interactions between yeasts and the different microbial groups which occur during the ripening process was also studied.

1.2 MANUFACTURE OF BLUE VEINED CHEESE VARIETIES.

The diversity in the varieties of blue veined cheeses produced all over the world is reflected based on the different ways in which these cheeses are manufactured and ripened. Despite the differences, all blue veined cheese varieties have the same basic steps in common during the production (Fig. 1). The difference in production is mainly attributed to two factors, namely the variety of cheese produced and the scale of production.

1.2.1 Preparation of milk

Whole milk from cows is separated into cream and skim milk fractions. The skim milk fraction is pasteurised, cooled to about 30°C and pumped into a cheese vat. Milk in its raw state, without being pasteurised, may also be used for manufacture, which is similar to Roquefort-cheese (Vivier et al., 1994).

The cream fraction may optionally be bleached by adding benzoyl peroxide (0.002% v/m). This is done to assure a white colour in the finished cheese's body, forming a contrast with the blue veining caused by the mould, and is mostly done for aesthetic reasons. The cream is pasteurised and homogenised. The homogenisation of the cream serves to increase the surface area of the milk fat globules, and thus helps to facilitate lipolysis during ripening which contributes to the ripening and flavour formation of the cheese (Nichol, 2000). The treated cream is then added to the pasteurised skim milk in the cheese vat.

1.2.2 Acid development

The acid development during the processing of the cheese is facilitated through the addition of starter cultures to the milk in the vat. Starter cultures are defined as bacteria selected for their ability to produce lactic acid as a result of lactose fermentation and for their contribution towards flavour development by the production of volatile compounds and desirable proteolytic and lipolytic activity (Farkye, 2000).

These cultures primarily consist of lactic acid bacteria (LAB), although other types of bacteria can also be included. Lactic acid bacteria can broadly be divided into two groups, namely the mesophiles and thermophiles. The mesophilic group consists of members of the genera *Lactococcus* and *Leuconostoc*, while the thermophilic group consists of members of the genera *Streptococcus* and *Lactobacillus* (Farkye, 2000).

During the production of blue veined cheese varieties, 0.3 – 2.0% of active mesophilic and thermophilic lactic starter cultures are added to the milk in the cheese vat. The milk is kept at 30°C for 1h allowing the starter culture to produce, primarily, lactic acid resulting in a decline in the pH of the milk (Nichol, 2000).

1.2.3 Addition of coagulant and additives

After 1hr of acidification of the milk, a suitable enzymatic coagulant, like rennet, which consists mainly of chymosin is added to the milk. If necessary, calcium chloride is added before the addition of the coagulant to aid in the formation of the coagulum. The rennet dosage applied usually consists of 30ml per 100g of milk or 0.03% (v/v). The addition of rennet results in the coagulation of milk and a curd is formed within 30 to 75 min after adding the coagulant, while kept at 30°C (Pernodet, 1986).

The enzymatic coagulation of the milk is a two-phase process. During the primary phase the casein micelles are destabilised by the hydrolysis of k-casein at the Phe105-Met106 bond.

Subsequently these destabilised casein micelles undergo aggregation during the second stage to form a gel network in which fat globules are entrapped (Brulé and Lenoir, 1986).

1.2.4 Cutting the curd

When the curd reaches a desirable firmness it is cut into cubes with a size of about 10-20mm with wire knives. This allows the drainage of whey from the curd (synergism) during the next stage of manufacture.

1.2.5 Stirring of curd

During this stage syneresis of whey from the curd takes place. The cubes remain in the whey to allow additional acid development, to aid syneresis. The curd is usually held at 30°C until the titratable acidity of the whey reaches 0.03%, usually within an hour. The curd is also stirred gently every 5 min to aid syneresis. Control of syneresis is very important to control the moisture content of the finished product. For blue veined cheese varieties syneresis is limited to achieve the desired high moisture content.

1.2.6 Draining of whey

Immediately before draining the whey, the temperature of the curd in the milkvat is raised to 33°C and maintained for about two minutes. All the whey is drained and the curd is gently trenched.

1.2.7 Mould inoculation

An inoculum mixture, consisting of salt and *Penicillium roqueforti* spore powder, is added to the trenched curd and mixed either mechanically, or manually for five minutes.

1.2.8 Moulding and pressing

During the moulding stage the treated curd is placed into perforated, circular stainless steel hoops or moulds specifically designed for blue veined cheese production. The moulds are open-ended and rest on drainage mats.

The next stage of production is pressing. Characteristic for blue veined cheese is the fact that it is self-pressed, meaning the curd is fused by its own weight and no mechanical pressure is applied. The temperature and humidity of the pressing room must be kept at 20-25°C and 90-95% RH, respectively during pressing. The moulds are turned every 15 minutes for the first 2 hours and are then allowed to drain overnight in the pressing room.

1.2.9 Salting

The following day the curd is removed from the moulds and salting takes place. Salting can be performed either by dry salting or by brine salting. During dry-salting, salt is rubbed onto the surface of the curd, whereas during brine salting, the curd is immersed in a brine solution with a concentration of 18% (w/v). During the salting-stage the cheese is stored at 16°C and 85% RH.

1.2.10 Piercing

After salting is completed, all flat surfaces of the cheese are pierced by machine, leaving about 50-55 holes per side of the flat surface of the cheese. The pierced holes encourage the growth of *Penicillium roqueforti* throughout the body of the cheese and facilitate the escape of CO_2 that is produced.

1.2.11 First Ripening

The pierced cheese is then stored at 10-13°C and 95% RH for a period ranging from about one month up to four months. It may be turned once during this period to facilitate the uniform development of the mould throughout the cheese body.

1.2.12 Second Ripening

After the first ripening period the surface of the cheese is cleaned and the cheese wrapped in plastic bags. The cheese is stored at 2 - 4°C until ready for distribution. Final ripening takes place during distribution.

1.3 THE OCCURRENCE OF MICROORGANISMS DURING PROCESSING AND RIPENING OF BLUE VEINED CHEESE VARIETIES.

All the microorganisms found on the surface and interior of the cheese are collectively referred to as the microflora of the cheese. The microflora can be divided into two groups, namely the primary microflora and the secondary microflora. The primary microflora comprises those microorganisms added during the cheese-making process for specific purposes, like the lactic acid starter bacteria and mould. The secondary microflora includes microorganisms that develop spontaneous on and in the cheese. This group is not added during the cheese-making process but rather develop through natural contamination from the environment, surfaces and processes (Beresford et al., 2001). A difference between the interior and the exterior of the cheese exists with regards to the composition and number of microorganisms. Yeasts, micrococci and coryneform bacteria occur at higher concentrations on the exterior of the cheese than in the interior. This can be explained by the fact that the available oxygen is higher on the exterior than in the interior of the cheese and yeasts, micrococci and corynebacteria proliferate under aerobic conditions (Choisy et al., 1986). Furthermore, the exterior of the cheese is more exposed to environmental influences making it prone to microbial contamination (van den Tempel and Jakobsen, 1998).

1.3.1 Primary microflora present in blue veined cheese varieties.

1.3.1.1 Lactic acid starter bacteria

Lactic acid bacteria (LAB) applied in the dairy industry can broadly be divided into two groups, namely mesophiles and thermophiles (Stanley, 1998).

Mesophilic LAB consists of *Lactococcus* and *Leuconostoc* species. These are applied during the cheese fermentation process which has process-temperatures ranging from 20-40^{o}C. Thermophilic LAB are used in fermentation processes which have process-temperatures of 30-50^{o}C. This group mainly includes *Streptococcus thermophilus*; *Lactobacillus delbrueckii* subsp. *bulgaricus*; *Lactobacillus delbrueckii* subsp. *lactis*;

Lactobacillus delbrueckii subsp. *delbrueckii* and *Lactobacillus helviticus* (Stanley, 1998; Farkye, 2000). The main function of lactic acid bacteria is to metabolise the disaccharide, lactose present in milk to lactic acid. The lactic acid then accumulates in the milk and consequently lowers the pH. The reduced pH is necessary for the consecutive stages in the cheese-making process (Kosikowski, 1970).

The main species of mesophilic lactic acid bacteria of importance to the dairy industry is *Lactococcus lactis*. The species can be divided into three subspecies, namely *Lactococcus lactis* subsp. *lactis* , *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis*. *Lactococcus lactis* subsp. c*remoris* has been preferred over *Lactococcus lactis* subsp. *lactis* in starter cultures because of the association of the latter with unwanted flavour production (Stanley, 1998). *Lactococcus lactis* subsp *lactis* is also characterised by its high production of lactic acid. This mainly contributes to its preferred use as a starter organism. *Lactococcus lactis* subsp. *diacetylactis* being heterofermentative, is characterised by its ability to metabolise citrate, present in milk, to diacetyl and carbon dioxide (Farkye, 2000). A combination of the above bacteria is usually applied as a cheese starter culture.

Leuconostocs comprise the other group of the mesophilic LAB and they are often used in conjunction with lactococci to enhance flavour production (Choisy et al., 1986). *Leuconostoc mesenteroides* subsp. *cremoris* is the main strain used in the dairy industry, while *Leuconostoc lactis* is used less frequently.

Leuconostoc mesenteroides subsp. *cremoris* produces high levels of CO_2 and is often employed in the production of blue veined cheese to promote open texture which allows for the better penetration and growth of the blue mould in the cheese (Stanley, 1998).

The thermophilic LAB used most commonly as starters comprise *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Even though they can be used for the production of cheese they are seldom applied because of their higher optimum temperature (Farkye, 2000). They are more often employed in the production of yoghurt where the production process favours their use.

Lactic acid bacteria play a more important role during the manufacture process of blue veined cheese varieties than in the actual ripening, when compared to the other organisms. This is mainly due to the fact that they die off within 2 to 3 weeks after manufacture because of the high acidity and salt concentration (Marth and Yousef, 1991). Their precise contribution has not yet been established (Stanley, 1998). It has been suggested that lactic acid bacteria contribute to the ripening of cheese either directly on indirectly in three ways: (i) they help to provide a suitable environment for enzymatic and non-enzymatic reactions to take place; (ii) through their lactose and citrate metabolism they produce molecules that contribute to the flavour and (iii) lactic acid bacteria trapped in the interior of the cheese die off and lyse, releasing proteolytic enzymes into the interior. These enzymes break down the milk protein to release peptides, amino acids and volatile flavour compounds (Coghill, 1979).

1.3.1.2 Moulds

Penicillium roqueforti is the mould used in the production of blue veined cheese varieties and is primarily responsible for the ripening and characteristic flavour and taste of blue veined cheese varieties (Beresford et al., 2001).
Although different strains are used in the dairy industry they all exhibit good lipolytic and proteolytic activity and vary in colour from dark blue to light green (Nichol, 2000).

This species is able to grow at low ripening temperatures (4-10^{o}C), can tolerate wide pH values (pH 4-6) as well as salt levels and grows well at low oxygen levels (Stanley, 1998). These characteristics contribute to its growth and spread through the open structure of the cheese during the ripening period. The two most important biochemical reactions that takes place during cheese ripening is lipolysis and proteolysis (Coghill, 1979). Both reactions occur more extensively in blue veined cheese than in other cheese varieties (Gripon, 1987). Lipolysis is essential for the proper flavour development of blue veined cheese. Coghill (1979) stated that the most important lipase enzymes in the ripening of blue veined cheeses are those derived from the mould *Penicillium roqueforti*, although lipases from other organisms present in the cheese also contribute to the process. Both extra- and intra-cellular lipases have been found in *Penicillium roqueforti*, contributing to the degradation of lipids to free fatty acids (Coghill, 1979). The accumulated fatty acids are then metabolised via the fatty acid β-oxidation

pathway to methyl ketones and other by-products, including secondary alcohols, methyl- and ethyl esters (Coghill, 1979). The methyl ketones produced by *Penicillium roqueforti* represent the major flavour component of blue veined cheese and are responsible for the characteristic taste of the cheese variety while the other by-products also contribute to the flavour profile (Stanley, 1998).

Another major biochemical reaction that takes place during ripening, namely proteolysis, occurs extensively in blue veined cheese. Sources of proteolytic enzymes include the microflora of the cheese. These enzymes are either excreted actively by the microorganisms or are released during the lysis of them (Farkye, 2000; Nichol, 2000). Proteolysis is essential for the development of the characteristic texture of blue veined cheese, which is soft, smooth and full flavour (Stanley, 1998).
Proteolysis not only contributes to the texture of the cheese during ripening but also to the flavour and aroma. Peptides and amino acids formed during proteolysis serve as precursors to flavour compounds like aldehydes, alcohols and esters (Coghill, 1979).

1.3.2 Secondary microflora present in blue veined cheese varieties.

A wide range of microorganisms which are not added as starter cultures, develop on the surface and in the matrix of ripening cheese. The type and concentration of these microorganisms depend on the type of cheese and the technologies applied during the manufacture of the cheese. These organisms are referred to as secondary microflora, comprising mainly yeasts, non-starter bacteria and moulds.

1.3.2.1 Yeasts associated with blue veined cheese varieties.

Fleet and Mian (1987) conducted a survey on the occurrence and growth of yeasts in all the major dairy products including milk, cream, butter, yoghurt, ice-cream and cheese. In this survey it was found that yoghurt and cheese had the highest yeast counts, often exceeding 10^4 cfu.ml^{-1} or cfu.g^{-1} of products (Fleet and Mian, 1987*)*. The high incidence of yeasts was mainly attributed to the low pH levels of these fermented products, storage at refrigeration temperatures and in the case of cheese high salt- and low

moisture contents, therefore creating a selective environment for the growth of yeasts (Fleet, 1990).

Yeasts occur without exception in almost all types of cheese, ranging from the very hard types like Parmesan to soft cheeses like Feta and Cottage cheese and may occur in very high numbers (Welthagen and Viljoen, 1998a). Yeast counts in different cheeses will vary, which is to be expected if one considers that different types of cheeses select for the occurrence and growth of different yeast species (Welthagen and Viljoen, 1999). Fleet and Mian (1987) found that of the 23 samples of Australian cheddar cheese, 48% had yeast counts in the range of 10^4 – 10^6 cells.g^{-1} and of the 19 samples of Cottage cheese, 37% had yeast counts in the range of 10^5-10^7 cells.g^{-1}. Viljoen and Greyling (1995) found that the curd and whey from Gouda cheese also had high numbers of yeast, 11.4% of the 35 samples had yeast counts higher than 10^6 cfu.g^{-1}, 14% of the samples had counts in the range of 10^5 – 10^6 cfu.g^{-1}, 17.14% of the samples had counts in the range of 10^3 – 10^4 and 57.18% of the samples had yeast populations less than 10^3 cfu.g^{-1}.

Roostita and Fleet (1996) found that of 85 Camembert samples studied, 54% had yeast populations higher than 10^6 cfu.g^{-1}. Nooitgedacht and Hartog (1988) also revealed 62% of their samples of Camembert had yeast populations exceeding counts of 10^6 cfu.g^{-1}. Similar findings were reported on Brie cheese (Nooitgedacht and Hartog, 1988).

1.3.2.1.1 Diversity of the yeasts present in cheese.

It is impossible to ascribe a general composition of the yeast flora associated with any given cheese, as each type of cheese has its own specific characteristics which cause a selective effect on the growth of yeasts in the cheese. Therefore the species present are just as varied and diverse as the specific variety of cheese (Marth and Yousef, 1991). It is important to remember that yeasts originate in cheese as contaminants of the cheese making process and that they are not usually added as starter cultures. The main sources of yeast contaminating cheese include the environment, the process equipment, starter culture, rennet, salt and added fungal cultures. Consequently the yeast flora can vary within each lot of the same type of cheese attributed to varying environmental factors (Fleet, 1990; Marth and Yousef, 1991).

Welthagen and Viljoen (1998a) found that the various yeast strains isolated from 67 cheeses represented both ascomycetous and basidiomycetous yeasts and this is the case for most cheeses, including hard-, semi-hard and soft-cheeses. Fleet and Mian (1987) showed that fermented products had the highest counts of *Candida famata, Kluyveromyces marxianus, Candida diffluens* and *Saccharomyces cerevisiae*. In South African Cheddar cheese *Debaryomyces hansenii* is the most frequently occurring yeast species with *Saccharomyces cerevisiae, Yarrowia lipolytica* and *Kluyveromyces marxianus* also being isolated consistently (Welthagen and Viljoen, 1998b).

Viljoen and Greyling (1995) indicated that South African Gouda mainly comprises species of *Debaryomyces hansenii, Cryptococcus albidus* and *Trichosporon beigelii*. Other yeast species isolated less frequently, include *Candida diffluens*, *Kluyveromyces marxianus* and *Yarrowia lipolytica*.

Nooitgedagt and Hartog (1988) revealed that Brie and Camembert primarily include species of *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces lactis* and *Candida* spp., whereas Roostita and Fleet (1996) found *Debaryomyces hansenii, Candida lipolytica, Candida kefir, Candida catenulata* and the asporogenous form of *Debaryomyces hansenii*, *Candida famata*, to predominate in Australian Camembert.

From the above surveys it can be seen that the yeast flora of different cheeses vary greatly with respect to their composition. However, based on these studies on the diversity of yeasts, some yeast species are isolated consistently irrespective of the cheese type. This is most likely because cheese, irrespective of the type, supplies a universal growth environment which encourages the growth of yeast (Choisy et al., 1986).

Blue veined cheeses show a high incidence of yeasts. In a survey of blue veined cheeses by de Boer and Kuik (1987), 87% and 77% of Gorgonzola- and Roquefort-cheese samples respectively had yeast populations exceeding 10^6 cfu.g^{-1} while some samples had counts in the range of 10^7 – 10^8 cfu.g^{-1}. Australian blue veined cheese frequently showed yeast populations exceeding 10^6 cfu.g^{-1} (Roostita and Fleet, 1996).

In a survey of blue veined cheese by de Boer and Kuik (1987), *Debaryomyces hansenii* was found to be the most frequently occurring species, with *Kluyveromyces marxianus, Saccharomyces cerevisiae, Yarrowia lipolytica* and *Candida* spp. also being present. Roostita and Fleet (1996) also found *Debaryomyces hansenii* to be the most frequently occurring species with *Candida catenulata, Cryptococcus albidus, Candida lipolytica* and *Kluyveromyces marxianus* being present on a regular basis. Van den Tempel and Jakobsen (1998) isolated the yeasts associated with Danablu revealing that the species occurring most frequently were *Candida famata, Candida catenulata, Candida lipolytica, Zygosaccharomyces spp.* and *Trichosporon cutaneum*. Besançon et al. (1992) found the yeast flora associated with the surface of Roquefort cheese to be representatives of *Dabaryomyces hansenii, Candida famata, Kluyveromyces lactis, Candida sphaerica* and other *Candida* species.

Eliskases-Lechner (1998) found 100% of samples (12) of blue veined cheese to contain yeasts present at levels higher than 10^5 cfu.g^{-1} on the exterior and 83% of samples had yeasts present at levels higher than 10^5 cfu.g^{-1} in the interior. The most frequently occurring yeast species in these samples were *Debaryomyces hansenii*, *Kluyveromyces marxianus* and *Yarrowia lipolytica* (Eliskases-Lechner, 1998).

Wojtatowicz et al. (2001) analysed Rokpol cheese (typical polish blue veined cheese) and found yeast populations ranging between 10^5 – 10^9 cfu.g^{-1}. Yeast populations on the exterior generally exceeded 10^7 cfu.g^{-1}, while those in the interior were 10 to a hundred times lower (Wojtatowicz et al., 2001). The yeast species found to occur most frequently in Rokpol cheese were, *Candida famata* (asporogenous form of *Debaryomyces hansenii*) and *Candida sphaerica* (asporogenous form of *Kluyveromyces marxianus* ssp. *lactis*). Other yeast species detected less frequently on Rokpol were, *Candida intermedia*; *Saccharomyces kluyveri*; *Candida kefyr* and *Candida lypolytica*. Minervini et al. (2001) analysed Gorgonzola-style cheese and found all samples to contain a mean yeast count exceeding 10^5 cfu.g^{-1}. Again, *Candida famata* was found to be the most frequently occurring yeast species. This species usually occurred in mixed culture with *Candida inconspicua* and *Candida zeylanoides* (Minervini et al., 2001).

From the above surveys of blue veined cheese types it is clear that certain species of yeasts are consistently isolated from blue veined cheeses irrespective of the type,

representing the basic yeast flora. This basic yeast flora consists of *Debaryomyces hansenii*, *Kluyveromyces marxianus, Kluyveromyces lactis, Yarrowia lipolytica* and several *Candida spp*, including *Candida famata, Candida sphaerica, Candida lipolytica* and *Candida catenulata* (de Boer and Kuik, 1987; Besancon et al., 1992; Roostita and Fleet, 1996; Eliskases-Lechner, 1998; van den Tempel and Jakobsen, 1998; Minervini et al., 2001; Wojtatowicz et al., 2001).

Debaryomyces hansenii occurs the most frequently in blue veined cheese, and other types of dairy products (Fleet, 1990). Several factors contribute to its frequent isolation, like its ability to grow at low a_w and low temperatures, its lipolytic and proteolytic activity, and its high salt tolerance (Welthagen and Viljoen, 1998b). Blue veined cheese is ripened at low storage temperatures, has a high fat and protein content and also has a high salt concentration. These factors encourage the growth and proliferation of *Debaryomyces hansenii. Debaryomyces hansenii* is responsible for the formation of slime on the surface and it has been found that its presence prolongs the survival of the lactic acid bacteria (Wyder, 1998). Furthermore, *Debaryomyces hansenii* utilises both lactic- and acetic acid aerobically and anaerobically, contributing to the de-acidification of the surface of the cheese (Wyder, 1998).

The ability of *Kluyveromyces marxianus* to assimilate and ferment lactose, the primary monosaccharide present in cheese, is the key factor contributing to its frequent occurrence in cheese (Roostita and Fleet, 1996). Other factors of significance in determining the occurrence of *Kluyveromyces marxianus* in blue veined cheese include weak utilisation of lactic and citric acid, and of proteins and fats (Roostita and Fleet, 1996).

Kluyveromyces marxianus contributes to the structure and flavour of blue veined cheese varieties by fermenting lactose, resulting in the formation of carbon dioxide gas creating the open structure of blue veined cheese (Wyder, 1998). During the fermentation of lactose the species also produces flavour compounds contributing to the flavour development in blue veined cheeses (Wyder, 1998).

Yarrowia lipolytica possesses strong extracellular lipolytic and proteolytic activities which are the primary factors that contribute to its frequent occurrence in blue veined

cheese (Welthagen and Viljoen, 1999). Other factors encouraging its prevalence in blue veined cheese include its strict aerobic nature, utilisation of lactic acid and the ability to grow at ripening temperatures (Wyder, 1998).

Saccharomyces cerevisiae is frequently isolated from blue veined cheese, usually at low numbers, despite its inability to survive in cheese with a high salt-concentration, as is the case for most blue veined cheeses (Roostita and Fleet, 1996; Welthagen and Viljoen, 1999). The reason for its presence remains unknown. The occurrence, growth and survival has most likely to do with its ability to utilise protein and the products of fat breakdown from other species (Roostita and Fleet, 1996). *Saccharomyces cerevisiae* lacks the ability to utilise lactose and citric acid (Welthagen and Viljoen, 1999) and only weakly utilises lactic acid, with no lipase or protease activity (Welthagen and Viljoen, 1999).

1.3.2.1.2 Yeasts contribution to cheese ripening

There is no doubt that yeasts contribute to the ripening of blue veined cheese. The exact nature of the contribution still has to be elicited, although much is already known (Choisy et al., 1986). Yeasts produce enzymes which positively contribute to the texture, flavour and aroma of the cheese (Coghill, 1979).

Yeast species of the genera *Candida*, *Debaryomyces, Rhodotorula* and *Yarrowia* contain intracellular lipolytic activity causing the liberation of fatty acids. These fatty acids are further metabolised and contribute to the flavour and aroma (Coghill, 1979). *Yarrowia lipolytica* is commonly recognised as the species with the greatest lipolytic activity and it contributes positively to the flavour and aroma of cheese. Is has been shown that the ripening of cheese can be accelerated and the quality improved by the addition of *Yarrowia lipolytica* to the cheese (Devoyod, 1990).

Yeasts also contribute to proteolysis in cheese. Species of the genera *Trichosporon* and *Debaryomyces* contain endo-cellular proteolytic activity while species of the genera *Kluyveromyces, Candida, Debaryomyces* and *Yarrowia* contain extra-cellular proteolytic activity (Choisy et al., 1986; Devoyod, 1990). The proteolytic activity of 162 yeast strains isolated from Camembert was evaluated and the activity was found to be comparable to

that of *Penicillium camemberti*, the primary ripening agent in Camembert cheese (Choisy et al., 1986). *Kluyveromyces marxianus* var. *marxianus* is regarded as the species with the greatest proteolytic activity (Choisy et al., 1986). It produces three proteases which contribute to the ripening of blue veined cheese through their proteolytic activity (Choisy et al., 1986). The lactose fermenting species, *Kluyveromyces marxianus* var *marxianus* and var *lactis* also produce ethanol, acetaldehyde and esters which contribute to the development of aroma in blue veined cheese (Choisy et al., 1986).

Yeast species also contribute indirectly to the ripening of blue veined cheese by being able to ferment lactose, resulting in the production of carbon dioxide (Wyder, 1998). The carbon dioxide produced prevents the fusion of the curd granules thereby contributing to the 'open' texture that is characteristic of the cheese. This allows the mould *Penicillium roqueforti* to penetrate and colonise the cheese, resulting in a better and more spread aroma and flavour (Wyder, 1998).

Another major effect of yeasts on the ripening of cheese is the utilisation of lactic acid. The yeasts are able to utilise the lactic acid leading to an increase in pH which consequently encourages the growth of bacterial species, like *Brevibacterium linens*. This species further contributes to the ripening of the cheese through its proteolytic and lipolytic activities (Devoyod, 1990). During the salting stage of blue veined cheese processing, the cell walls of non-salt-tolerant yeasts that occur on the surface are destroyed leading to the release of cellular contents (Devoyod, 1990). The appearance of nucleotides, peptides, vitamins and other metabolites explains the growth of micrococci, which also contributes to the ripening of blue veined cheese (Devoyod, 1990).

1.3.2.2 Non-starter bacteria

The non-starter bacterial microflora of blue veined cheese consists mainly of two groups of bacteria, namely micrococci and coryneform bacteria. These organisms grow mainly on the surface of the cheese because of their aerobic nature but can also occur in the matrix of the cheese, at much lower concentrations.

1.3.2.2.1. Coryneform bacteria

The coryneform bacteria are a taxonomically diverse collection of unrelated bacteria (Addis, 2002). The common property that groups them is pleomorphism (Crombach, 1974). The group can broadly be divided into two, namely medical related and non-medical related species (Crombach, 1974; Addis, 2002).

The non-medical group consists mainly of genera isolated from soil, dairy products and animals (Crombach, 1974). Some dairy strains have the capacity to cause human infections, though this happens rarely (Addis, 2002). Bacteria in this group are Gram-positive aerobes and require a neutral pH in the environment for optimal growth. Some members of this group grow well at storage temperatures (10^{o}C), eventhough their optimal temperature ranges between 20-30^{o}C. Members of the group are generally salt-tolerant explaining their prevalence in cheese. They contribute to cheese flavour through their proteolytic and lipolytic activity.

The two genera of coryneform bacteria that occur most frequently in blue veined cheeses are *Arthrobacter* and *Brevibacterium* (Reps, 1993). Members of the genus *Corynebacterium* also occur to a lesser extent (Gobbetti, 2000). Species of the genus *Arthrobacter* are capable of casein hydrolysis (Reps, 1993) and contribute to the ripening of the cheese, although the exact nature of the contribution is unclear and needs further studying. *Brevibacterium* species contribute to the ripening of cheese through the synthesis of proteolytic and lipolytic enzymes (Reps, 1993). *Brevibacterium linens* contains both extra- and intra-cellular proteases. The species also degrades amino acids resulting in the release of volatile sulphur-containing compounds (Weimer, 1998). Coryneform bacteria in cheese originate as post pasteurisation contaminants, mainly from milk and soil (Choisy et al., 1986).

The species of coryneform bacteria most frequently isolated from blue veined cheese comprise of *Brevibacterium linens*, and *Brevibacterium erythrogenus* (Reps, 1993). Although *Brevibacterium linens* is frequently isolated, it is not always the most dominant species. The number of *Brevibacterium linens* depends on pH and the temperature at which ripening takes place (Reps, 1993). Studies by Hartley and Jezeski (1954) showed

that strains of *Brevibacterium erythrogenus* are the most dominant coryneform bacteria in the blue veined cheese whereas *Brevibacterium linens* only accounted for a small percentage.

It was found that at ripening temperatures of 8–10^{o}C, strains of *Brevibacterium erythrogenus* develop to become dominant while at ripening temperatures of 13–15^{o}C, *Brevibacterium linens* strains appeared to be dominant (Hartley and Jezeski, 1954). *Brevibacterium linens* only develops on the surface of the cheese once the pH of the cheese is above 5.85 (Reps, 1993).

1.3.2.2.2. Micrococci

Members of the genus *Micrococcus* have recently undergone a massive taxonomic reclassification. Members have been relocated into six genera of which some are new (Garcia-Lopez et al., 2000). The old taxonomy is still used frequently and therefore will also still be applied to avoid confusion.

Micrococci originate as post pasteurisation contaminants, mainly from the milk and brine (Choisy et al., 1986). Members of this group belongs to the genus *Micrococcus* and are aerobic, salt tolerant and also have the ability to grow at low storage temperatures. Microccocci occur on the surface of the cheese, as well as in the interior or matrix of the cheese (Reps, 1993). Species commonly occurring in cheese include, *Micrococcus varians, Micrococcus caseolyticus* and *Micrococcus freudenreichii* (Stanley, 1998). Investigations have shown that they play a role in the ripening of cheese through their proteolytic and lipolytic activity (Addis, 2002). The extent to which they contribute to ripening in mould ripened cheese is uncertain. Extracellular proteases are produced by several species in the genus, but their contribution to cheese ripening remains questionable as their optimum activity falls in the alkaline pH range, being almost negligible at pH values below 5.5. Both intra- and extra-cellular lipolytic strains have been described at their optimal activity in the alkaline pH range.

1.4 SOURCES OF CONTAMINATION OF YEASTS DURING THE RIPENING OF BLUE VEINED CHEESE VARIETIES.

Laws have been enacted in many countries around the world which state that milk has to be pasteurised for the manufacture of cheese, including blue veined cheese (Purko et al., 1951). The pasteurisation process kills all pathogenic organisms and also lowers the number of wild lactic acid bacteria present in the milk. All yeasts naturally present in the raw milk are also killed (Farkye, 2000). Consequently, milk used for the manufacture of cheese contains no natural yeasts. Yeasts however become representative in blue veined cheese during processing. This is indicative of the fact that these yeasts originate as post pasteurisation contaminants from the dairy environment (Devoyod, 1990; Fleet, 1990).

Sources in the dairy environment responsible for yeast contamination include: air, surfaces, workers, brine and additives. The air in the dairy environment is usually responsible for a small proportion of the contaminating yeasts. Viljoen and Greyling (1995) showed that air at the dairy plants had an insignificant count of contaminating yeasts. The low incidence was verified by Welthagen and Viljoen (1998b, 1999). Surfaces responsible for yeast contamination include the floors, walls and equipment. Welthagen and Viljoen (1998b, 1999) indicated that surfaces accounted for the highest number of yeast contaminants in the environment. The contribution towards yeast contamination however varies from dairy plant to dairy plant and is also substantially influenced by sanitation practices. In the survey by Viljoen and Greyling (1995) workers hands and aprons had the lowest number of yeast counts.

It has been shown by many researchers that the brine used in the salting of cheese is the main source of post pasteurisation yeast contamination (Seiler and Busse, 1990; Viljoen and Greyling, 1995). Each dairy's brine has a characteristic yeast flora. The brines are contaminated by the cheese itself and by dairy equipment (Seiler and Busse, 1990). Although research has been done on the brine of different cheeses, limited research has been done on the brine used during the processing of blue veined cheese. Van den Tempel and Jakobsen (1998) found that brine used in blue veined cheese

production contains a high concentration of yeasts, ranging from 10^4 to 10^6 cfu.ml^{-1}. Species frequently identified include *Debaryomyces hansenii* and *Yarrowia lipolytica* (Seiler and Busse, 1990; van den Tempel and Jakobsen, 1998). Both species have a high tolerance to salt, which explains their dominance in the brine (Seiler and Busse, 1990; van den Tempel and Jakobsen, 1998).

1.5 PHYSICAL FACTORS INFLUENCING MICROBIAL GROWTH.

1.5.1 Temperature

The ripening of blue veined cheese can generally be divided into two parts. During the first part of ripening the cheese is held at 8-10^{o}C and 85-95% RH, usually for 4 weeks, depending on the specific variety (Morris, 1964; Marth and Yousef, 1991). During the second part, the cheese is moved to a second incubating room, at 2-4^{o}C, usually for 16-18 months, again depending on the specific variety (Morris, 1964; Marth and Yousef, 1991). After ripening, the cheese is packed and distributed. Vidal-Leira (1979) determined the T_{max} of nearly 600 yeast strains, which represent over 100 species. Based on the results it was concluded that 98% of the yeasts examined were mesophiles with a T_{max} value ranging between 24 and 48^{o}C. Only a small portion (2%) were true psychrophiles with a T_{max} below 24^{o}C. Species in the mesophilic group comprised those commonly isolated from blue veined cheese during ripening. The fact that mesophiles are able to survive at the relatively low ripening temperatures can be explained by the fact that these values represent their maximum growth temperatures and is not indicative of their minimal growth temperature.

1.5.2 Water-activity

Water-activity plays an important role in the occurrence and growth of yeasts in food products (Deak and Beuchat, 1996). The water activity of food mainly depends on the concentration and type of solute(s) present in the food (Deak and Beuchat, 1996). These solutes can be added or can be present naturally. Yeasts are more tolerant to reduced a_w than bacteria and are able to grow in foods with a reduced a_w (Deak and Beuchat, 1996).

Blue veined cheese normally has a higher salt concentration and therefore a reduced a_w which renders a competitive advantage to yeasts compared to undesired bacteria, especially since the majority of yeasts associated with blue veined cheese originate from cheese brines which have a 20–25% salt concentration (Seiler and Busse, 1990).

These brines further contribute to the selection of yeasts with a high salt tolerance which would favour their growth during ripening.

1.5.3 Oxygen

Yeasts are basically aerobic organisms and although fermentation is a noticeable feature for some species many other species are strictly non-fermentative aerobes (Deak and Beuchat, 1996). Yeast can be divided into three categories based on their fermentative capabilities, namely non-fermentative, facultatively fermentative and obligatory fermentative (Deak and Beuchat, 1996). Cheese, like most other foodstuffs, consists of two areas with regards to its oxygen demand. The surface of the cheese is exposed to air and therefore is an aerobic environment (Fleet, 1999). The interior is an anaerobic environment although a small amount of oxygen might be present (Fleet, 1999). This greatly influences the type of yeasts present in the two parts of the cheese. On the surface one would mainly find non-fermentative and facultatively fermentative yeasts like *Debaryomyces hansenii* and *Yarrowia lipolytica* (Fleet, 1999). In the interior obligatory fermentative and facultatively fermentative yeasts like *Kluyveromyces marxianus* will dominate (Fleet, 1999).

Part of blue veined cheese production involves packaging the cheese in plastic bags followed by a period of ripening. These bags are not flushed with nitrogen gas, as is the case for other foodstuffs, because it would inhibit the growth of coryneform bacteria which contribute to the ripening. During this period however the environment with respect to oxygen becomes micro-aerophilic and cause species which are non-fermentative to die off (Fleet, 1990).

1.6 CHEMICAL FACTORS INFLUENCING MICROBIAL GROWTH.

1.6.1 Nutrients

Like most microorganisms, yeasts need a carbon source for their energy requirements. Yeasts are able to utilise a wide range of carbon-sources (Deak and Beuchat, 1996), the most common of these sources are sugars. Yeasts are able to utilise only a few sugars, mostly hexoses and oligosaccharides, although others can also be utilised (Deak and Beuchat, 1996). The only sugar that occurs naturally in cheese in significant quantities, is the disaccharide lactose and only a few yeast species are able to utilise it as a carbon-source, like *Kluyveromyces marxianus* and *Candida pseudotropicalis* (Devoyod, 1990). During ripening, cheese usually contains greater concentrations of organic acids, like lactic acid and citric acid, produced by lactic acid bacteria present in the curd (Farkye, 2000). The acid produced can be utilised by the yeasts as a carbon source. This utilisation of lactic acid plays a very important role in the succession of microorganisms during cheese ripening as discussed earlier. Polyhydroxy alcohols, like glycerol, are also produced by yeasts through the EM-pathway and can be utilised by yeasts as a carbon source (Spencer et al., 1997). Yeast species also utilise free fatty acids, their esters and triglycerides as carbon sources, although these species are not abundant (Spencer et al., 1997).

Both organic and inorganic nitrogen compounds can be utilised as nitrogen sources (Deak and Beuchat, 1996). Few species are able to utilise proteins extracellularly. Peptides formed during ripening through proteolysis are therefore transported into the cell and utilised. Almost all yeasts are able to utilise proteins in this way and furthermore they are also able to utilise amino acids, amines, urea and inorganic ammonium salts as nitrogen sources (Deak and Beuchat, 1996).

These compounds do not however occur frequently in cheese. Only certain yeast species are able to utilise nitrate, which is added to certain cheese varieties in the form of KNO_3 or $NaNO_3$ (Beresford et al., 2001)

Other than carbon and nitrogen sources, micro-elements and growth factors are also needed by yeasts for growth (Deak and Beuchat, 1996). Most of the inorganic micro-elements needed by yeasts occur in sufficient quantities in foodstuffs (Deak and Beuchat, 1996). Inorganic micro-elements present in milk provide adequate micro-elements to support yeast growth in the cheese curd. Many yeast species are able to synthesise growth factors like vitamins.

1.6.2 pH levels

The pH of any ripening cheese is mainly determined by the type and concentration of acids derived from the breakdown of lactose by the starter cultures (Beresford et al., 2001). Ripening cheese contains mainly organic acids and fewer inorganic acids (Deak and Beuchat, 1996). The main organic acids found in cheese during ripening are lactic, acetic and propionic acids, of which lactic acid occurs in the greatest concentration (Beresford et al., 2001). Yeasts are able to tolerate a wide pH range and grow actively at values between 3 and 10 (Fleet, 1990; Deak and Beuchat, 1996). The optimum pH ranges between 4.5 and 6.5, as they prefer a slightly acidic environment (Deak and Beuchat, 1996) and therefore the pH levels in the cheese will stimulate yeast growth. The ability of the yeasts to survive and grow at these low pH values are not yet clearly understood. It most probably has to do with its dependence on an active transport system which removes H^+ from the interior of the cell and thus prevents acidification of the cell (Deak and Beuchat, 1996).

1.7 PROCESSING FACTORS GOVERNING THE GROWTH OF YEASTS DURING CHEESE RIPENING.

Many blue veined cheese varieties exist all over the world and are mostly bound by region. Roquefort is arguable the best known blue veined cheese variety and is produced in France (Besançon et al., 1992). The cheese is made from unpasteurised ewe's milk and contains a diverse microflora (Choisy et al., 1986). This is in contrast to locally produced cheese, which is produced from pasteurised milk.

During the cheese-making process lactic acid bacteria convert lactose to lactic acid. This process continues during moulding after production, up untill the cheese is salted (Beresford et al., 2001). Yeasts develop rapidly during the first 24 hours after production because environmental conditions favour their growth (Choisy et al., 1986; Devoyod, 1990; Lopez-Diaz et al., 1995; Beresford et al., 2001). On the surface of the ripening cheese the numbers of micrococci decrease rapidly mainly due to the lowered pH of the cheese (Choisy et al., 1986). The cheese is then transferred into a cold ripening room and consequently the microbial activity slows down. During this period, the total bacterial load remains practically the same whereas the yeasts increase in numbers. The yeast numbers reach a maximum of 5×10^7 cells.g^{-1} on the exterior and 10^5 cells.g^{-1} in the interior prior to salting (Choisy et al., 1986). The viability of the yeasts at this stage is based on the metabolisation of glucose and galactose due to the breakdown of lactose and the lactic acid produced by the lactic acid bacteria (Choisy et al., 1986).

The next stage is salting, which greatly affects the microflora on the exterior of the cheese resulting in a decrease of microbial numbers (Seiler and Busse, 1990).

In the interior of the cheese, the effect of the salt is less severe because the salt moves according to a gradient from the exterior to the interior and therefore the effect will only be noticeable after 10 days when the salt reaches the interior in greater quantities (Besançon et al., 1992).

After salting the cheese is ripened for the second period. During the second stage of ripening the number of yeasts on the exterior of the cheese increases sharply, mainly because the exterior of the cheese was exposed to salt tolerant yeasts within the brine (Seiler and Busse, 1990). The yeast population reaches its maximum number, of about 10^9 cells.g^{-1}, during this period. Yeasts contribute directly to the ripening of the cheese during this period through their metabolic activity and by changing the environment of the cheese to allow the growth of other secondary microflora that contribute to ripening. The yeasts accomplish the latter by utilising the lactic acid, thereby increasing the pH, and by producing certain growth factors, like vitamins (Purko et al., 1951; Lubert and Frazier, 1955). After the subsequent rise in pH and production of growth factors, members of the secondary microflora develop and their numbers increase sharply, most notably micrococci and coryneform bacteria. These organisms are pH sensitive and can only develop once an enhanced pH is achieved due to yeast growth. During packaging and distribution the concentration of yeasts and secondary bacteria remain constant on the exterior, whereas in the interior, the numbers of secondary bacteria decrease. This seems to be related to the growth of *Penicillium roqueforti* (Choisy et al., 1986).

Not all blue veined cheese varieties are produced from raw milk and famous varieties such as Gorgonzola and Danublu are produced from pasteurised milk (de Boer and Kuik, 1987). When pasteurised milk is used for cheese production the development of yeasts and secondary bacteria during ripening is completely different from that of cheese produced from raw milk. The main reason is that most of the natural flora present in raw milk is killed during the pasteurisation process and the secondary flora therefore originates exclusively from post pasteurisation contamination. (Seiler and Busse, 1990; Welthagen and Viljoen, 1998b; 1999). Gobbetti et al. (1997) studied the development of the secondary microflora during the ripening of Gorgonzola cheese. Pasteurised milk is used for the manufacture of this cheese variety and salting takes place 10 hrs after production. After one day of ripening the yeast population was already established on the surface of the cheese with a count of 5.14 log cfu.g^{-1}. In the interior of the cheese, the yeast count was even higher at 5.32 log cfu.g^{-1} (Gobbetti et al., 1997). The yeast species originated mainly from the brine used during salting. The high yeast count in the interior was unexpected since yeasts are not able to penetrate into the curd during brining. The yeast count on the surface of the cheese increased for the next 36 days of ripening to reach a maximum of 7.63 log cfu.g^{-1}. Similar findings were

obtained in the interior of the cheese where the yeast count reached a maximum of 7.71 log cfu.g^{-1}. After 36 days to the end of ripening the yeast counts remained the same, with only minor fluctuations for both the exterior and interior (Gobbetti et al., 1997).

1.8 INTERACTIONS BETWEEN YEASTS AND OTHER MICRO-ORGANISMS.

Cheese can be considered as a dynamic ecosystem which harbors all the major microbial groups (Fleet, 1999). These microbial groups constantly undergo dynamic changes and interact with each other in varied and diverse ways (Addis, 2002). The domination of one group of microorganisms at one stage is heavily dependant on the prevailing environmental conditions at that stage and changing conditions results in the successional development of different microbial groups (Fleet, 1999) This successional development, due to interactions between the different groups, is very important for the proper maturation of the cheese. The different biochemical reactions that the groups of microorganisms undergo during these interactions contribute to the flavour, aroma and appearance of each type of cheese (Viljoen, 2001).

Yeast interactions have been found in a variety of cheeses (Jakobsen and Narvhus, 1996). Like any interaction between microbial groups, yeast interactions can be classified as positive, neutral or negative (Addis, 2002; Viljoen, 2001). Positive interactions can be defined as stimulating growth of a group as found between yeasts and the secondary bacteria. The positive effect is based on yeast species ability to utilise lactic acid (Fleet, 1990) resulting in the deacidification of the cheese surface and a subsequent increase in the pH on the cheese surface (Corsetti et al., 2001). Consequently, less acid-tolerant organisms like micrococci, *Brevibacterium linens, Arthrobacter* and *Corynebacterium* spp. will develop (Corsetti et al., 2001). The interaction between certain yeast species and the mould used in ripening blue veined cheese varieties, namely *Penicillium roquefortii*, is also regarded as positive. Fermentative yeast species such as *Kluyveromyces marxianus* ferments lactose, while CO_2 is produced (Wyder, 1998) causing the formation of small holes in the interior of the curd, which facilitates the establishment of the mould within the cheese (Choisy et al., 1986).

Negative interactions can be defined as interactions which inhibit the growth of a group, or at worse, causes cell death (Addis, 2002). A typical example of a negative interaction

in cheese is the inhibition or even elimination of undesired microorganisms by yeasts (Jakobsen and Narvhus, 1996). Studies showed that *Debaryomyces hansenii* inhibits the germination and therefore the growth of *Clostridium butyricum* and *Clostridium tyrobutyricum* (Wyder, 1998). Although the exact nature of the inhibition is not yet clear it is probably connected to the depletion of lactic and acetic acid and the production of a 'killer toxin' by *Debaryomyces hansenii* (Fleet, 1990; Jakobsen and Narvhus, 1996; Wyder, 1998). Another example of a negative interaction, which is frequently overlooked, is the parasitism of both bacteria and yeasts by viruses. Bacteriophages attack the bacterial flora which often leads to devastating results, either during production or ripening (Addis, 2002).

Neutral interactions can be defined as interactions which have no affect on the participants (Addis, 2002). An example of a neutral interaction is the synergistic growth of both yeasts and starter cultures during cheese ripening, where both groups continue to multiply and no inhibition of either group is observed (Viljoen, 2001).

1.8.1 Interaction between yeasts and *Penicillium roqueforti*.

Few studies have been carried out on the interactions between yeasts and *Penicillium roqueforti* during the ripening of blue veined cheese varieties (Hansen et al., 2001). From these limited studies it has been shown that yeasts may contribute positively to cheese maturation based on positive and negative interactions with *Penicillium roqueforti* (Kronborg Hansen and Jakobsen, 1998; van den Tempel and Nielsen, 2000; Hansen and Jakobsen, 2001; Hansen et al., 2001).

The positive and negative interactions between yeasts and *Penicillium roqueforti* vary between yeast species. It would seem as though the effect of a specific yeast species towards *Penicillium roqueforti* is the same no matter what the strain of *Penicillium roqueforti*. (Kronborg Hansen and Jakobsen, 1998; Hansen et al., 2001). Positive interactions usually include enhanced growth of *Penicillium roqueforti*, thicker and more velvety mycelia, a more intense blue colour and an enhanced effect on the metabolic activity of the mould (Kronborg Hansen and Jakobsen, 1998; Hansen and Jakobsen, 2001). Negative interactions include inhibition of growth and sporulation of *Penicillium roqueforti*.

Kronborg Hansen and Jakobsen (1998) found that *Debaryomyces hansenii* showed a limited contribution regarding interaction towards *Penicillium roqueforti*, being almost non- existent whereas *Kluyveromyces lactis* showed pronounced inhibition of growth and sporulation of *Penicillium. roqueforti*. The nature of this inhibition is still not fully understood but it is known that *Kluyveromyces lactis* produces killer toxins (Kronborg Hansen and Jakobsen, 1998). *Saccharomyces cerevisiae* was the only yeast species that showed any positive interaction towards *Penicillium roqueforti* based on increased growth and sporulation (Kronborg Hansen and Jakobsen, 1998). A synergistic effect in the degradation of casein by *Saccharomyces cerevisiae* and *Penicillium roqueforti* was also found and *Saccharomyces cerevisiae* could therefore contribute to the ripening of blue veined cheese (Kronborg Hansen and Jakobsen, 1998). This synergistic effect could also possibly explain the stimulating effect of *Saccharomyces cerevisiae* on *Penicillium roqueforti* although this still has to be proven.

Van den Tempel and Nielsen (2000) in contrast to Kronborg Hansen and Jakobsen (1998) found *Debaryomyces hansenii* to have a varying effect on *Penicillium roqueforti*. They studied the effect of several environmental conditions on the interactions between yeasts and moulds related to blue veined cheese production. Minor inhibitions of *Penicillium roqueforti* towards *Debaryomyces hansenii* were found at 21% oxygen levels. Stimulation of *Penicillium roqueforti* by *Debaryomyces hansenii* occurred at 25% CO_2 and 0.3% O_2 based on increased radial growth. These positive interactions can possibly be explained by the ability of *Debaryomyces hansenii* to modify the environment to benefit *Penicillium roqueforti* (van den Tempel and Jakobsen, 2000). At atmospheric conditions comprising normal atmospheric oxygen levels minor inhibition of *Penicillium roqueforti* by *Debaryomyces hansenii* occurred based on decreased radial growth (van den Tempel and Nielsen, 2000).

Hansen et al. (2001) studied the interactions between a dairy strain of *Saccharomyces cerverisiae* and *Penicillium roqueforti*. Their results confirmed those of Kronborg Hansen and Jakobsen (1998), that *Saccharomyces cerevisiae* showed positive interactions with *Penicillium roqueforti*. These positive interactions included enhanced growth and sporulation of *Penicillium roqueforti* and a more intense blue-green colour of the conidia (Hansen and Jakobsen, 2001; Hansen et al., 2001). This was however not

the only positive interaction, as *Saccharomyces cerevisiae* also had an enhanced effect on the proteolytic activity of *Penicillium roqueforti*. The positive contribution of *Saccharomyces cerevisiae* could also further be detected in the softer texture of the cheese inoculated with the yeast (Hansen et al., 2001).

These interactions, between the above mentioned yeast species and *Penicillium roqueforti* should be considered when selecting a yeast species as a starter culture in blue veined cheese manufacture. More studies should be conducted with a wider range of yeast species to examine the interactions between yeasts found in blue veined cheese and *Penicillium roqueforti*.

1.8.2 Interactions between yeasts and secondary bacteria.

1.8.2.1 Interaction between yeasts and coryneform bacteria.

The positive interactions between yeasts and coryneform bacteria are well known and understood (Purko et al., 1951; Lenoir, 1984; Devoyod, 1990; El Soda, 2000). Yeasts cause a stimulating effect on the growth of coryneform bacteria. Yeasts aid in the deacidification of the cheese surface, which leads to an increase in the pH on the surface and the subsequent development of coryneform bacteria, such as *Arthrobacter* spp., *Brevibacterium* spp. and *Corynebacterium* spp. (Reps, 1993; Corsetti et al., 2001). The yeasts decrease the acidity of the curd by metabolising the lactic acid, formed by the starter culture during production, as a carbon source (El Soda, 2000). During this process the yeasts completely oxidise lactic acid to CO_2 and H_2O, which in turn leads to an increase in the pH on the cheese surface (Beresford et al., 2001; Corsetti et al., 2001). The yeasts also deaminate amino acids to their corresponding keto-acid and NH_3 (Corsetti et al., 2001). These products, of alkaline metabolism, further increase the pH on the cheese surface (Purko et al., 1951; Eliskases-Lechner and Ginzinger, 1995; Beresford et al., 2001). Both metabolic actions cause the pH of the curd to increase, which facilitates the development and growth of coryneform bacteria which failed to grow in an environment that is more acid than pH 5.85 (Purko et al., 1951; Bockelmann and Hoppe-Seyler, 2001).

Yeasts also stimulate the growth of coryneform bacteria by producing certain bacterial growth factors (Purko et al., 1951; Fleet, 1990; Viljoen, 2001). The most important of these growth factors are vitamins and amino acids (Lenoir, 1984; El Soda, 2000). The vitamins include pantothenic acid, niacin, riboflavin and biotin (Purko et al., 1951; Valdes-Stauber et al., 1997; Weimer, 1998; El Soda, 2000). Pantothenic acid seems to be the most important of these (Purko et al., 1951). Another stimulating effect on coryneform bacteria by yeasts comprises the release of their cellular contents during cell death which are nutrient rich and could be utilised (Addis, 2002).

1.8.2.2. Interaction between yeasts and micrococci.

Yeasts show a positive interaction towards micrococci, although the exact nature of the stimulation is not well known or studied. Yeasts stimulate the growth of micrococci in a similar way as for coryneform bacteria, by increasing the pH of the curd through its lactic acid and alkaline metabolism products (Lubert and Frazier, 1955). Furthermore micrococci are also stimulated by growth factors found in yeast autolysates (Lubert and Frazier, 1955; Corsetti et al., 2001). These growth factors include, nucleotides, peptides and vitamins (Corsetti et al., 2001).

Addis et al. (2001) studied the interactions between yeasts and micrococci isolated from blue veined cheese during ripening. The predominant yeast species isolated were *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus* and *Saccharomyces cerevisiae.* The interactions between these yeast species and four strains of *Micrococcus* spp. revealed that none of the above mentioned yeast species inhibited any of the *Micrococcus* spp (Addis et al., 2001). The same researchers also found that none of the *Micrococcus* spp. were inhibitory towards any of the above mentioned yeast species (Addis et al., 2001). In fact, it was found that *Yarrowia lipolytica* enhanced the growth of one of the *Micrococcus* spp (Addis et al., 2001).

1.9 YEASTS AS STARTER CULTURES IN THE MANUFACTURE OF BLUE VEINED CHEESE VARIETIES.

Traditionally yeasts have been used as starter cultures in the production of kefir and related products (Wyder, 1998) and are also employed in the fermentation of whey (Jakobsen and Narvhus, 1996). Many attempts have been made to incorporate selected yeasts in the production of blue veined cheese. These yeasts were usually incorporated into the cheese in one of two forms, namely whole yeast cells or enzyme preparations from selected yeasts.

Several factors, including the cheese itself, the brine, the environmental conditions and the treatment of the milk, select towards a uniform and well defined yeast-flora (Fleet, 1990; Jakobsen and Narvhus, 1996). This leads to a situation where wild yeasts from the environment serve as starter culture, although not deliberate. It would be preferable to replace these 'wild' yeasts from the environment with a known yeast species that could be applied as an additional starter culture, with known characteristics.

Although companies produce and sell yeast starter cultures for use in the manufacture of commercial cheese, these starter cultures are not generally used in cheese production. The main reason for this is the fact that not much is known about the role of yeasts in the ripening of different types of cheese and it is only recently becoming apparent that yeasts play an integral part in the ripening of these cheeses (Devoyod, 1990).

Yeast should be selected as starter cultures based on biochemical, physiological and technological characteristics. These characteristics should include: i) proteolytic and lipolytic properties which contribute to the flavour profile; ii) the ability to form aroma compounds which contribute to the flavour profile; iii) the fermentation of residual lactose; iv) the assimilation of residual lactate and
v) positive interactions with the primary starter cultures, the *Penicillium* starter cultures and the secondary flora, eg. *Brevibacterium* spp. (Fleet, 1990; Jakobsen and Narvhus, 1996).

Yarrowia lipolytica possesses strong lipolytic and proteolytic properties (Roostita and Fleet, 1996; Wyder, 1998). It expresses proteolytic activity through extracellular proteases and proteolytic enzymes, like peptidases, i.e. aminopeptidases, and carboxypeptidases, located in the interior of the yeast (Roostita and Fleet, 1996). These would be of greater importance to cheese ripening if they could be released by cell lysis (Wyder, 1998). Furthermore, the species is able to express these activities at temperatures as low as 0°C, making it suitable as starter culture for the ripening of blue veined cheese varieties which are usually ripened a few degrees above 0°C (Welthagen and Viljoen, 1999).

Yarrowia lipolytica contributes to the ripening of blue veined cheese by the formation of aroma and flavour compounds based on lipolysis producing free fatty acids which are then further converted to aroma and flavour compounds (Martin *et al.*, 1999). The species is also an exceptionally strong utiliser of lactate (Fleet, 1999) and other organic acids like citric acid (Roostita and Fleet, 1996), especially on the surface of the curd where it causes the pH to increase (Fleet, 1999).

Yarrowia lipolytica is involved in a number of positive interactions with the microflora of ripening cheese, especially the bacteria. Guerzoni *et al.* (1998) showed that the species had no negative effect on the growth of lactic acid bacteria and that some strains even stimulated their growth (Guerzoni *et al*., 1998). It has also been shown that the production of aroma compounds by bacteria may be enhanced when they are associated with *Yarrowia lipolytica* (Martin *et al.* 1999).

Debaryomyces hansenii also possesses strong proteolytic and lipolytic properties (Choisy et al., 1986; Wyder, 1998). It contains intracellular proteolytic activity expressed through intracellular proteases which preferably hydrolyse caseins at an optimum pH of 5.8 (Choisy et al., 1986; Wyder, 1998; Roostita and Fleet, 1996). In addition, extracullular proteinases, i.e. aminopeptidases and carboxypeptidases were also detected in these species (Wyder, 1998). The lipolytic activity of *Debaryomyces hansenii* can be attributed to intracellular lipases, at an optimum pH of 8 (Choisy et al., 1986). This activity causes the liberation of fatty acids which play an important role in the formation of flavour and aroma. The proteolytic activity of *Debaryomyces hansenii* leads to the formation of precursors of aroma, mainly amino acids. These amino acids

contribute to the formation of aroma and flavour during the ripening of the cheese (Lenoir, 1984). The species further contributes to the ripening of blue veined cheese by breaking down larger peptides into smaller peptides and amino acids, therefore removing the larger, 'bitter' peptides from the flavour profile (Wyder, 1998). When *Debaryomyces hansenii* is co-cultured with lactic acid bacteria the proteolytic activity of the species is greater than when both are cultured separately (Wyder, 1998).

Debaryomyces hansenii is also an exceptionally strong utiliser of lactate (Fleet, 1999). It is able to utilise residual lactate both aerobically and anaerobically and preferably the L(+) isomer of this compound (Wyder, 1998). This utilisation is especially strong on the surface of the curd which leads to an increase in pH levels (Fleet, 1999).

Kluyveromyces marxianus is another yeast species recognised for its high proteolytic activity (Wyder, 1998). This species is well known for its strong extracellular proteolytic activity, but also has a relatively strong intracellular proteolytic activity (Roostita and Fleet, 1996). The species also possess lipolytic activity, although not as strong as its proteolytic activity (Choisy et al., 1986; Fleet and Mian, 1987). The species further has the unique characteristic of being able to utilise lactose directly, producing CO_2.

The CO_2 produced prevents fusion of the curd particles and consequently promotes the open structure of blue veined cheese. This allows better growth and penetration of the mould, *Penicillium roqueforti* (Devoyod, 1990). During the utilisation of lactose, *Kluyveromyces marxianus* not only produces CO_2, but also ethanol and acetaldehyde. These compounds contribute to the flavour profile of blue veined cheese (Wyder, 1998), however, the production of fruity volatile compounds can potentially contribute negatively to the flavour profile of cheese (Martin *et al*, 1999).

Van den Tempel and Jakobsen (2000) evaluated the application of *Debaryomyces hansenii* and *Yarrowia lipolytica* as potential starter cultures for the production of Danablu and found *Debaryomyces hansenii* to show good growth. The species was also able to assimilate lactate, citric acid, lactose and galactose (van den Tempel and Jakobsen, 1998; 2000). Based on these actions, *Debaryomyces hansenii* was able to modify the micro-environment in the cheese to benefit *Penicillium roqueforti* and protect the cheese against unwanted carbohydrate fermentation (van den Tempel and

Jakobsen, 2000). The role of *Yarrowia lipolytica* as potential starter was strongly related with its ability to induce early lipolysis and proteolysis. Guerzoni et al. (1998) also studied the role of *Yarrowia lipolytica* as potential starter culture in the production of milk products and found the yeast to show good growth during the ripening period. No inhibition of inoculated or naturally occurring lactic acid bacteria occurred and it was found that *Yarrowia lipolytica* stimulated the growth of these bacteria (Guerzoni et al., 1998).

Hansen et al. (2001) studied the application of *Saccharomyces cerevisiae* from dairy origins as potential starter culture in the production of Mycella, a Danish Gorgonzola style cheese. Positive interactions were noted between *Saccharomyces cerevisiae* and *Penicillium roquefortii* based on accelerated growth and sporulation of the mould. *Saccharomyces cerevisiae* also had a positive effect on the proteolytic activity of *Penicillium roqueforti* as indicated by capillary electrophoresis, resulting in a softer texture (Hansen et al, 2001).

1.10 REFERENCES

Addis, E.P.H. (2002) The microbial ecology of Australian Camembert and Blue-veined cheeses during the maturation process. PhD dissertation. University of New South Wales.

Addis, E.P.H., Fleet, G.H., Cox, J.M. and Leung, T. (2001) The growth, properties and interactions of yeasts and bacteria associated with the maturation of Camembert and blue-veined cheeses. *Int. J. Food Microbiol*. **69**, 25-36.

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

Besançon, X., Smet, C., Chabalier, C., Rivemale, M., Reverbel, J.P., Ratomahenina, R. and Galzy, P. (1992) Study of surface yeast flora of Roquefort cheese. *Int. J. Food Microbiol*. **17**, 9-18

Bockelmann, W. and Hoppe-Seyler, T. (2001) The surface flora of bacterial smear-ripened cheeses from cow's and goat's milk. *Int. Dairy J*. **11**, 307-314

Brulé, G. and Lenoir, J. (1986) The coagulation of milk. In Cheesemaking-science and technology (Ed A. Eck) pp. 1-21. New York. Lavoisier Publishing.

Choisy C., Desmazeaud, M., Gripon, JC., Lamberet, G., Lenoir, J. and Tourneur, C. (1986) Microbiological and biochemical aspects of ripening. In Cheesemakning (Ed A. Eck) pp.62-89. New York. Lavoisier Publishing.

Coghill, D. (1979) The ripening of blue vein cheese: A review. *Aust. J. Dairy Technol.* **34,** 72-75.

Corsetti, S., Rossi, J. and Gobbetti, M. (2001) Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *Int. J. Food Microbiol*. **69**, 1-10.

Crombach, W.H.J. (1974) Relationship among coryneform bacteria from soil, cheese and sea fish. *Antonie v. Leeuwenhoek* **40**, 347-359.

De Boer, E., Kuik, D. (1987) A survey of the microbiological quality of blue-veined cheeses. *Neth. Milk Dairy J*. **41**, 227-237.

Deak, T. and Beuchat, L.R. (1996) Metabolic activities. In Handbook of Food Spoilage Yeasts (Eds T. Deak and L.R. Beuchat) pp.37-45. Boca Raton. CRC Press.

Devoyod, J.J. (1990) Yeasts in cheese-making. In Yeast Technology (Eds JFT Spencer and D.M. Spencer) pp.228-240. Berlin. Springer-Verlag.

Eliskases-Lechner, F. and Ginzinger, W. (1995) The yeast flora of surface-ripened cheeses. *Milchwissenschaf*t. **50**, 458-462.

Eliskases-Lechner, F. (1998) Yeasts in selected cheese varieties – Occurrence, identity and biochemical characteristics. In Yeasts in the dairy industry : positive and negative aspects (Eds M. Jakobsen, J. Narvhus & B.C. Viljoen) pp.88-97. Brussels. International Dairy Federation.,

El Soda, M. (2000) Cheese - role of specific groups of bacteria. In Encyclopedia of Food Microbiology Vol. 1 (Eds R.K. Robinson, C.A. Batt and P.D. Patel) pp.393-397. San Francisco. Academic Press.

Farkye, N.Y. (2000) Cheese: microbiology of cheese-making and maturation. In Encyclopedia of Food Microbiology. Vol. 1 (Eds R.K. Robinson, C.A. Batt and P.D. Patel) pp. 381-387. San Francisco. Academic Press.

Fleet, GH. (1990) Yeasts in dairy products. *J. Appl. Bacteriol*. **68**, 199-211

Fleet, GH. and Mian MA. (1987) The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiol*. **4**, 145-155

Fleet, GH. (1999) Microorganisms in food ecosystems. *Int. J. Food Microbiol*. **50**, 101-117

Garcia-Lopez, ML., Santos, JA. and Otero, A. (2000) Micrococcus. In Encyclopedia of Food Microbiology Vol. 2 (Eds R.K. Robinson, C.A. Batt and P.D. Patel) pp. 1344-1350. San Francisco. Academic Press

Gobbetti, M. (2000) *Arthrobacter*. In Encyclopedia of Food Microbiology Vol. 1 (Eds R.K. Robinson, C.A. Batt and P.D. Patel) pp 54-61. San Francisco. Academic Press.

Gobbetti, M., Burzigotti, R., Smacchi, E., Corsetti, A. and De Angelis, M. (1997) Microbiology and biochemistry of Gorgonzola cheese during ripening. *Int. Dairy J*. **7**, 519-529

Gripon, J.C. (1987) Mould-ripened Cheeses. In Cheese: Chemistry, Physics and Microbiology Vol 2, (Ed P.F. Fox) pp.121-150. London. Elsevier Applied Science.

Guerzoni, ME., Gobbetti, M., Lanciotto, R., Vannini, L. and Chaves Lopez, C. (1998) *Yarrowia lipolytica* as potential ripening agent in milk products. In Yeasts in the Dairy Industry: Positive and Negative Aspects aspects (Eds M. Jakobsen, J. Narvhus & B.C. Viljoen) pp.23-33. Brussels. International Dairy Federation.

Hartley, C.B. and Jezeski, J.J. (1954) The microflora of blue cheese slime. *J. Dairy Sci.* **37**, 436-445.

Hansen, T.K. and Jakobsen, M. (2001) Taxonomical and technological characteristics of *Saccharomyces cerevisiae* spp. associated with blue veined cheese. *Int. J. Food Microbiol*. **69**, 59-68

Hansen, T.K., van den Tempel, T., Cantor, M.D. and Jakobsen, M. (2001) *Saccharomyces cerevisiae* as a starter culture in Mycella. *Int. J. Food Microbiol*. **69**, 101-111.

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

Kosikowski, F.V. (1970). Cheese and fermented milk foods**.** pp. 255-278. New York, Kosikowski Publishers.

Kronborg Hansen, T. andJakobsen, M. (1998) Interactions between *Penicillium roqueforti* and yeast of importance in the production of Danish blue cheese. In Yeasts in the Dairy Industry: Positive and Negative Aspects (Eds M. Jakobsen, J. Narvhus & B.C. Viljoen) pp.50-58. Brussels. International Dairy Federation.

Lenoir, J. (1984) The surface flora and its role in the ripening of cheese. *IDF Bulletin* **171**., 3-20.

Lopez-Diaz, T.M., Alonso, C., Santas, J., Garcia, M.L. and Moreno, B. (1995) Microbiological changes during manufacture and ripening of a naturally ripened blue cheese (Valdeon, Spain). *Milchwissenschaft* **50,** 381-384.

Lubert, D.J. and Frazier, W.C. (1955) Microbiology of the surface ripening of brick cheese. *J. Dairy Sci.* **38**, 981-990

Marth, E.H. and Yousef, A.E. (1991). Fungi and Dairy Products. In Handbook of Applied Mycology Vol 3 (Eds D.K. Arora, K.G. Mukerji, E.H. Marth, M. Dekker) pp. 376-414. New York. Marcel Dekker Inc.

Martin, N., Savonitto, S., Molimard, P., Berger, C., Brousse, M. and Spinnler, HE. (1999) Flavor generation in cheese curd by coculturing with selected yeast, mold and bacteria. *J. Dairy Sci.* **82**, 1072-1080

Minervini, F., Montagna, G., Spilotros, G., Monaci, L., Santacroce, M.P. and Visconti, A. (2001) Survey on mycoflora of cow and buffalo dairy products from Southern Italy. *Int. J. Food Microbiol*. **69**, 141-146

Morris, T.A. (1964) The manufacture of blue-vein cheese in Queensland. *Aust. J. Dairy Technol.* January-March, 9-18.

Nichol, A.W. (2000) Cheese: Mould-ripened varieties. In Encyclopedia of Food Microbiology Vol 1 (Eds R.K. Robinson, C.A. Batt, P.D. Patel) pp. 387-393. San Francisco. Academic Press.

Nooitgedacht, A.J. and Hartog, B.J. (1988) A survey of the microbiological quality of Brie and Camembert cheese. *Neth. Milk Dairy J.* **42**, 57-72.

Pernodet, G. (1986) Comparative technology of the different types of curd. In Cheesemaking-Science and Technology (Ed A. Eck) pp. 219-247. New York. Lavoisier Publishing.

Purko, M., Nelson, W.O. and Wood, W.A. (1951) The associative action between certain yeasts and Bacterium linens. *J. Dairy Sci.* **34**, 699-706

Reps, A (1993) Bacterial surface-ripened cheeses. In Cheese: Chemistry, Physics and Microbiology Vol 2 (Ed P.F. Fox) pp.151-184. London. Chapman and Hall.

Roostita, R. and Fleet, G.H. (1996) The occurrence and growth of yeasts in Camembert and Blue-veined cheese. *Int. J. Food Microbiol*. **28**, 393-404.

Seiler, H. and Busse, M. (1990) The yeasts of cheese brines. *Int. J. Food Microbiol*. **11**, 289-304.

Spencer, J.F.T., Spencer, D.M. and Figueroa, L.I.C. (1997) Yeasts as living objects: yeast nutrition. In Yeasts in natural and artificial habitats (Eds J.F.T. Spencer and D.M. Spencer) pp.68-79. Berlin. Springer Verlag.

Stanley, G. (1998) Cheeses. In Microbiology of Fermented Foods Vol 1 (Ed B.J.B. Wood) pp 263-307. London. Blackie Academic & Professional.

Van den Tempel, T. and Jakobsen, M. (1998) Yeasts associated with Danablu. *Int. Dairy J*. **8**, 25-31.

Van den Tempel, T. and Jakobsen, M. (2000) *Debaryomyces hansenii* and *Yarrowia lipolytica* as potential starter cultures for production of blue cheeses. *Int. Dairy J.* **10**, 263-270.

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

Valdés-Stauber, N., Scherer, S. and Seiler, H. (1997) Identification of yeasts and coryneform bacteria from the surface microflora of brick cheeses. *Int. J. Food Microbiol.* **34**, 115-129

Vidal-Leira, M. (1979) Distribution of the maximum temperature for growth amoung yeasts. *Mycologia* **71**, 493-501.

Viljoen, BC. and Greyling, T. (1995) Yeasts associated with Cheddar and Gouda making. *Int. J. Food Microbiol.* **28**, 79-88.

Viljoen, B.C. (2001) The interaction between yeasts and bacteria in dairy environments. *Int. J. Food Microbiol*. **69**, 37-44.

Vivier, D., Ratomahenina, R. and Galzy, P. (1994) Characteristics of micrococci from the surface of Roquefort cheese. *J. Appl. Bacteriol*. **76**, 546-552.

Weimer, B. (1998) *Brevibacterium*. In Encyclopedia of Food Microbiology Vol. 1 (Eds R.K. Robinson, C.A. Batt, P.D. Patel) pp. 308-314. San Francisco. Academic Press.

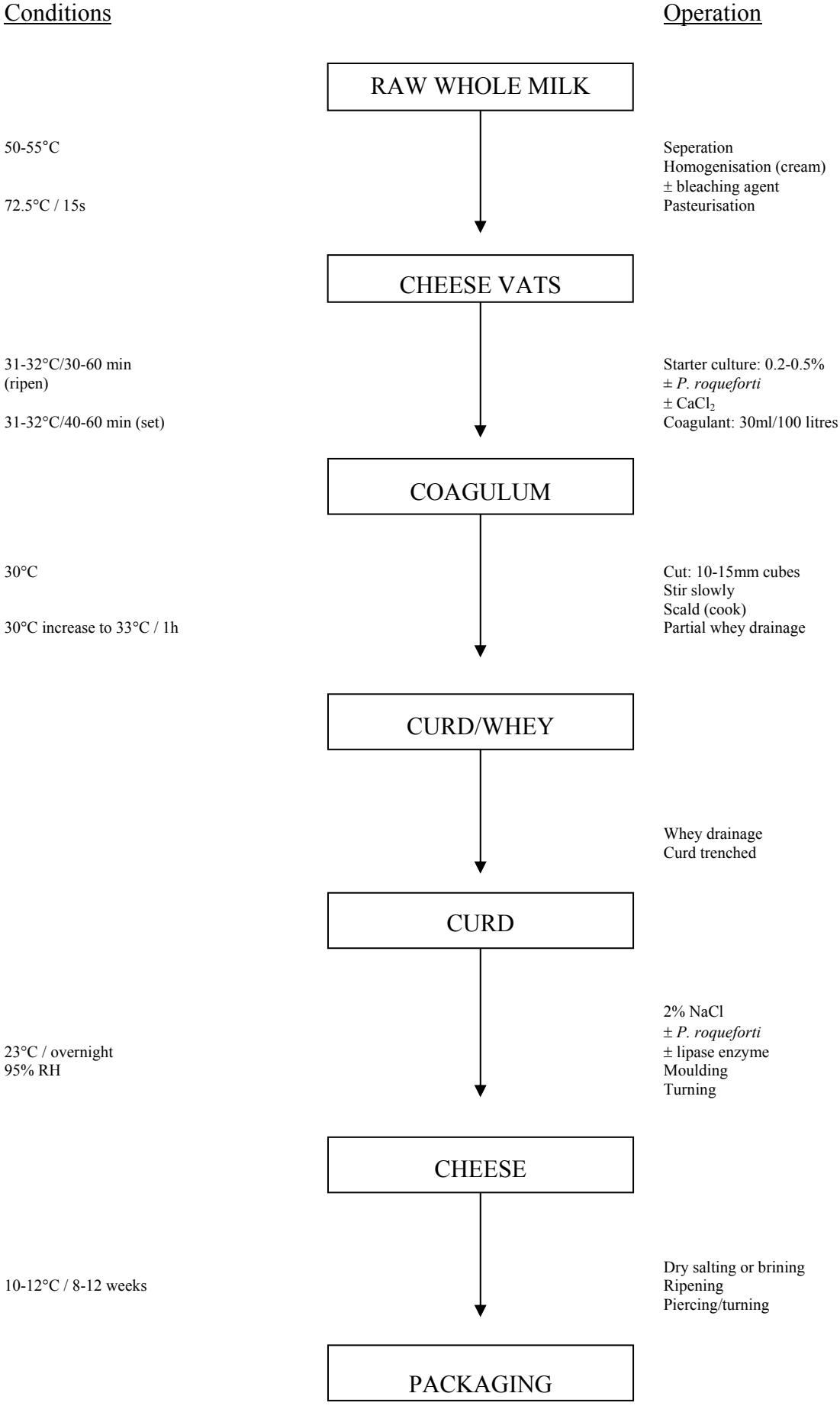
Welthagen, J.J. and Viljoen, B.C. (1998a) The presence of yeasts in different types of cheese. In Yeast in the dairy industry: Positive and negative aspects (Eds R.K. Robinson, C.A. Batt, P.D. Patel) pp.78-88. Brussels. International Dairy Federation.

Welthagen, JJ. and Viljoen, BC. (1998b) Yeast profile in Gouda cheese during processing and ripening. *Int. J. Food Microbiol*. **41**, 185-194

Welthagen, JJ. and Viljoen, BC. (1999) The isolation and identification of yeasts obtained during the manufacture and ripening of cheddar cheese. *Food Microbiol*. **16**, 63-73.

Wojtatowicz, M., Chrzanowska, J., Juszczyk, P., Skiba, A. and Gdula, A. (2001) Identification and biochemical characteristics of yeast microflora of Rokpol cheese. *Int. J. Food Microbiol*. **69**, 135-140.

Wyder, M.T. (1998) Identification and characterisation of the yeast flora in kefyr and smear ripened cheese – contribution of selected yeasts to cheese ripening. PhD dissertation, Diss. ETH No. 12842., Zurich, Swiss Federal Institute of Technology.


Conditions
Operation
RAW WHOLE MILK
50-55°C
Seperation
Homogenisation (cream)
± bleaching agent
72.5°C / 15s
Pasteurisation
CHEESE VATS
31-32°C/30-60 min
(ripen)
Starter culture: 0.2-0.5%
± P. roqueforti
± CaCl2
31-32°C/40-60 min (set)
Coagulant: 30ml/100 litres
COAGULUM
30°C
Cut: 10-15mm cubes
Stir slowly
Scald (cook)
30°C increase to 33°C / 1h
Partial whey drainage
CURD/WHEY
Whey drainage
Curd trenched
CURD
2% NaCl
± P. roqueforti
23°C / overnight
95% RH
± lipase enzyme
Moulding
Turning
CHEESE
Dry salting or brining
10-12°C / 8-12 weeks
Ripening
Piercing/turning
PACKAGING

CHAPTER 2

Statistical comparison of ten media for the enumeration and isolation of yeasts from blue veined cheese varieties.

ABSTRACT

During the ripening process of blue veined cheese, different microbial groups interact and contribute to the final product. One of the most important of these groups are yeast. Further studies are needed to clarify their specific contribution to the ripening process. In order to accomplish this, a suitable and satisfactory enumeration medium is needed. Consequently, ten selective media were evaluated for their potential to inhibit and suppress the growth of moulds and bacteria without affecting the yeasts. Based on statistically compared data, no significant difference could be found amongst the ten media, except for one. Further studies were performed on the three media considered to be the most effective, MEA + Ox, MEA + NaCl and MEA + BP based on qualitative results. Accordingly, the three selected media were evaluated based on their ability to support the growth of the five most frequently occurring yeast species in blue veined cheese. No significant difference was obtained between two of the three media. MEA + NaCl however, was unable to support the growth of two of the five most dominant yeast species. MEA + Ox and MEA + BP proved to be superior for the enumeration and isolation of yeasts from blue veined cheese. DRBC, RBC and DG18 proved satisfactory regarding the enumeration of yeasts, whereas OGGY, MEA + SP and molybdate containing media are not recommended.

Key words: Selective media, Yeasts, Moulds, Enumeration

2.1 INTRODUCTION

Commercial blue veined cheese produced in most countries, like South Africa, is manufactured from pasteurised bovine milk with the addition of cultures of starter bacteria and moulds (Addis et al., 1998). Ripened dairy products, like blue veined cheese, contain a diverse collection of microorganisms, consisting mainly of moulds, yeasts and bacteria either added or originating as contaminants (Roostita and Fleet, 1996; van den Tempel and Jakobsen, 1998). The added lactic acid bacteria and moulds main function is to contribute to the ripening of the product assuring flavour and aroma development. It has been shown however that yeasts are a vital part of the ripening flora of blue veined cheese, making a substantial contribution to the final flavour and aroma of the product (de Boer and Kuik, 1987; Besançon et al., 1992; Roostita and Fleet, 1996; van den Tempel and Jakobsen, 1998). Further studies are however needed to quantify their specific and important contribution to the ripening process of blue veined cheese.

In order to evaluate the contribution of yeasts to the final product, precise and accurate yeast enumeration results are needed and consequently appropriate selective media need to be evaluated. Selective yeast media currently applied for general purposes are not satisfactory for the enumeration of yeasts in the presence of high numbers of moulds (Roostita and Fleet, 1996; Welthagen and Viljoen, 1997). Clarification of the suitability of selective media for the enumeration of yeasts in a high mould environment will not only aid in eliciting the role of yeasts in the ripening of blue veined cheese, but also contribute to quality management programs (Beuchat, 1992), standardisation for routine processes in the industry and research in the academic field.

Several factors unique to blue veined cheeses and the ripening should be considered during the development and evaluation of media for the enumeration and isolation of yeasts. Factors like the overgrowth of yeasts by moulds, elevated salt concentration, the complexity and diversity of the microflora present all play a substantial role on the final enumeration results (Addis et al., 1998). The overgrowth of yeasts by spreading moulds, results in lower counts of yeasts, worsen by the fact that both are sensitive to

the same antimycotic compounds added as inhibitors in the media (Deak, 1992). Several antimycotic compounds have been applied, with variable success, in restricting the spreading of moulds (Deak, 1992).

The different antimycotic compounds applied include dyes like rose bengal and dichloran, often used in conjunction to enhance their effectiveness. The most frequently used selective media containing these antimycotic compounds are, Dichloran-Rose Bengal-Chloramphenicol-Agar (DRBC), Rose Bengal-Chloramphenicol-Agar (RBC) and Dichloran-18%-Glycerol-Agar (DG18). DRBC and RBC are often used for the enumeration of yeasts in foods containing high concentrations of moulds despite inferior results indicated by RBC (Bovill et al., 2001). DRBC-agar, a modified formula developed by King et al. (1979), is recommended as a general purpose medium for enumeration of yeasts, (Deak et al., 2001) originally developed to restrict the growth of rapidly growing fungi, such as *Rhizopus* and *Mucor* species (Addis et al., 1998). Photo-degradation of rose bengal, leading to cytotoxic compounds, inhibits the growth of yeasts leading to the underestimation of yeasts (Beuchat, 1992). DG18-agar, originally developed to isolate moderately xerophilic fungi from foods with a low a_w (Hocking and Pitt, 1980), also found application in the enumeration of yeasts from foods with a high concentration of moulds, retarding the growth of mould colonies (Deak et al., 2001).

Salts, like sodium- and calcium propionate, sodium chloride and biphenyl also enhance the recovery of yeasts and are therefore a suitable adjunct for yeast selectivity (Rale and Vakil, 1984). Sodium chloride, acting by lowering the a_w which facilitates the recovery of yeasts, has been successfully used in determining the viable mold counts in different foods (Mislivec and Bruce, 1988). The added NaCl inhibits spreading, and slows down the growth of moulds, but reacts with agar to produce a soft, granular medium causing problems with inoculation during spread plating (Hocking and Pitt, 1980). Biphenyl was originally used for the isolation of yeasts from cider factories where it has been shown to have an inhibitory effect on the growth and spreading of moulds (Addis et al., 1998). This has been broadened and used with great success in enumerating yeasts from foods with a high concentration of moulds.

Recently, another group of antimycotic compounds consisting of antibiotics was introduced. Previously no anti-fungal antibiotic was readily available, until oligomycin

was applied by Sheridan (1992) as an adjunct to yeast extract-glucose-chloramphenicol agar (YGCA) in order to inhibit moulds, with good success. The application of the antibiotic was refined by Eliskases-Lechner and Prillinger (1996) and showed great promise in inhibiting the spreading of moulds. Unrelated chemicals like ox-bile (ox-gal), molybdic acid and gentamycin have also been evaluated with variable success (Mossel et al., 1975; Rale and Vakil, 1984; Beuchat et al., 1992).

2.2 MATERIALS AND METHODS

2.2.1 Samples

A South African produced Danish-style blue veined cheese representative of the same batch was purchased from a local supermarket and immediately analysed for microbial status.

2.2.2 Media

Dichloran Rose Bengal Chloramphenicol Agar (DRBC-agar; Oxoid, Basingstoke, England, CM727); Rose Bengal Chloramphenicol Agar (RBC-agar; Biolab, C107); Dichloran Glycerol 18 Agar (DG18-agar; Oxoid, Basingstoke, England, CM729) and Oxytetracycline Glucose Yeast Agar (Merck, Darmstadt, Germany, 10877) were prepared according to the manufactures instructions. Chloramphenicol (Fluka, Buchs, Germany, 23275) was added, where indicated, at a concentration of 100μg/ml after filter sterilisation using a 0.22μm filter. Analytical grade glycerol (Fluka, Buchs, Germany) was added to DG18-agar to reduce the a_w as indicated by the manufacturers. In order to temper OGYYA medium, 0.1g oxytetracycline (filter sterilised) and 0.05g gentamycin (filter sterilised) were added aseptically.

Molybdate agar was prepared according to Atlas (1993). A modified version of the medium (Molybdate agar + sodium propionate at 0.125%; Molybdate+SP) was also prepared by adding 12.5ml of a sterile 10% sodium propionate solution prior to use. Sodium propionate was prepared and kept in a 10% stock solution, sterilised by autoclaving.

Malt Extract agar (MEA; Merck, Darmstadt, Germany, C10) supplemented with 7.5% NaCl (w/v) (Merck, Darmstadt, Germany, 6400) was prepared according to the method of Mislivec and Bruce (1988), substituting MEA for PDA. Malt Extract agar (MEA) supplemented with 0.05% biphenyl (w/v) (BDH, Poole, England) was prepared according to the method of Addis et al. (1998). The biphenyl was dissolved in ethanol.

Malt Extract agar (MEA), supplemented with 12.5ml of a sterile 10% sodium propionate solution was prepared according to the manufacturers instructions. Malt Extract agar (MEA), supplemented with 0.04% ox-bile (w/v) (Oxoid, Basingstoke, England, L50) was prepared as described by Roostita and Fleet (1996).

2.2.3 Examination of cheese sample

10g cheese sample diluted in 90ml sterile peptone water was homogenised in sterile plastic bags (Whirl-pak, Nasco) with the aid of a Colworth 400 stomacher (London, UK) for 2 min. Further dilutions were prepared as required in 9ml sterile peptone water and 0.1 ml of the aliquots spread plated onto fresh plates of the media described above. This was performed in triplicate. Inoculated plates were incubated upside down in the dark without being disturbed at 25 ± 2°C for 5 days. Data were recorded concerning the counts obtained, the size and shape of the colonies and ease of count. The entire process of plating out on the relevant media was repeated five times.

2.2.4 Comparison of media using yeast strains.

Five yeast strains were used: *Debaryomyces hansenii* UOFS Y – 0219, *Yarrowia lipolytica* UOFS Y – 1138, *Torulaspora delbrueckii* UOFS Y – 0227, *Kluyveromyces marxianus* UOFS Y – 0866 and *Saccharomyces cerevisiae* UOFS Y – 2169.

Yeast strains were streaked on Malt Extract agar (MEA) and incubated at 25 ± 2°C for 5d, after being examined microscopically for purity. After purity was confirmed strains were inoculated into 9ml sterile peptone water. Selected dilutions (0.1ml) for each strain were spread plated onto MEA+Ox, MEA+NaCl and MEA+BP in triplicate. Plates were incubated at 25 ± 2°C for 5d.

After incubation plates containing between 15 and 150 colonies were selected for enumeration. Data were recorded concerning the counts, the size and shape of the colony and the ease of count. The process was repeated five times.

2.2.5 Statistical analysis of results

Results were statistically analysed by means of Analysis of Variance (ANOVA). Means were compared by using the Student-Neumann-Keuls test. A significant F-value of $p \leq 0.05$ was employed (Schefler, 1979).

2.3 RESULTS AND DISCUSSION

2.3.1 Mould counts

Mould counts in the cheese samples investigated represented an average of $\log_{10}$ 6.00 $\pm$ 0.20, ranging from a minimum of $\log_{10}$ 2.50 to a maximum of $\log_{10}$ 6.15 as obtained using MEA + NaCl and Molybdate + SP respectively (Table1).

Statistical analysis (ANOVA) of the results obtained with media as factors and a probability level of 0.05 showed no significant difference between the media for the suppression of moulds, except for MEA + NaCl, which was significantly different ($p < 0.05$) compared to the other nine media. MEA + NaCl was able to suppress the growth and spread of moulds better than any of the other nine media, resulting in a low number of moulds recovered (Table1). The remaining selective media did not suppress the growth or inhibit the spread of moulds, resulting in counts that showed no significant difference ($p >0.05$).

Numerous studies have shown that differences exist between media, but the data generated from different media are not significant (Koburger and Farhat, 1975). Based on the current data obtained differences in appearances and size of colonies exist relating to the ability of the media to suppress the growth and spread of moulds, but the difference in enumerating results was not significant, except for one of these media. The use of a diverse range of media for the suppression of the growth and spread of moulds during the enumeration and isolation of yeasts from blue veined cheese is therefore not incorrect, as most media tested showed no significant difference and a need to standardise to one medium cannot be motivated based on these results.

Differences in the visual quality could be detected and therefore the qualitative results between the different enumeration media differed dramatically. These differences can be defined as larger colonies and colonies with less overgrowth. This is very important when considering a medium for the isolation and enumeration of microorganisms, as visual quality greatly determines the easiness of count of colonies recovered.

Spreading of moulds occurred on all media except two, namely MEA + NaCl and MEA + BP. The addition of biphenyl resulted in sufficient inhibition of mould colonies, as was the case with the reduced a_w due to the addition of NaCl. Limited spreading of moulds occurred on DRBC, RBC, DG18 and MEA + Ox which did not interfere with the enumeration of yeasts. Molybdate, Molybdate + SP and OGGY were unable to inhibit the spreading of moulds and often fused with yeast colonies. The diameter of the mould colonies was restricted on most media, ranging from small to medium in size. The only exceptions were MEA + SP, Molybdate and Molybdate + SP where the diameter of the mould colonies were very large.

2.3.2 Yeast counts

Yeast counts in the cheese samples investigated ranged from a minimum of $\log_{10}$ 4.97 to a maximum of $\log_{10}$ 6.34 with an average of $\log_{10}$ 5.30 $\pm$ 0.20.

Statistical analysis (ANOVA) of the results obtained, with media as factors and a probability level of 0.05, showed no significant difference between the media for the recovery of yeasts (Table1). All the media performed equally adequate for the recovery of yeasts. While differences existed between media for the recovery of yeasts, none of these differences were significant. The formulation of the media tested was of such a nature that it only suppressed the growth of moulds and inhibited their spreading. No additional compounds were added to increase the recovery of yeasts. It is therefore not surprising that there is no significant difference between media in the recovery of yeasts.

Evidence exists suggesting that rose bengal added to media suppresses the growth and inhibits the spreading of moulds, but also inhibits some yeasts (Deak, 1992). No such evidence could be found from this study to support this view as there was no significant difference in the recovery of yeasts from media containing rose bengal, when compared to other media.

In terms of colony appearance most media performed satisfactory. Yeast colonies appeared clear and separate, with no clumping, making enumeration easy. The only

exception was media containing molybdate. On these media yeast colonies appeared having a dark colour and blended with the dark colour of the medium, making enumeration difficult. In terms of colony size most media exhibited yeast colonies moderate in size, making enumeration easy. The only exception was MEA + BP where yeast colonies tended to be small in size making enumeration more tedious, but still acceptable. Media containing molybdate again proved to be a problem since clear yeast colonies could not be distinguished from the background. Bacterial growth was not detected on any of the media indicating that the antibiotics used, either alone or in conjunction with other anti-bacterial factors such as reduced $a_{w,}$ were effective.

DRBC agar gave satisfactory results in terms of qualitative and quantitative criteria. Only slight overgrowth of yeasts occurred. The combination of rose bengal and dichloran proved effective in inhibiting the spreading of moulds, although not totally effective (Addis et al., 1998). Although RBC-agar only contains one antimycotic compound, namely rose bengal, no significant difference between media containing one or more antimycotic compounds was found. DG18-agar was found to be equally effective as DRBC- and RBC-agar, and no significant difference could be detected between these media. The lowered a_w of DG18-agar combined with dichloran proved effective in inhibiting the spreading of moulds, although again not totally effective as yeasts were slightly overgrown.

Although no significant difference could be detected between MEA + NaCl, MEA + BP and MEA + Ox and the other media, these three media proved to be most satisfactory. Despite the development of mould colonies, the colonies were small and no overgrowth of the yeasts was experienced. Yeast counts obtained from MEA + NaCl seemed not to be influenced by the high concentration of NaCl. This was expected, as the yeasts were recovered from blue veined cheese, which normally contains a high concentration of NaCl. A significant difference in results exists regarding the recovery of five dominant dairy associated yeasts on the three media (Table 2). MEA + NaCl totally inhibited the recovery of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* and therefore proved inadequate as a general purpose medium for the enumeration of yeasts in a mould environment, while MEA + Ox and MEA + BP proved to be adequate.

OGGY and MEA + SP proved unsatisfactory when compared with the other media and cannot be recommended for the enumeration and isolation of yeasts from blue veined cheese. This is mainly due to the fact that overgrowth of yeasts occurred to a large extend, making enumeration almost impossible. This is in contrast with findings from other researchers (Deak, 1992; Addis et al., 1998) who reported that OGGY was a suitable medium for the enumeration and isolation of yeasts from foods containing moulds.

2.4 CONCLUSION.

Although there was no significant difference between the ten media based on the quantitative results, it is evident from the qualitative results that MEA + Ox and MEA + BP were superior for enumeration and isolation of yeasts from blue veined cheese when compared to the other media. The addition of biphenyl or ox-gal proved effective in suppressing the growth and inhibiting the spreading of moulds. Although MEA + NaCl also proved highly effective in suppressing the growth and inhibiting the spreading of moulds, it totally inhibited the growth of two of the five most frequent occurring yeast species in blue veined cheese. This makes it unsuitable for the ecological studies of the yeast flora from blue veined cheese.

DRBC, RBC and DG18 performed satisfactory and no significant difference based on yeast recovery could be detected between the media. They all suppressed the growth and inhibited the spreading of moulds, although slight spreading of the moulds did occur. This did not occur to the extent that it greatly interfered with the enumeration of yeasts. OGGY, MEA + SP and molybdate containing media performed unsatisfactory and cannot be recommended for the enumeration of yeasts from blue veined cheese.

2.5 REFERENCES

Addis, E.P.H., Fleet, G.H. and Cox, J.M. (1998) Evaluation of methods for the isolation and enumeration of yeasts in mould-ripened cheese. In Yeasts in the dairy industry : positive and negative aspects **(**Eds M. Jakobsen, J. Narvhus and B.C. Viljoen) pp.109-116. Brussels. International Dairy Federation.

Atlas, R.M. (1993) Handbook of Microbiological Media. (Ed L.C. Parks) p.614, CRC Press. London.

Besançon, X., Smet, C., Chabalier, C., Rivemale, M., Reverbel, J.P., Ratomahenina, R. and Galzy, P. (1992) Study of surface yeast flora of Roquefort cheese. *Int. J. Food Microbiol*. **17**, 9-18.

Beuchat, L.R. (1992) Media for detecting and enumerating yeasts and moulds. *Int. J. Food Microbiol*. **17**, 145-158.

Bovill, R., Bew, J. and Robinson, S. (2001) Comparison of selective media for the recovery and enumeration of probiotic yeasts from animal feed. *Int J. Food Microbiol.* **67**, 55-61.

De Boer, E. and Kuik, D. (1987) A survey of the microbiological quality of blue-veined cheeses. *Neth. Milk Dairy J*. **41**, 227-237.

Deak, T. (1992) Media for enumerating spoilage yeasts - collaborative study. In Modern Methods in Food Mycology (Eds R.A. Samson, A.D. Hocking, J.L. Pitt and A.D. King) pp. 31-38. Amsterdam. Elsevier Scientific Publications.

Deak, T., Chen, J., Golden, D.A., Tapia, M.S., Tornai-Lehoczki, J., Viljoen,B.C. Wyder, M.T. and Beuchat, L.R. (2001) Comparison of dichloran glycerol (DG 18) agar with general purpose mycological media for enumerating food-spoilage yeasts. *Int. J Food Microbiol.* 67, 49-53.

Eliskases-Lechner, F., Prillinger, H. (1996) Inhibition of mould growth by oligomycin during quantative analysis of yeasts. *J. Dairy Science.* **63**, 483-488.

Hocking, A. and Pitt, J. (1980) Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. *Appl. Environ. Microbiol*. **39**, 488-492.

King, A.D., Hocking, A.D. and Pitt, J.I. (1979) Dichloran-rose bengal medium for enumeration and isolation of molds from foods. *Appl. Environ. Micobiol.* **37**, 959-964.

Koburger, J.A. and Farhat, B.Y. (1975) Fungi in foods. IV. A comparison of media to enumerate yeasts and moulds. *J. Milk Food Technol*. **38**, 466-468.

Mislivec, P.B. and Bruce, V.R. (1988) The use of sodium chloride for determining viable mold counts and mold flora in foods. *J. Food. Prot*. **51**, 770-772.

Mossel, D.A.A., Vega, C.L. and Put, H.M.C. (1975) Further studies on the suitability of various media containing antibacterial antibiotics for the enumeration of moulds in food and food environments. *J. Appl. Bacteriol*. **39**, 15-22.

Rale, V.B. and Vakil, J.R. (1984) A note on an improved molydate agar for the selective isolation of yeasts from tropical fruits. *J. Appl. Bacteriol*. **56**, 409-413.

Roostita, R. and Fleet, G.H. (1996) The occurrence and growth of yeasts in Camembert and Blue-veined cheese. *Int. J. Food Microbiol*. **28**, 393-404.

Schefler, W.C. (1979) Statistics for biological sciences, 2nd edn. Addison-Wesley. London.

Sheridan, P.M. (1992) Oligomycin as a selective agent in a medium to determine yeast populations of blue veined cheese. *J. Dairy Science*. **75,** suppl. 1, 131.

Van den Tempel, T. and Jakobsen, M. (1998) Yeasts associated with Danablu. *Int. Dairy J*. **8**, 25-31.

Welthagen, JJ. and Viljoen, BC. (1997) Comparison of ten media for the enumeration of yeasts in dairy products. *Food Research Int*. **30**, 207-211.

Table 1. Mean populations of yeasts and moulds recovered from blue veined cheese on ten media.

Media	Yeast		Mould	
	Mean[a] counts (log_{10} cfu.g^{-1})	SD	Mean[a] counts (log_{10} cfu.g^{-1})	SD
DRBC	**5.47**	**1.20**	**6.11**	**0.23_{c}**
RBC	**5.50**	**1.08**	**6.12**	**0.24_{c}**
DG18	**5.32**	**1.18**	**5.87**	**0.37_{c}**
Molybdate agar	**5.28**	**1.30**	**6.12**	**0.18_{c}**
Molybdate + SP	**5.17**	**1.40**	**6.15**	**0.15_{c}**
MEA + Ox	**5.30**	**1.28**	**5.97**	**0.22_{c}**
MEA + NaCl	**5.40**	**1.18**	**2.50**	**2.32_{b}**
MEA + SP	**5.20**	**1.51**	**6.07**	**0.16_{c}**
MEA + BP	**6.34**	**0.74**	**4.83**	**2.71_{bc}**
OGGY	**4.97**	**1.60**	**6.01**	**0.15_{b}**

[a] Mean values of triplicate samples from five trials

SD - Standard deviation of the mean within each sample of the same media from five trials.

Means with different letters (subscripts) in the same column differ significantly (P<0.05)

DRBC = Dichloran Rose Bengal Chloramphenicol agar; RBC = Rose Bengal Chloramphenicol agar; DG18 = Dichloran Glycerol 18 agar; Molybdate + SP = Molybdate agar + Sodium propianate; MEA + Ox = Malt Extract agar + Ox-bile; MEA + NaCl = Malt Extract agar + sodium chloride; MEA + SP = Malt Extract agar + sodium propionate; MEA + BP = Malt Extract agar + biphenyl; OGGY = Oxytetracycline Gentamycin Glucose Yeast extract agar.

Table 2. Mean populations of yeasts recovered on three selective media.

Yeasts	Mean[a] populations (log_{10} cfu.g^{-1}) Selective medium					
	MEA + Ox	SD	MEA + NaCl	SD	MEA + BP	SD
Saccharomyces cerevisiae	**4.89**	**2.74_c**	**0.00**	**0.00_b**	**6.14**	**0.25_c**
Debaryomyces hansenii	**6.83**	**0.13_c**	**6.46**	**0.12_b**	**6.41**	**0.22_b**
Candida famata	**7.14**	**0.05_c**	**6.56**	**0.09_b**	**7.04**	**0.07_c**
Yarrowia lipolytica	**6.31**	**0.18**	**6.22**	**0.10**	**6.22**	**0.14**
Kluyveromyces marxisanus	**6.59**	**0.09_c**	**0.00**	**0.00_b**	**0.00**	**0.27_c**

[a] Mean values of triplicate samples from five trials.

SD - Standard deviation of the mean within each sample of the same media from five trials.

MEA + Ox = Malt Extract agar + Ox-bile; MEA + NaCl = Malt Extract agar + sodium chloride; MEA + BP = Malt Extract agar + biphenyl.

CHAPTER 3

Development of yeast and lactic acid bacteria populations during the processing and ripening of blue veined cheese.

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Development of yeast and lactic acid populations during the processing and ripening of blue veined cheese.

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ABSTRACT

Samples of a variety of blue veined cheeses were analysed during the manufacture and ripening. The aim of the research was to quantify the yeasts and lactic acid bacteria present at the different stages of processing, to determine the origin of the contaminating yeasts and lactic acid bacteria, as well as estimate yeast diversity. Contact- and air samples were taken randomly during the manufacture process at selected points within the cheese plant. Samples from the raw milk, pasteurised milk and during processing were taken on regular intervals and the yeasts isolated were identified. Cheese samples were surveyed during the different ripening periods, including the inner and outer core of the samples.

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 source of post-pasteurisation contamination, as very few yeasts were isolated from the milk and cheese making process itself. Counts of lactic acid bacteria from air samples tended to be low, while counts from equipment and surfaces were higher. The highest number of lactic acid bacteria were detected from brine samples. 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 yeasts isolated from the dairy environment were identified as *Debaryomyces hansenii, Torulaspora delbrueckii, Candida versatilis, Candida antillancae, Cryptococcus albidus,* and *Dekkera anomala*. The most abundant yeasts isolated from the inner and outer core of the ripening cheese were identified as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida versatilis* and *Cryptococcus albidus*. During and at the end of the ripening period, yeasts were present in high numbers, making their occurrence meaningful.

Key words: Blue cheese, Yeasts, Lactic acid bacteria, Identification

INTRODUCTION

The occurrence of yeasts in dairy products like cheese is not unexpected, as these products have variable properties encouraging the proliferation of yeasts. These properties include: high acidity, storage at low temperature, low moisture content and elevated salt concentrations (Fleet, 1990; Viljoen, 2001). The significance of yeasts in cheese is not well understood despite being a major component of the microflora that contribute to the ripening and flavour development (Fleet, 1990; Beresford et al., 2001). Their presence is of major importance as they can be beneficial or detrimental, causing spoilage and in the most extreme cases be a concern to public health (Fleet & Mian, 1987; Jakobsen & Narvhus, 1996).

Blue veined cheese varieties have unique physical and chemical properties which select for the growth and prevalence of specific yeast species (Roostita & Fleet, 1996; van den Tempel & Jakobsen, 1998; Addis et al., 2001). These properties include: high fat and protein concentration, 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.

Yeast species most frequently isolated from blue veined cheeses include: *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus*, *Kluyveromyces lactis* and *Candida* spp. (de Boer & Kuik, 1987; Besancon et al., 1992; Roostita & Fleet, 1996; van den Tempel & Jakobsen, 1998). These species form an integral part of the microflora of blue veined cheese and contribute to the ripening, 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 the pH on the surface of the cheese, resulting in the development of the secondary microflora, like *Brevibacterium linens* (Devoyod, 1990; Wyder & Puhan, 1999).

Traditionally raw milk was used for the manufacture of blue veined cheese varieties, which is still the case for some varieties (de Boer & Kuik, 1987). Currently, however, cheese processors prefer pasteurised milk. 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 & Kuik, 1987). The improved milk quality also resulted in a less diverse population of yeasts, formerly considered as undesirable and a sign of poor hygiene (Jakobsen & Narvhus, 1996; Fleet, 1990). All the yeasts present in the cheese originate as post pasteurisation contaminants as they are not thermoresistant and are therefore not able to survive pasteurisation temperatures (Fleet, 1990; Devoyod, 1990). Several sources within the dairy environment are responsible for the contamination of the milk and cheese during processing. The primary sources include the brine and the factory surfaces, which include the floors, walls and equipment (Seiler & Busse, 1990; Welthagen & Viljoen, 1999; Leclercq-Perlat et al., 2000).

It has been shown that brine contains a high concentration of yeast populations acting as the primary source of contamination (Seiler & Busse, 1990). Brine used for the production of blue veined cheese can contain yeast populations ranging from 10^4 cfu.ml^{-1} up to 10^6 cfu.ml^{-1} (van den Tempel & Jakobsen, 1998). Because of its high salt content, the brine selects for a specific microflora, and consequently yeasts like *Debaryomyces hansenii* and *Yarrowia lipolytica* are frequently encountered, exhibiting high tolerance towards salt (van den Tempel & Jakobsen, 1998; Leclercq-Perlat et al., 2000).

Factory surfaces, floors, walls and equipment contribute to contamination during the manufacturing process (Welthagen & Viljoen, 1999). The floors and walls are usually responsible for the highest number of contaminating yeasts (Welthagen & Viljoen, 1998). The contribution of these surfaces to contamination varies however from factory to factory, depending on the sanitising practices of the factory.

Other sources contributing to a lesser extend to post-pasteurisation contamination include: the air and workers hands and aprons (Welthagen & Viljoen, 1999; Viljoen & Greyling, 1995).

The main function of the lactic acid bacteria, added as a starter, is to produce lactic acid during the manufacture process that serves to decrease the pH of the cheese (Devoyod, 1990). Starter cultures consisting of different species of lactic acid bacteria are used during the manufacturing process. These are defined cultures and consist of

lactic acid bacteria strains specifically selected for their positive contribution towards the cheese making process. Although lactic acid bacteria occur naturally in the environment, in significant numbers, their contribution during the manufacturing process is negligible when compared to the contribution of the defined starter cultures that are used. Despite the various sources of yeast contamination in the immediate environment, the development of the yeasts during processing and maturation is governed by the properties prevailing in the cheese and its vicinity. Lactic acid bacteria produce lactic acid during the manufacture process, thereby lowering the pH. This offers a selective environment for yeast growth, being unfavourable for most bacteria (Devoyod, 1990; Beresford et al., 2001). Similarly the high salt concentration in the brine, storage at low temperature and lower water-activity offer the 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 in cheeses counts as high as 10^8 cells.g^{-1} were detected without any visible effects.

The growth and development of yeasts during the ripening process on the exterior and interior of the cheese differ dramatically within blue veined cheese (Dovoyod, 1990). A 100 fold difference in yeast population size was encountered between the exterior and the interior, with the exterior exhibiting higher yeast populations (Devoyod, 1990). The composition of the yeast populations also differs between the exterior and the interior of blue veined cheeses. The interior yeast population showed a less diverse group compared to the exterior and comprises only a few species like *Kleyveromyces marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Candida versatilis* (Devoyod, 1990). The yeast population representative of the exterior comprises a wider diversity and in addition includes strains of *Candida sake, Candida intermedia* and *Yarrowia lipolytica* (Devoyod, 1990). Populations remain viable until retail although minor differences may be expected (Choisy et al., 1986).

Despite the frequent references to the occurrence and numbers of yeasts in blue veined cheeses, no studies have been indicated on the development of yeasts during processing and ripening. Consequently in this study we endeavored to determine the origin of both contaminating yeasts and lactic acid bacteria in a blue veined cheese plant and their proliferation during processing and maturation.

MATERIALS AND METHODS

Blue veined cheese manufacture

Blue veined cheeses (Gorgonzola-style and Danish Blue-style) were manufactured on four occasions at a commercial cheese factory in the Western Cape region of South Africa. The procedure for cheese making was carried out as described by Kosikowski (1982).

Sampling methods and selection of isolates

On all four occasions, several surfaces (Table1) were sampled in duplicate by means of RODAC contact plates (Favero et al., 1968) using De Mann 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 the isolation of yeasts. Surfaces that could not be sampled by means of RODAC contact plates were sampled by means of sterile swabs. Appropriate serial dilutions were prepared from these swabs and plated onto the same media as mentioned above for enumeration. Air was sampled by using standard settle plates (90mm Petri dishes) containing the same medium with an exposure time of 5 min (Welthagen & Viljoen, 1999).

Samples were also taken during the manufacturing of the blue veined cheeses, at selected points as indicated in Table 2. Liquid samples (1ml) were diluted in 9ml sterile peptone water. For solid samples 10g portions with 90ml sterile peptone water were homogenised in a Colworth 400 stomacher (London, UK) for 2 min.

Further decimal dilutions of the suspensions were performed as required. Aliquots (0,1ml) of the dilutions were spread inoculated over the surface of plates containing the media. The plates for the yeast counts were aerobically incubated at 25°C for 96h, and at 25°C for 48h for the enumeration of lactic acid bacteria. Yeast colonies were isolated from plates with the highest dilution, containing between 30 and 300. The yeast isolates were sub-cultured on Malt Extract agar (MEA) (Merck, C10 - pH 5,4) for 48h at 25ºC

and checked for purity. The pure cultures were stored at 4ºC on Yeast Malt Extract agar (YM) (Wickerham, 1951) slants during the period of investigation, until characterisation.

Sampling during ripening

Gorgonzola-style (13) and Danish Blue-style (26) cheeses from the same batch were kept under controlled conditions at 4ºC at the site of cheese production and sampled directly after processing at consecutive intervals on a weekly basis during ripening over a 13 week period for Gorgonzola-style and 26 weeks for Danish Blue-style. Cheese samples were prepared for microbiological analysis by opening the cheese aseptically with a sterile tier. For each sample, surface and centre, 10g were aseptically weighed into 90ml sterile peptone water in a sterile plastic bag (Whirl-pak, Nasco) and homogenised in a Colworth 400 stomacher (London, UK) for 2min. Further decimal dilutions were performed in duplicate as required, for microbiological assays, in 9ml sterile peptone water and spread plated on the media described above.
Similar incubation procedures were applied as described earlier.

Sample analysis

All plates containing between 30 and 300 colony forming units (cfu) from the highest dilution (or the highest number if below 30), were enumerated and the mean values determined from duplicate samples. Results are the mean values of duplicate plate samples originating from duplicate cheese samples from the same batch.

Yeast Identification

Individual yeast isolates were identified by conducting physiological, sporulation and morphological tests as described by Kurtzman & Fell, (1998). Data were interpreted using the keys of Kurtzman & 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 & Yarrow, 1984). Additional tests performed included growth at 37°C, in 50% D-glucose medium and urea hydrolysis. Assimilation of nitrogen compounds, as performed by means of auxanographic method (Lodder & 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 malt extract agar (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 malt extract agar (Biolab, Merck, Darmstadt) and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium were examined on corn meal agar according to the Dalmau plate technique (Wickerham, 1951).

RESULTS AND DISCUSSION

Yeast development during processing

Enumeration of samples from the dairy environment associated with blue veined cheese manufacture (Table 1) showed similar results to those obtained during the making of Gouda, Cheddar and other cheeses (Fleet & Mian, 1987; Welthagen & Viljoen, 1998).

Air samples, in the vicinity of processing equipment, revealed no yeasts from the production and brine rooms, and low numbers from the cheese ripening rooms. In contrast to the low yeast counts observed from air samples, higher counts of lactic acid bacteria were present, especially in the brine room (Table 1). The low number of yeasts obtained on the air plates exposed to the environment corresponds to earlier data (Viljoen & Greyling, 1995; Welthagen & Viljoen, 1998, 1999) 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 growth of yeasts and lactic acid bacteria (<10 cfu/ 25cm). 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 & Viljoen, 1998). These microbial numbers, however, increased during processing and successive batches (Welthagen & Viljoen, 1999). Building surfaces, including the floors, walls and doors as well as the shelves in the second ripening room exhibited high numbers of yeasts (>100 cfu/25cm), whereas low numbers (<10 cfu/25cm) were detected on the surfaces in the processing room (Table 1). The higher yeast counts observed in the

second ripening room is attributed to the use of wooden shelves. These maintain higher loads of microbial contamination due to increased difficulty for proper cleaning. Movement between the rooms, as indicated by the high yeast counts on the doors leading to the ripening rooms (>300 cfu/25cm) also plays an important role in the spreading of yeasts within the plant. Welthagen & Viljoen (1998) also found that surfaces, like walls and floors, contained a high number of yeasts and therefore make an important contribution to yeast contamination. Counts of lactic acid bacteria tended to be low on building surfaces the only exception being the floor of the brine room, the doors and the shelving used in the ripening rooms. The most likely reason for the higher counts of lactic acid bacteria in the brine room is the handling of the exposed cheese during the brine stage. The high counts of lactic acid bacteria on the doors can once again be attributed to the movement between the different rooms. The high counts of lactic acid bacteria from the shelves can be attributed to the fact that these are difficult to clean properly and therefore harbour higher loads of lactic acid bacteria.

The brine (>10^5 cfu.ml^{-1}) and the vicinity of the brine baths (>50 cfu/25cm) were responsible for enhanced yeast contamination (Table 1). The brine also contained high numbers of lactic acid bacteria, although counts were never as high as those of the yeasts. The high incidence of yeasts associated with the brine has been shown to be a major source of yeast contamination (Seiler & Busse, 1990). Some of these yeasts, like *Debaryomyces 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 ripening period (Welthagen & 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 & Busse, 1990; Welthagen & Viljoen, 1998).

Yeasts isolated from the dairy environment included, *Debaryomyces hansenii*, *Torulapsora delbrueckii, Dekkera anomala, Cryptococcus albidus, Candida versatilis, Candida zeylanoides* and *Dekkera bruxellensis* (Table 3). The occurrence of *Debaryomyces hansenii* on all occasions during environmental samplings was expected as the species has been isolated from the dairy environment by numerous researchers (Fleet & Mian, 1987; Welthagen & 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 in limited numbers (Welthagen & Viljoen, 1998). Despite the presence of *Dekkera anomala* and *Dekkera bruxellensis* on the walls and equipment, these species have not been isolated previously from the dairy environment, and were not recovered during processing or maturation. Therefore, their presence might be regarded as accidental (Deak & 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 2). Counts of yeasts generally tended to increase slightly towards the end. The highest count recorded was 70 $cfu.g^{-1}$ during moulding at the end of the process. The low yeast count could be argued by the fact that yeasts are not added as part of the starter culture and are killed during pasteurisation. Counts of lactic acid bacteria increased during the manufacture process up until the end and reached a maximum (Table 2). This is to be expected as the conditions during manufacture favours their growth and proliferation. Most important of these being an abundance of substrate, namely lactose, while the process temperature further contributes by being at the optimum for the starter culture.

The only yeast species isolated during the processing stage was a representative of *Torulaspora delbrueckii*.

Yeast development during the ripening period

Danish Blue-style

The initial number of yeasts on the surfaces of the cheese directly after processing revealed no yeasts, but rapidly increased to numbers in excess of 10^7 $cfu.g^{-1}$ reaching a maximum of $2{,}39 \times 10^8$ $cfu.g^{-1}$ within 28 days (Fig. 1). Yeasts on the interior remained absent during the initial four weeks after processing, followed by a substantial increase in yeast numbers, reaching counts as high as 10^5 $cfu.g^{-1}$ within the next two weeks (Fig. 1). These excessive changes in yeast numbers during the initial stages may be attributed to the temperature (8-10ºC) during the first ripening period, which favours the

growth of yeasts (Fleet, 1990) and suppresses the growth of lactic acid bacteria (Choisy et al., 1986). The high acidity of the curd, caused by fermentation of lactose by lactic acid bacteria, also favours the growth of yeasts (Fleet, 1990) while osmotolerant yeasts originating as contaminants from the brine and the environment establish on the exterior of the cheese (Seiler & Busse, 1990).

During the ripening period, a substantial difference (>1 log unit) in the number of yeast populations on the exterior compared to the interior of the cheese (Fig. 1) was evident. The high incidence of yeasts on the exterior was due to the direct contact with the environment and therefore the yeasts, originating as contaminants from sources like the brine, surfaces and workers (Devoyod, 1990) colonised on the surface. Yeasts on the surface are furthermore exposed to aerobic conditions, whereas the yeasts in the interior have limited access to oxygen (Fleet, 1990; Deak & Beuchat, 1996) and consequently the former has a competitive advantage reaching higher numbers (Devoyod, 1990). The development of yeasts during the ripening period exhibited a similar trend between the exterior and the interior, but at lower numbers for the interior.

According to literature, the proportion between the yeast numbers on the exterior and the interior during ripening is about 100 to 1 (Lenoir, 1984; Choisy et al., 1986). After the increase in yeast numbers at the initial stages of the ripening, the numbers remained more or less stable after 42 days (Fig. 1). Yeast numbers representative of the interior, gradually increased after 140 days, whereas the numbers on the surface on the other hand declined after 140 days prior to the end of ripening. At the end of the ripening period yeast numbers on the exterior were high (>10^5 cfu.g^{-1}), while in the interior counts exceeded 10^6 cfu.g^{-1}.

Table 4 shows the composition and proportionate representation of yeasts during the ripening period on the surface and the interior of the cheese. A total of 30 isolates were isolated from the interior and 34 from the surface (Table 4). The most predominant species on both was *Debaryomyces hansenii* representative of >50% of the isolates on the surface and interior. Other species encountered like *Torulaspora delbrueckii, Candida versatilis*, *Cryptococcus albidus* and *Saccharomyces cerevisiae* accounted for less than 20% on all occasions. *Debaryomyces hansenii* was also found to be the most frequently occurring species in blue veined cheese in literature (de Boer & Kuik, 1987;

Besancon et al., 1992; Roostita & Fleet, 1996). The predominance of *Debaryomyces hansenii* on the surface and in the interior of the cheese is attributed to the species salt resistance (Fleet, 1990), growth at low temperatures (Welthagen & Viljoen, 1999), poteolytic and lipolytic activity (Welthagen & Viljoen, 1998), the ability to utilise lactic and citric acid (Roostita & Fleet, 1996) and the frequent association with environmental samples (Table 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 (Table 4). This species has been typically found in the raw milk of several dairies and according to van den Tempel & Jakobsen (1998) the possibility might exist that it is not killed through pasteurisation. Despite the absence of yeasts in the pasteurised milk during our survey, the species has been isolated from the equipment (Table 3) and therefore it is possible that the species became established through post pasteurisation contamination. In a survey by Welthagen & Viljoen (1998) of several cheese varieties, ranging from hard- to soft-cheeses, it was found that *Torulaspora delbrueckii* was present in almost 40% of the samples.

Candida versatilis was found on the exterior and in the interior of the Danish Blue-style cheese, originating from the brine (Table 3). The presence of the species in blue veined cheese varieties is attributed to its ability to grow in high saline conditions and its varying ability to utilise lactic acid (Barnett et al., 1984). According to Barnett et al. (1990) it has been isolated from pickling brines. *Candida versatilis* has been found in cheese samples (Devoyod, 1990), but to a lesser extend.

Cryptococcus albidus was only found in the interior of the cheese. The most likely source of contamination is the processing equipment (Table 3) which corresponds with data presented by Welthagen & Viljoen (1999). The species has been recovered from different dairy products such as ice-cream, butter and cheese (Fleet & Mian, 1987; Welthagen & Viljoen, 1998) as well as blue veined cheese varieties where it predominated (Roostita & Fleet, 1996). Its ability to grow at low temperatures and its lipolytic activity mainly contribute to its presence (Welthagen & 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 & Fleet (1996) *Saccharomyces 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 veined cheese is probably based on the utilisation of protein and fat breakdown products from other species (Roostita & Fleet, 1996). Its origin is unknown.

Gorgonzola-style

Yeasts associated with Gorgonzola type blue veined cheese showed similar growth patterns as exhibited by the Danish style blue cheese (Figs. 1&2).

Yeasts present on the surface of Gorgonzola type blue veined cheese rapidly increased directly after processing reaching a maximum of 1.34 x 10^8 cfu.g^{-1} after 49 days of ripening (Fig. 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 x 10^6 cfu.g^{-1} after 49 days. Again the substantial increase in yeast numbers was attributed to the low temperatures (8-10°C), high acidity of the curd and the yeast contaminants originating from the environment. Similar to the Danish style blue veined cheese, yeasts on the surface were significantly higher compared to the interior.

After 49 days of ripening, yeast numbers on the exterior decreased gradually until the end of ripening at day 91 prior to packaging (Fig. 2). Yeasts were still present at substantial quantities of 2.4 x 10^5 cfu.g^{-1}. A similar decreasing profile was followed by the yeasts present in the interior except for a slight increase at the end of the ripening period resulting in a final count of 7.1 x 10^5 cfu.g^{-1}.

The most frequently occurring yeast species on the exterior of Gorgonzola-style blue veined cheese were representatives of *Debaryomyces hansenii*, *Candida versatilis*, *Trichosporon beigelii* and *Torulaspora delbrueckii*. In the interior, an enhanced diversity in the yeast population was noted, represented by *Debaryomyces hansenii, Torulaspora*

delbrueckii, Candida versatilis, Saccharomyces cerevisiae, Candida zeylanoides, Cryptococcus albidus and *Rhodotorula glutinis*.

Again, *Debaryomyces hansenii* clearly predominated on the exterior and interior of the cheese, represented by more than 30% of the population (Table 4). *Candida versatilis* was found to be 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 cheese. All of the above yeast species have also been isolated from the Danish style blue veined cheese. The only species not recovered from Danish style blue veined cheese, were *Trichosporon beigelii, Rhodotorula glutinis* and *Candida zeylanoides* (Table 4). *Trichosporon* species associated with dairy products have been frequently recovered from cheeses (Kaminarides & Anifantakis, 1998; Welthagen & Viljoen, 1998), raw and pasteurised milk (Roostita & Fleet, 1996), and the brine (Seiler & Busse, 1990) originated from the floors and walls, and equipment (Table 4). *Rhodotorula glutinis* and *Candida zeylanoides* have been isolated from cheeses (Viljoen & Greyling, 1995; Minervini et al., 2001) originating from the air, equipment surfaces (Welthagen & Viljoen, 1998; Welthagen & Viljoen, 1999) and brine (Seiler & 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 & Mian, 1987).

Results obtained clearly indicated that the development of yeasts within the blue veined 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 as obtained in Australian and European blue veined cheese varieties (Roostita & Fleet, 1996) were encountered. This can be attributed to the use of pasteurised milk in South African, Australian and certain European varieties (van den Tempel & Jakobsen, 1998). At the end of the ripening period, yeasts were present in both cheese varieties at high numbers, making their occurrence in South African blue veined cheese varieties meaningful. Only a few species proved to be dominant at all times, the selection of which is based on environmental influences or competition between species for survival.

REFERENCES

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

Barnett, J.A., Payne, R.W. & Yarrow, D. (1987). *Yeasts identification program*. Cambridge University Press.

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

Besancon, X., Smet, C., Chabalier, C., Rivemale, M., Reverbel, J.P., Ratomahenina, R. & Galzy, P. (1992). Study of yeast flora of Roquefort cheese. *International Journal of Food Microbiology, 17*, 9-18.

Choisy, C., Desmazeaud, M., Gripon, J.C., Lamberet, G., Lenoir, J. & Tourneur, C. (1986). Microbiological and biochemical aspects of ripening. In: A. Eck (Ed.), *Cheesemaking: Science and Technology* (pp. 62-100). Lavoisier Publishing Inc., New York and Paris.

Deak, T. & Beuchat, L.R. (1996). *Handbook of Food Spoilage Yeasts*. New York: CRC Press.

De Boer, E., & Kuik, D. (1987). A survey of the microbiological quality of Blue-veined cheeses. *Netherlands Milk and Dairy Journal, 41*, 227-237.

Devoyod, J.J. (1990). Yeasts in cheese-making. In J.F.T. Spencer & D.M. Spencer (Eds.), *Yeast Technology.* (pp. 229-240). Helderberg: Springer.

Favero, M.S., McDone, J.J., Robertson, J.A., Hoffman, R.F. & Edwards, R.W. (1968). Microbiological sampling of surfaces. *Journal of Applied Bacteriology*, *31*, 366 – 373.

Fleet, G.H. (1990). Yeasts in dairy products – A review. *Journal of Applied Bacteriology, 68*, 199-211.

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

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

Kaminarides, S.E. & Anifantakis, E.M. (1998). Evolution of the microflora of Kopanisti cheese during ripening. Study of the yeast flora. *Lait, 69*, 537-546.

Kreger–van Rij, N.J.W. (1984). *The Yeasts, A Taxonomic Study.* (3rd Ed.), Elsevier Science Publishers, Amsterdam.

Kosikowski, F.V. (1982). *Cheese and Fermented Milk Foods* (2nd ed.) New York: Brookendale.

Kurtzman, C. & Fell, J. (1998). *The Yeasts – a Taxonomic Study*. (4th ed.) Amsterdam: Elsevier.

Leclercq-Perlat, M.N., Oumer, A., Bergere, J.L., Spinnler, H.E. & Corrieu, G. (2000). Behaviour of *Brevibacterium linens* and *Debaryomyces hansenii* as ripening flora in controlled production of smear soft cheese from reconstituted milk: growth and substrate consumption. *Journal of Dairy Science, 83*, 1665-1673.

Lenoir, J. (1984). The surface flora and its role in the ripening of cheese. *International Dairy Federation Bulletin, 171*, 3- 20.

Lodder, J. & Kreger-van Rij, N.J.W. (1952). *The Yeasts, A Taxonomic Study.* North-Holland Publishing Co., Amsterdam.

Minervini, F., Montagna, M.T., Spilotros, G., Monaci, L., Santacroce, M.P. & Visconti, A. (2001). Survey on mycoflora of cow and buffalo dairy products from Southern Italy. *International Journal of Food Microbiology, 69,* 141-146.

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

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

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

van den Tempel, T. & Jakobsen, M. (1998). Yeasts associated with Danablu, *International Dairy Journal*, *8*, 25-31.

Van der Walt, J.P., Yarrow, D., (1984). Methods for the isolation, maintenance, classification and identification of yeasts. In: Kreger-van Rij, N.J.W. (Ed.), *The Yeasts – a Taxonomic Study,* (3rd ed.). Elsevier, Amsterdam.

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

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

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

Welthagen, J.J. & Viljoen, B.C. (1999). The isolation and identification of yeasts obtained during the manufacture and ripening of cheddar cheese. *Food Microbiology, 16*, 63-73.

Wickerham, L. J. (1951). Taxonomy of yeasts. *Technical Bulletin No. 129*, US Dept Agric., Washington DC.

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

Table 1. Enumeration of environmental dairy associated samples of yeasts and bacteria (results are the means of duplicate samples).

SAMPLE	YEASTS	BACTERIA
Air (cfu/90mm Petri dish)		
Production Room	0	2
Brine Room	3	22
1st Ripening Room	4	11
2nd Ripening Room	2	5
Equipment (cfu/25cm^2)		
Cheese vat	0	0
Net	75	0
Machinery	0	25
Table	126	0
Equipment (cfu.ml^{-1})		
Cheese Moulds	0	980
Humidifier outlet	0	212
Surfaces (cfu/25cm^2)		
Wall – Production	0	0
Wall – Brine	0	0
Wall – 1st Ripening	0	0
Wall – 2nd Ripening	27	0
Surfaces (cfu.ml^{-1})		
Floor – Production	0	0
Floor – Brine	52	1300
Floor – 1st Ripening	259	0
Door 1	1900	1070
Door 2	2105	2700
Shelf – 1st Ripening	0	880
Shelf – 2nd Ripening	375	1200
Brine (cfu.ml^{-1})		
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 2. Enumeration of lactic acid bacteria and yeasts during processing of blue veined cheese.

Elapsed time (hr)	Procedure	Lactic acid bacteria (cfu.ml^{-1} or g^{-1})	Yeasts (cfu.ml^{-1} or g^{-1})
00:00	Milk in vat	2500	0
00:25	Mold suspension	Not determined	0
01:00		1.09×10^5	0
01:30	Starter added	5.21×10^8	0
02:00		2.62×10^8	0
02:40		3.60×10^8	0
03:15		1.50×10^7	0
03:45	Curd cut	7.30×10^7	10
04:00	Stir starts	1.00×10^8	0
04:30		1.10×10^8	0
05:00		6.10×10^7	0
05:30		9.10×10^7	10
06:00		1.39×10^8	0
06:30		6.60×10^7	0
07:00		1.12×10^8	0
07:30		9.40×10^7	60
07:45	Moulding	6.18×10^8	70

Table 3. Yeasts associated with blue veined cheese manufacture according to source.

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 4. Distribution of yeast populations in interior/exterior of Danish-Blue style cheese and Gorgonzola style cheese.

DANISH-BLUE STYLE	INTERIOR	EXTERIOR
Yeast	**Population (%)**	**Population (%)**
Debaryomyces hansenii	53	50
Saccharomyces cerevisiae	10	18
Torulaspora delbrueckii	13	20
Candida versatilis	13	12
Cryptococcus albidus	10	ND

GORGONZOLA STYLE	INTERIOR	EXTERIOR
Yeast	**Population (%)**	**Population (%)**
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

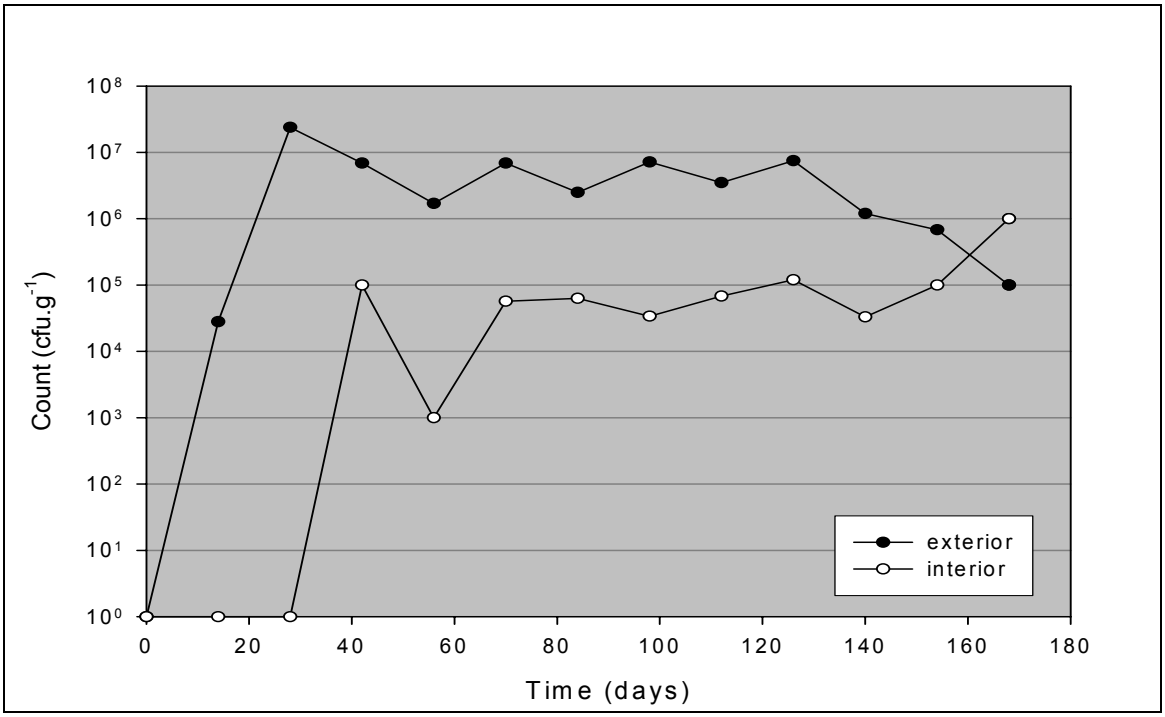

Fig. 1. Survival of yeasts in the interior and exterior of Danish Blue type cheese during ripening.

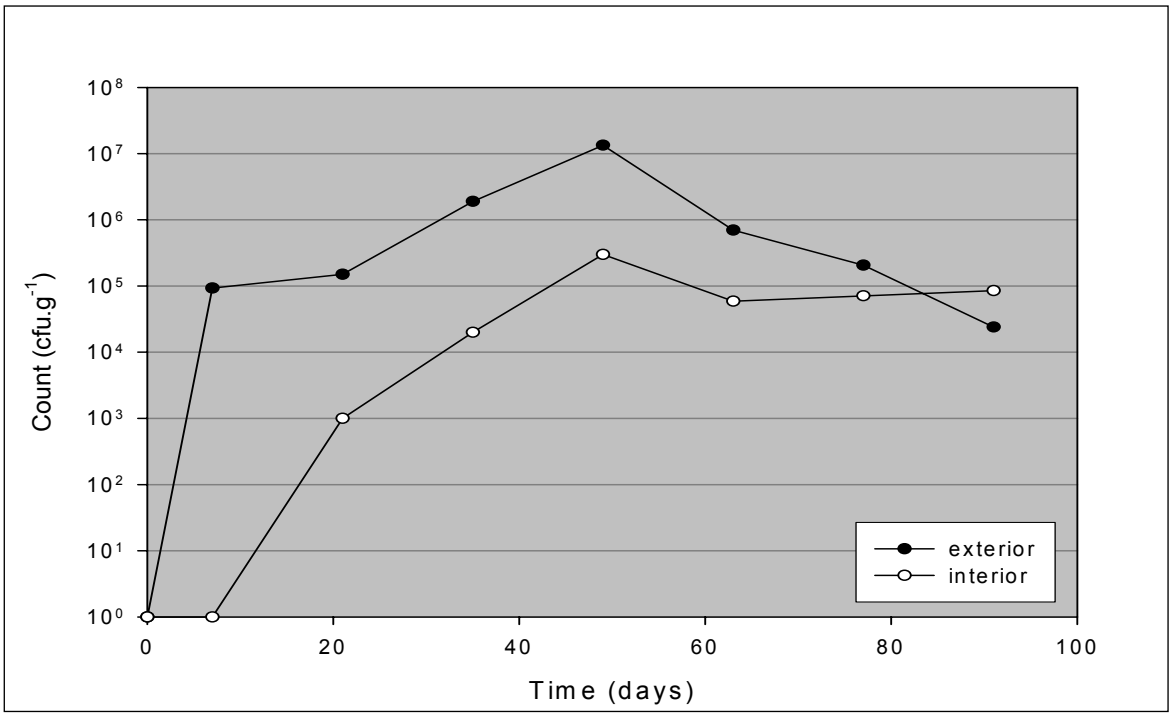

Fig. 2. Survival of yeasts on the interior and exterior of Gorgonzola type cheese during ripening.

CHAPTER 4

Growth, survival and interactions between yeasts, moulds and bacteria during the ripening of blue veined cheese.

ABSTRACT

The growth and survival of three microbial groups namely yeasts, micrococci and coryneform bacteria were followed throughout the ripening period of a locally produced blue veined cheese similar to Roquefort. Representative members of each microbial group were isolated to study the interactions between the yeasts and the bacterial groups. The interactions that occur between these microbial groups play an important role in the ripening and final quality of the cheese. All three microbial groups dominated throughout the ripening period and were present in meaningful numbers at the end of ripening. Parallel development of the populations on the exterior and in the interior was noted for all three microbial groups. Yeasts isolated were identified as *Debaryomyces hansenii; Torulaspora delbrueckii; Candida versatilis; Dekkera anomala; Cryptococcus albidus; Candida saitoana* and *Saccharomyces kluyveri*. The majority of interactions between the yeasts and *Penicillium roqueforti* were neutral, whereas *Cryptococcus albidus* and *Saccharomyces kluyveri* showed negative interactions. Interactions between the yeasts and bacteria were also predominantly neutral, with few exceptions. *Debaryomyces hansenii* exhibited the most stimulatory effects on bacterial species whereas *Torulaspora delbrueckii* represented the most antagonistic effects.

Key words: Interactions, Yeasts, Moulds, Secondary bacteria, Antagonistic

4.1 INTRODUCTION

The ripening of cheese is a complex process, relying on both the growth and biochemical interactions between the different microbial groups found within the ripening cheese to produce a cheese of suitable quality (Fleet, 1999; Addis et al., 2001).

The different microbial groups present during the ripening of blue veined cheeses are quite diverse and include: the primary microflora, namely the lactic acid bacteria (LAB), *Penicillium roqueforti* and the secondary microflora, which are mainly representatives of yeasts and certain genera of bacteria (Addis et al., 2001; Beresford et al., 2001). The primary microflora is inoculated during the production stage. Lactic acid bacteria contribute to the production by producing lactic acid and also play a role during the maturation of cheese (Lopez-Diaz et al., 2000). *Penicillium roqueforti* is the primary ripening agent during the maturation of blue veined cheese varieties and is also inoculated during the production stage (Coghill, 1979; Gripon, 1987). The secondary microflora is not added and originates as post-pasteurisation contaminants during and after the production stage. Most likely sources of contamination include brine solutions, the surrounding atmosphere and contact with equipment and workers (Viljoen and Greyling, 1995; Welthagen and Viljoen, 1998a, 1999; Fleet, 1999).

It has been found by many researchers that yeasts occur throughout the ripening process in different types of cheese and continue to proliferate until the end, often reaching numbers of 10^6 – 10^9 cfu.g^{-1} (Fleet and Mian, 1987; Nooitgedacht and Hartog, 1988; Roostita and Fleet, 1996; Welthagen and Viljoen, 1998a; Fleet, 1999). There is therefore no doubt that yeasts contribute to the ripening of cheese, although the exact nature of the contribution still has to be elicited (Choisy et al., 1986).

Another major component representative of the secondary microflora include certain genera of bacteria belonging to two main groups, namely coryneform bacteria and micrococci. The coryneform bacteria are a taxonomically diverse group of unrelated bacteria, the common property being pleomorphism (Crombach, 1974; Addis, 2002). The two genera of coryneform bacteria that occur most frequently in blue veined cheese

are *Arthrobacter* and *Brevibacterium*, while members of the genus *Corynebacterium* also occur to a lesser extent (Reps, 1993; Gobbetti, 2000). Micrococci originate as post pasteurisation contaminants mainly from the milk and brine (Choisy et al., 1986). Members of this group belong to the genus *Micrococcus*. The exact nature of the contribution of these bacteria to the ripening of cheese has not been studied in much detail, but it is known that they play an important role (Beresford et al., 2001).

It is expected that during the maturation process the different microbial groups present will undergo interactions as the environment is not static but keeps changing dynamically (Fleet, 1999; Addis, 2002). The outcome of these interactions determines the population levels of the different microbial groups present at any time, depending on the type of interaction. Interactions between the different microbial groups can be defined as positive, neutral or negative. Positive interactions stimulate growth while negative interactions inhibit growth and neutral interactions have neither a positive nor a negative effect (Viljoen, 2001; Addis, 2002).

Both yeasts and *Penicillium roqueforti* occur in blue veined cheese varieties in high concentrations and interactions between the two groups are therefore expected (Kronborg Hansen and Jakobsen, 1998; van den Tempel and Nielsen, 2000). Positive interactions usually include enhanced growth of *Penicillium roqueforti*, thicker and more velvet mycelia, a more intense blue colour of *Penicillium roqueforti* and an enhanced effect on the metabolic activity, while negative interactions include inhibition of growth and sporulation of *Penicillium roqueforti* (Kronborg Hansen and Jakobsen, 1998; Hansen and Jakobsen, 2001).

Fermentative yeast species like *Kluyveromyces marxianus* stimulate the growth of *Penicillium roqueforti* through the production of CO_2 during the fermentation of lactose (Coghill, 1979). The CO_2 helps in establishing the open structure, characteristic for blue veined cheese varieties, helping the mould to penetrate the cheese and establish itself (Choisy et al., 1986). Positive interactions between *Penicillium roqueforti, Debaryomyces hansenii* and *Saccharomyces cerevisiae* have been demonstrated (Kronborg Hansen and Jakobsen, 1998; van den Tempel and Nielsen, 2000; Hansen and Jakobsen, 2001; Hansen et al., 2001). Inhibition of *Penicillium roqueforti* by *Kluyveromyces lactis* has been observed being very species specific (Kronborg Hansen

and Jakobsen, 1998). Yeast-bacterial interactions are common in ripening cheese especially related to interactions between yeasts and the lactic acid bacteria used as starter cultures and are well documented and understood (Lenoir, 1984; Devoyod, 1990; Gadaga, 2001; Viljoen 2001; Addis, 2002). The interactions between yeasts and one of the major bacterial groups present during the ripening of blue veined cheese varieties, namely coryneform bacteria, is also well documented and understood (Purko et al., 1951; Lenoir, 1984; Devoyod, 1990; El Soda, 2000). The yeasts aid in the deacidification of the cheese surface by utilising lactic acid resulting in an increase in the pH on the surface and subsequently coryneform bacteria, such as *Arthrobacter* spp., *Brevibacterium* spp. and *Corynebacterium* spp., develop (Reps, 1993; Corsetti et al., 2001). Yeasts stimulate the growth of coryneform bacteria by producing bacterial growth factors such as vitamins and amino acids (Purko et al., 1951; Lenoir, 1984; Fleet, 1990; El Soda, 2000; Leclercq-Perlat et al., 2000; Viljoen, 2001).

The interactions between yeasts and micrococci, the other major bacterial group present during the ripening of blue veined cheese varieties, are not well known nor understood (Addis, 2002). Yeasts stimulate the growth of micrococci in a similar way as experienced for coryneform bacteria, by increasing the pH on the surface of the curd through its lactic acid and alkaline metabolism products (Lubert and Frazier, 1955).

Micrococci are also stimulated by growth factors found in yeast autolysates which include nucleotides, peptides and vitamins (Lubert and Frazier, 1955; Corsetti et al., 2001).

The aim of this study was to supplement the limited collection of literature concerning the interactions between yeasts and secondary bacteria in ripening blue veined cheese varieties. These microbial groups play an important role in the ripening of blue veined cheese varieties and an understanding of how they interact with each other will assist in understanding the complex ripening process that takes place in blue veined cheese varieties and the role of the different microbial groups during this process. More knowledge will also aid in the development of proper starter cultures.

4.2 MATERIALS AND METHODS

4.2.1 Samples

Locally produced blue veined cheese similar to Roquefort, representative of the same batch, was obtained from the manufacturer and sampled weekly for the duration of the ripening period of 12 weeks. Samples were taken by means of a sterile cheese tier and placed in sterile Whirl-pak bags (Nasco, USA). Analysis was usually performed within 48h after sampling.

4.2.2 Sampling methods

For all samples, 10g portions were homogenised in 90ml sterile peptone water (Biolab) in Whirl Pak bags (Nasco, USA) in a Colworth 400 stomacher (London, UK) for 2min and the liquid portion diluted. Further decimal dilutions of the suspensions were prepared as required. Aliquots (0.1ml) of the dilutions were spread inoculated in duplicate over the surface of MSA-plates (Biolab) (micrococci), DRBC (Oxoid, Basingstoke, UK) agar plates (yeasts), LGCS-agar plates (Toolens and Koning-Theune, 1970) (coryneform bacteria) and MEA-plates (Biolab) (moulds). MSA, DRBC agar and MEA were prepared according to the manufacturer's instructions. The LGCS-agar was prepared by adding 3g Lab-Lemco beef extract (Oxoid, Basingstoke, UK); 5g Bacto-peptone (Difco, Michigan, USA); 5g glucose (Biolab); 55g NaCl (Merck, Darmstadt, Germany); 5g $CaCO_3$ (Merck, Darmstadt, Germany); 20g agar (Merck, Darmstadt, Germany) and 10ml of a 0.01% cyclohexamide (Sigma, London, UK) solution to 1L of distilled water. MSA-plates were incubated aerobically at 30°C for 3 days, while DRBC-; MEA- and LGCS-agar plates were incubated aerobically at 25°C for 5 days. After incubation the colonies on the plates were enumerated.

4.2.3 Sample Analysis

Plates containing between 30 and 300 colony forming units (cfu) or the highest number if below 30 were counted and the means determined from duplicate plates.

Yeast colonies were visually selected from plates of DRBC agar. The representative yeast isolates were subcultured on DRBC agar to inhibit the growth of moulds until pure. Pure cultures were stored at 4°C on MEA slants during the period of investigation.

Bacterial colonies were isolated from the plates with the help of a Harrison's disc (Harrigan and McCane, 1976). Selected colonies were subcultured on nutrient agar (Oxoid, Basingstoke, UK) plates until pure. Pure cultures were stored at 4°C on nutrient agar slants during the period of investigation.

Pure cultures of *Penicillium roqueforti* were obtained by visually selecting mould colonies from plates of MEA and subculturing on MEA plates. Pure cultures were stored at 4°C on MEA slants during the period of investigation.

4.2.4 Identification

The yeast isolates were identified using the methods described by Kreger-van Rij (1984) and the computerised identification system of Barnett et al. (1990).

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, in 50% D-glucose medium and urea hydrolysis. Assimilation of nitrogen compounds, as 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 malt extract agar (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 malt extract agar and on Dalmau plates (Kreger-van Rij, 1984). The formation of pseudomycelium and true mycelium were examined on corn meal agar according to the Dalmau plate technique (Wickerham, 1951).

4.2.5 Chemical analysis

The pH was determined at weekly intervals during sampling for the entire ripening period. The pH of the homogenised cheese samples was measured at 24°C with a HI 9321 Microprocessor pH meter (HANNA Intruments, Germany) as described by the methods of Kosikowski (1982).

4.2.6 Microbial interactions

The spot-on-lawn assay (Piddock, 1990) was used to study the interactions between yeasts and moulds, and between yeasts and bacterial cultures. The basal medium used was MYGP agar, applied for both mould-yeast interactions and yeast-bacterial interactions. The medium was prepared by adding to 1L distilled water, 3g malt extract (Difco, Michigan, USA), 3g yeast extract (Difco, Michigan, USA), 10g glucose (Merck, Darmstadt, Germany) 5g Bacto-peptone (Difco, Michigan, USA) and 20g agar (Merck, Darmstadt, Germany). After preparation the solution was divided into 20ml aliquots, sterilised and kept in a molten (50°C) state. Inoculum species were diluted in SPO-solution to 10^5-10^6 cfu.ml^{-1}. Cell counts were estimated by means of microscopy. The SPO-solution was prepared by adding to 1L of distilled water 1g Bacto-peptone (Difco, Michigan, USA), 8.5g NaCl (Merck, Darmstadt, Germany) 0.3g $Na_2HPO_4.2H_2O$ (Merck, Darmstadt, Germany) and 10ml Tween 80 (Merck, Darmstadt, Germany).

The species being tested was seeded at 10^5-10^6 cfu.ml^{-1} into the molten media and poured into a Petri dish. After cooling and solidification the agar medium was spot-inoculated on the surface with another species. The seed and spot inoculum species were grown up as fresh cultures on either MEA-plates for yeasts and moulds or nutrient agar plates for bacteria. Inoculated plates were incubated at either 25°C for 4 days for yeasts and moulds or 30°C for bacteria. Inhibition of the seeded organism by the spot-inoculated organism was indicated by a clear zone surrounding the growth of the spot culture. Stimulated growth of the seeded organism was evident by increased biomass density surrounding the spot culture.

4.3 RESULTS AND DISCUSSION

4.3.1 Growth of yeasts.

Yeasts predominated during the ripening period and were still present at the end of the ripening period in meaningful numbers (Fig. 1). Growth of yeasts initiated at the second week of ripening on the exterior and on the fourth week in the interior. Yeasts on the exterior of the cheese reached a higher concentration compared to the concentration of yeasts in the interior during the ripening period. The most likely reasons for this is the increased amount of available oxygen on the surface and yeasts accumulated onto the surface during the manufacture process (Devoyod, 1990; van den Tempel and Jakobsen, 1998). Yeasts continued to increase on the exterior up until the 8^{th} week of ripening after which the numbers of yeasts stabilised. Yeasts continued to increase in the interior of the cheese up until the 8^{th} week of ripening followed by a stabilisation in numbers, except for a small decrease during the last two weeks of ripening. These results correspond with results obtained elsewhere (Devoyod, 1990; Lopez-Diaz et al., 1995; van den Tempel and Jakobsen, 1998; Wojtatowicz et al., 2001). The proliferation of yeasts on the exterior correlated well with the increase in pH on the exterior (Fig. 4). An increase in pH was evident after the third week of ripening due to the utilisation of lactic acid by the yeasts. The pH continued to increase throughout the ripening period until the end where it reached a maximum. Similar trends in pH values were reported by others (Besançon et al., 1992; Gobbetti et al., 1997; Nichol, 2000).

Yeasts isolated during the ripening period were identified as *Debaryomyces hansenii, Torulaspora delbrueckii, Candida versatilis, Dekkera anomala, Cryptococcus albidus, Candida saitoana* and *Saccharomyces kluyveri*. *Debaryomyces hansenii* has been found to be the dominant yeast species in blue veined cheese varieties and usually occur in high concentrations (Fleet, 1990; Welthagen and Viljoen, 1998b; Beresford et al., 2001).

Torulaspora delbrueckii, Candida versatilis, Dekkera anomala and *Cryptococcus albidus* are also frequently encountered at lower concentrations (Viljoen and Greyling, 1995; Roostita and Fleet, 1996; Welthagen and Viljoen, 1998a; Cosentino et al., 2001).

The occurrence of *Saccharomyces kluyveri* was unexpected due to the high salt concentration present in blue veined cheese, the species has however been isolated from Rokpol (polish blue veined cheese) cheese at high concentrations (Wojtatowicz et al., 2001). *Candida saitoana* does not frequently occur in cheese but has been isolated by Lopandic (2002, unpublished data) from milk products. The species is commonly used as probiotic yeast in animal feed (Bovill et al., 2001).

4.3.2 Growth of secondary bacteria.

The growth curve of micrococci during the ripening period is illustrated in Fig. 2. During the initial stages of ripening micrococci were absent due to the low pH which occurs on the cheese surface during the early stages. Higher numbers only appeared once the yeasts have sufficiently neutralised the surface of the curd (Lopez-Diaz et al., 1995). Between the second and third week of ripening the concentration of micrococci increased dramatically, in excess of 10^6 $cfu.g^{-1}$. This increase correlated with the substantial increase in the concentration of yeasts on the exterior (Fig.1) due to the neutralisation of the cheese surface by the yeasts (Fig. 4). From the third week on until the end of the ripening period the numbers of micrococci stabilised with only minor fluctuations. The substantial numbers were a result of these bacteria being salt-tolerant, having an aerobic metabolism and growing well at ripening temperatures (Lopez-Diaz et al., 1995; Menendez et al., 2001). These results support those found by other authors (Nunez, 1978; Choisy et al., 1986; Lopez-Diaz et al., 1995; Garcia et al., 2002).

During the ripening period counts of micrococci tended to be similar between the exterior and the interior, except at the end when the concentration of micrococci on the exterior converged with those in the interior. This is not uncommon and has been found to occur by other authors (Lopez-Diaz et al., 1995; Menendez et al., 2001).

Despite the frequent occurrence of coryneform bacteria in blue veined cheese varieties the exact population levels and development in mould-ripened cheeses are still unclear (Addis, 2002). During the initial stages of ripening the presence of coryneform bacteria were not detected (Fig. 3) due to the low pH on the surface of the ripening cheese (pH < 5). After two weeks of ripening the concentration of yeasts increased drastically, resulting in an enhanced pH, facilitating the growth of the coryneform bacteria.

Between the second and third week of ripening the concentration of coryneform bacteria increased substantially for both the exterior and the interior. During the remainder of the ripening period the concentration of coryneform bacteria on the exterior revealed moderate fluctuations and at the end of the ripening period were present in meaningful numbers (>10^6 cfu.g^{-1}). During the corresponding period the concentration of coryneform bacteria in the interior exhibited a decrease in accordance with results found elsewhere (Leclercq-Perlat, 2000; Beresford et al., 2001; Bockelmann, 2001).

4.3.3 Microbial Interactions.

4.3.3.1 Yeast-mould interactions.

The results of the interaction studies between the individual isolated yeasts and *Penicillium roqueforti* are summarised in Table 1. Five of the seven yeast species tested against *Penicillium roqueforti* showed a neutral interaction, whereas two of the yeast species, *Cryptococcus albidus* and *Saccharomyces kluyveri*, showed a negative interaction towards *Penicillium roqueforti*. The negative interaction was characterised by an inhibition zone of the mould around the spot-inoculated culture of the yeast species. No positive interactions were detected between any of the yeast species and the mould.

Kronberg Hansen and Jakobsen (1998) found that *Debaryomyces hansenii* showed very little interaction towards *Penicillium roqueforti*, if any. This was true for both whole cells and cell-free extracts. Van den Tempel and Nielsen (2000) studied the effect of various environmental conditions on the interaction between yeasts and *Penicillium roqueforti* and found minor inhibition and stimulation of *Penicillium roqueforti* by *Debaryomyces hansenii* at varying atmospheric conditions. At conditions prevailing during cheese ripening they found slight inhibition of *Penicillium roqueforti* by *Debaryomyces hansenii*. Van den Tempel and Jakobsen (2000) also found minor inhibition of *Penicillium roqueforti* by *Debaryomyces hansenii*. No data exists that demonstrate interactions between any of the remaining yeast species and *Penicillium roqueforti* other than studies between *Saccharomyces cerevisiae* and *Penicillium roqueforti* showing positive results characterised by faster growth and better sporulation (Hansen and Jakobsen, 2001; Hansen et al, 2001).

4.3.3.2 Yeast-bacterial interactions.

Results of the interactions studied between the yeast species and micrococci isolated are shown in Table 2. The majority (83%) of interactions were neutral, while a minority of interactions (17%) were negative and only a small percentage (0.4%) was positive.

Interactions between *Debaryomyces hansenii* and the bacteria were predominantly neutral, although some interactions were shown to be negative and one positive interaction occurred. Interactions between *Torulaspora delbrueckii* and the bacteria were predominantly negative indicating that this specie was the most antagonistic towards the bacteria. Interactions between *Candida versatilis, Cryptococcus albidus, Saccharomyces kluyverii* and the bacteria were predominately neutral and only a few negative interactions occurred. *Dekkera anomala* and *Candida saitoana* showed only neutral interactions.

Addis et al. (2001) studied the interactions between four yeast species, (*Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus* and *Saccharomyces cerevisiae*), 15 strains of *Sthapylococcus* spp. and 4 strains of *Micrococcus* spp. and found no inhibitory interactions between the yeasts and bacteria. Two strains of *Debaryomyces hansenii* had a positive effect on the bacteria characterised by enhanced growth. Lubert and Frazier (1955) found that the film yeasts from brick cheese stimulated the growth of *Micrococcus* spp. by increasing the pH and by secreting bacterial growth factors.

Results obtained based on interactions between the yeast species and coryneform bacteria isolated are shown in Table 3. The majority of interactions (80%) were neutral. The remainder of interactions were equally positive and negative. Interactions between *Debaryomyces hansenii* and the coryneform bacteria were predominately neutral although the species exhibited enhanced stimulatory effects towards coryneform bacteria and a few negative interactions. Interactions between *Torulaspora delbrueckii* and the coryneform bacteria were mostly neutral although a few negative interactions occurred. This species was again the most antagonistic towards the coryneform bacteria, although relatively few negative interactions occurred. *Candida versatilis*, *Dekkera anomala*, *Cryptococcus albidus* and *Saccharomyces kluyveri* showed mostly

neutral interactions towards the coryneform bacteria although limited positive and negative interactions also occurred. *Candida saitoana* displayed no positive or negative interactions towards any of the coryneform bacteria isolated.

Leclercq-Perlat et al. (2000) co-inoculated *Debaryomyces hansenii* and *Brevibacterium linens* in experimental cheese and found that an increased pH due to the utilisation of lactate by the yeasts initiated the growth of the bacterium. Corsetti et al. (2001) found that several yeasts liberated certain unknown bacterial growth factors, stimulating different groups of bacteria such as lactic acid bacteria and coryneform bacteria.

Positive interactions between the yeast species and the bacterial isolates can be ascribed to the increase in pH caused by the yeasts, as well as growth factors secreted by the yeasts (Lubert and Frazier, 1953; Corsetti et al., 2001). The yeast genera most frequently isolated from blue veined cheese furthermore often posses the ability to produce ‘killer’ toxins (Young, 1987). These ‘killer’ toxins and other anti-microbial compounds are also responsible for the negative interactions observed towards the bacterial isolates, although further studies are needed to verify the exact cause (Addis, 2002).

4.4 CONCLUSION

All three microbial groups studied dominated throughout the ripening period and were present in meaningful numbers at the end of the ripening period. Counts of organisms on the exterior surface of the cheese tended to be 10 to 100 times higher than those in the interior. The proliferation of yeasts on the exterior corresponded with the increase in pH on the exterior, indicative of lactate utilisation and as a consequent a decrease in the acidity on the exterior of the cheese. The proliferation of the secondary bacteria was encouraged by the increase in pH reflecting a typical synergestic interaction once the yeasts have suffienctly neutralised the surface of the cheese.

Five of the seven yeast species isolated showed a neutral interaction towards *Penicillium roqueforti* during the yeast-mould interaction studies. *Cryptococcus albidus* and *Saccharomyces kluyveri* showed a negative interaction towards *Penicillium roqueforti*. The majority of yeast-bacterial interactions were neutral. *Debaryomyces hansenii* was found to be the most stimulatory yeast species towards micrococci and coryneform bacteria isolates, while *Torulospora delbrueckii* was the most antagonistic. The positive interactions were most probably due to the increase in pH by the yeasts and the secretion of certain bacterial growth factors whereas the negative interactions were linked to the production of so called ‘killer’ toxins and other anti-microbial compounds by the yeasts. Further studies are needed to elicit the nature of the interactions that occur between yeast species and the secondary bacteria. This is important as both microbial groups contribute to the ripening of the cheese and a better knowledge and understanding of these interactions would assist in the development of proper starter cultures.

4.5 REFERENCES

Addis, E.P.H. (2002) The microbial ecology of Australian Camembert and Blue-veined cheeses during the maturation process. PhD dissertation. University of New South Wales.

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

Barnett, J.A., Payne, R.W. and Yarrow, D. (1990) Yeasts: characteristics and indentification, 2nd edition, Cambridge University Press, Cambridge.

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

Besançon, X., Smet, C., Chabalier, C., Rivemale, M., Reverbel, J.P., Ratomahenina, R. and Galzy, P. (1992) Study of surface yeast flora of Roquefort cheese. *Int. J. Food Microbiol*. **17**, 9-18

Bockelmann, W. and Hoppe-Seyler, T. (2001) The surface flora of bacterial smear-ripened cheeses from cow's and goat's milk. *Int. Dairy J.* **11**, 307-314.

Bovill, R., Bew, J. and Robinson, S. (2001) Comparison of selective media for the recovery and enumeration of probiotic yeasts from animal feed. *Int. J. Food Microbiol.* **67**, 55-61.

Choisy C., Desmazeaud, M., Gripon, JC., Lamberet, G., Lenoir, J. and Tourneur, C. (1986) Microbiological and biochemical aspects of ripening. In Cheesemakning (Eds A. Eck) pp.62-89. New York. Lavoisier Publishing.

Coghill, D. (1979) The ripening of blue vein cheese: A review. *Aust. J. Dairy Technol.* **34,** 72-75.

Corsetti, S., Rossi, J. and Gobbetti, M. (2001) Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *Int. J. Food Microbiol*. **69**, 1-10.

Cosentino, S., Fadda, M.E., Deplano, M., Mulargia, A.F. and Palmas, F. (2001) Yeasts associated with Sardinian ewe's dairy products. *Int. J. Food Microbiol.* **69**, 53-58.

Crombach, W.H.J. (1974) Relationship among coryneform bacteria from soil, cheese and sea fish. *Antonie v. Leeuwenhoek* **40**, 347-359.

Devoyod, J.J. (1990) Yeasts in cheese-making. In Yeast Technology (Eds JFT Spencer and D.M. Spencer) pp.228-240. Berlin. Springer-Verlag.

El Soda, M. (2000) Cheese - role of specific groups of bacteria. In Encyclopedia of Food Microbiology Vol 1 (Eds R.K. Robinson, C.A. Batt and P.D. Patel) pp.393-397. San Francisco. Academic Press.

Fleet, GH.(1990) Yeasts in dairy products. *J. Appl. Bact*. **68**, 199-211.

Fleet, G.H. (1999) Microorganisms in food ecosystems. *Int. J. Food Microbiol*. **50**, 101-117.

Fleet, GH. and Mian MA. (1987) The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiol*. **4**, 145-155.

Gadaga, T.H., Mutukumira, A.N. and Narvhus, J.A. (2001) The growth and interactions of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. *Int. J. Food Microbiol.* **68**, 21-32.

Garcia, M.C., Rodriguez, M.J., Bernardo, A., Tornadijo, M.E. and Carballo, J. (2002) Study of enterococci and micrococci isolated throughout manufacture and ripening of San Simon cheese. *Food Microbiol* **19**, 23-33.

Gobbetti, M., Burzigotti, R., Smacchi, E., Corsetti, A. and De Angelis, M.(1997) Microbiology and biochemistry of Gorgonzola cheese during ripening. *Int. Dairy J*. **7**, 519-529.

Gobbetti, M. (2000) *Arthrobacter*. In Encyclopedia of Food Microbiology Vol 1 (Eds R.K. Robinson, C.A. Batt and P.D. Patel) pp 54-61. San Francisco. Academic Press.

Gripon, J.C. (1987) Mould-ripened Cheeses. In Cheese: Chemistry, Physics and Microbiology Vol 2, (Ed P.F. Fox) pp.121-150. London. Elsevier Applied Science.

Hansen, T.K. and Jakobsen, M. (2001) Taxonomical and technological characteristics of *Saccharomyces cerevisiae* spp. associated with blue veined cheese. *Int. J. Food Microbiol*. **69**, 59-68.

Hansen, T.K., van den Tempel, T., Cantor, M.D. and Jakobsen, M. (2001) *Saccharomyces cerevisiae* as a starter culture in Mycella. *Int. J. Food Microbiol*. **69**, 101-111.

Harrigan, W.F. and McCance, M.E. (1976) Laboratory methods in food and dairy microbiology. London. Academic Press.

Kosikowski, F.V. (1982) Cheese and fermented milk products. 2nd edn Brooktondale, New York, F.V. Kosikowski Assoc.

Kreger-van Rij, N.J.W. (1984) The yeasts-A taxonomic study, 3rd edn Amsterdam, Elsevier.

Kronborg Hansen, T. and Jakobsen, M. (1998) Interactions between *Penicillium roqueforti* and yeast of importance in the production of Danish blue cheese. In Yeasts in the Dairy Industry: Positive and Negative Aspects (Eds M. Jakobsen, J. Narvhus & B.C. Viljoen) pp.50-58. Brussels. International Dairy Federation.

Leclercq-Perlat, M.N., Oumer, A., Bergere, J.L., Spinnler, H.E. and Corrieu, G. (2000) Behaviour of *Brevibacterium linens* and *Debaryomyces hansenii* as ripening flora in controlled production of smear soft cheese from reconstituted milk: growth and substrate consumption. *J. Dairy Sci.* **83**, 1665-1673.

Lenoir, J. (1984) The surface flora and its role in the ripening of cheese. *IDF Bulletin* **171**., 3-20.

Lodder, J. and Kreger-van Rij, N.J.W. (1952) The yeasts, a taxonomic study. Amsterdam. Elsevier North-Holland Publishing Co.

Lopez-Diaz, T.M., Alonso, C., Santas, J., Garcia, M.L. and Moreno, B. (1995) Microbiological changes during manufacture and ripening of a naturally ripened blue cheese (Valdeon, Spain). *Milchwissenschaft* **50,** 381-384.

Lopez-Diaz, T.M., Alonso, C., Roman, C., Garcia-Lopez, M. and Moreno, B. (2000) Lactic acid bacteria isolated from a hand-made blue cheese. *Food Microbiol.* **17**, 23-32.

Lubert, D.J. and Frazier, W.C. (1955) Microbiology of the surface ripening of brick cheese. *J. Dairy Sci.* **38**, 981-990.

Menendez, S., Godinez, R., Centeno, J.A. and Rodriguez-Otero, J.L. (2001) Microbiological, chemical and biochemical characteristics of 'Tetilla' raw cows-milk cheese. *Food Microbiol.* **18**, 151-158.

Nooitgedacht, A.J. and Hartog, B.J. (1988) A survey of the microbiological quality of Brie and Camembert cheese. *Neth. Milk Dairy J.* **42**, 57-72.

Nichol, A.W. (2000) Cheese: Mould-ripened varieties. In Encyclopedia of Food Microbiology Vol 1 (Eds R.K. Robinson, C.A. Batt, P.D. Patel) pp. 387-393. San Francisco. Academic Press.

Nunez, M. (1978) Microflora of Cabrales cheese: changes during maturation. *J. Dairy Research* **45**, 501-508.

Piddock, L.J.V. (1990) Techniques used for the determination of antimicrobial resistance and sensitivity. *J. Appl. Bacteriol.* **68**, 307-318.

Purko, M., Nelson, W.O. and Wood, W.A. (1951) The associative action between certain yeasts and Bacterium linens. *J. Dairy Sci.* **34**, 699-706.

Reps, A (1993) Bacterial surface-ripened cheeses. In Cheese: Chemistry, Physics and Microbiology Vol 2 (Ed P.F. Fox) pp.151-184. London. Chapman and Hall.

Roostita, R. and Fleet, G.H. (1996) The occurrence and growth of yeasts in Camembert and Blue-veined cheese. *Int. J. Food Microbiol*. **28**, 393-404.

Toolens, H.P. and Koning-Theune, W. (1970) A selective medium for the detection of *Brevibacterium linens* in cheese. *Milchwissenschaft* **25**, 79-83.

Van den Tempel, T. and Jakobsen, M. (1998) Yeasts associated with Danablu. *Int. Dairy J*. **8**, 25-31.

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

Van der Walt, J.P. and Yarrow, D. (1984) Methods for the isolation, maintainance, classification and identification of yeasts. In The yeasts – a taxonomic study 3rd edn (Ed N.J.W. Kreger-van Rij) pp. 45-104. Amsterdam. Elsevier.

Viljoen, B.C. (2001) The interaction between yeasts and bacteria in dairy environments. *Int. J. Food Microbiol*. **69**, 37-44.

Viljoen, BC. and Greyling, T. (1995) Yeasts associated with Cheddar and Gouda making. *Int. J. Food Microbiol.* **28**, 79-88.

Welthagen, J.J. and Viljoen, B.C. (1998a) The presence of yeasts in different types of cheese. In Yeast in the dairy industry: Positive and negative aspects (Eds R.K. Robinson, C.A. Batt, P.D. Patel) pp.78-88. Brussels. International Dairy Federation.

Welthagen, JJ. and Viljoen, BC. (1998b) Yeast profile in Gouda cheese during processing and ripening. *Int. J. Food Microbiol*. **41**, 185-194.

Welthagen, JJ. and Viljoen, BC. (1999) The isolation and identification of yeasts obtained during the manufacture and ripening of cheddar cheese. *Food Microbiol*. **16**, 63-73.

Wickerham, L.J. (1951) Taxonomy of yeasts. *U.S. Dept. Agr. Tech. Bull*., 1029.

Wojtatowicz, M., Chrzanowska, J., Juszczyk, P., Skiba, A. and Gdula, A. (2001) Identification and biochemical characteristics of yeast microflora of Rokpol cheese. *Int. J. Food Microbiol*. **69**, 135-140.

Young, T.W. (1987) Killer yeasts. In: The Yeasts Vol. 2 2nd edn. (Eds: A.H. Rose and J.S. Harrison) pp.131-164. London. Harrison Academic Press.

Table 1. Interactions between yeast isolates and *Penicillium roqueforti*.

Organism	Interaction
Debaryomyces hansenii	0
Torulaspora delbrueckii	0
Candida versatilis	0
Dekkera anomala	0
Cryptococcus albidus	-
Candida saitoana	0
Saccharomyces kluyveri	-

(+) positive interaction; (0) neutral interaction and (-) negative interaction.

Table 2. Interactions between yeast- and micrococci isolates.

Organism	Positive[a]	Neutral[b]	Negative[c]
Debaryomyces hansenii	1/38	27/38	10/38
Torulaspora delbrueckii	0/38	14/38	24/38
Candida versatilis	0/38	37/38	1/38
Dekkera anomala	0/38	38/38	0/38
Cryptococcus albidus	0/38	32/38	6/38
Candida saitoana	0/38	38/38	0/38
Saccharomyces kluyveri	0/38	35/38	3/38

[a] Number of isolates found positive over the total number of isolates examined against that strain.

[b] Number of isolates found to be neutral over the total number of isolates examined against that strain.

[c] Number of isolates found negative over the total number of isolates examined against that strain.

Table 3. Interactions between yeast- and coryneform bacteria isolates.

Organism	Positive[a]	Neutral[b]	Negative[c]
Debaryomyces hansenii	11/41	25/41	5/41
Torulaspora delbrueckii	0/41	31/41	10/41
Candida versatilis	4/41	39/41	2/41
Dekkera anomala	3/41	38/41	5/41
Cryptococcus albidus	6/41	29/41	6/41
Candida saitoana	0/41	41/41	0/41
Saccharomyces kluyveri	5/41	36/41	0/41

[a] Number of isolates found positive over the total number of isolates examined against that strain.

[b] Number of isolates found to be neutral over the total number of isolates examined against that strain.

[c] Number of isolates found negative over the total number of isolates examined against that strain

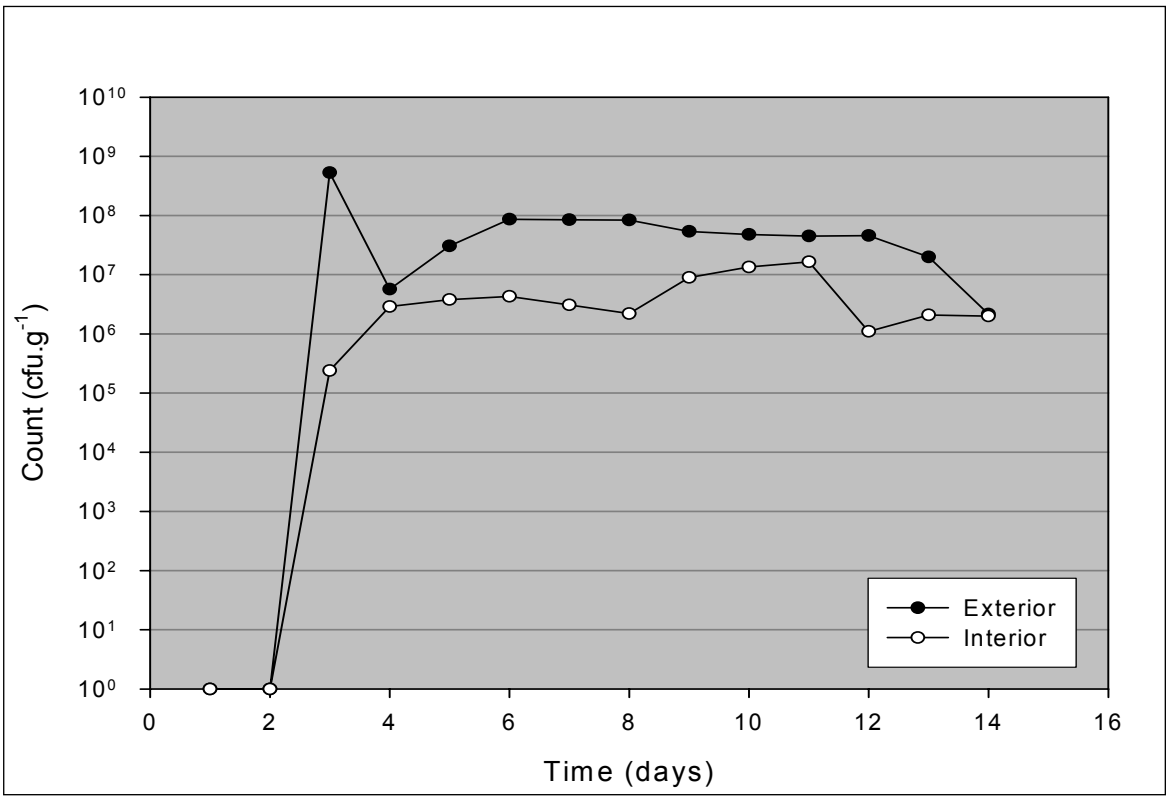

Fig. 1. Proliferation of yeasts during blue veined cheese ripening.

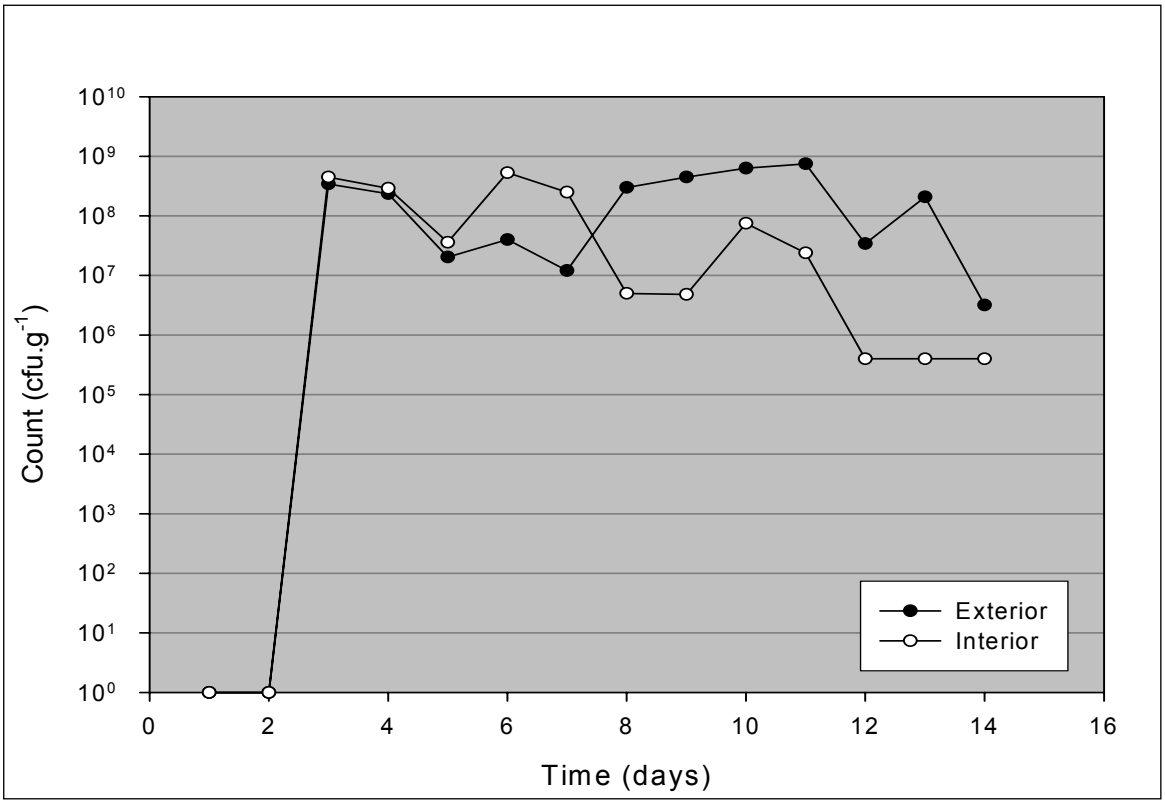

Fig. 2. Proliferation of micrococci during blue veined cheese ripening.

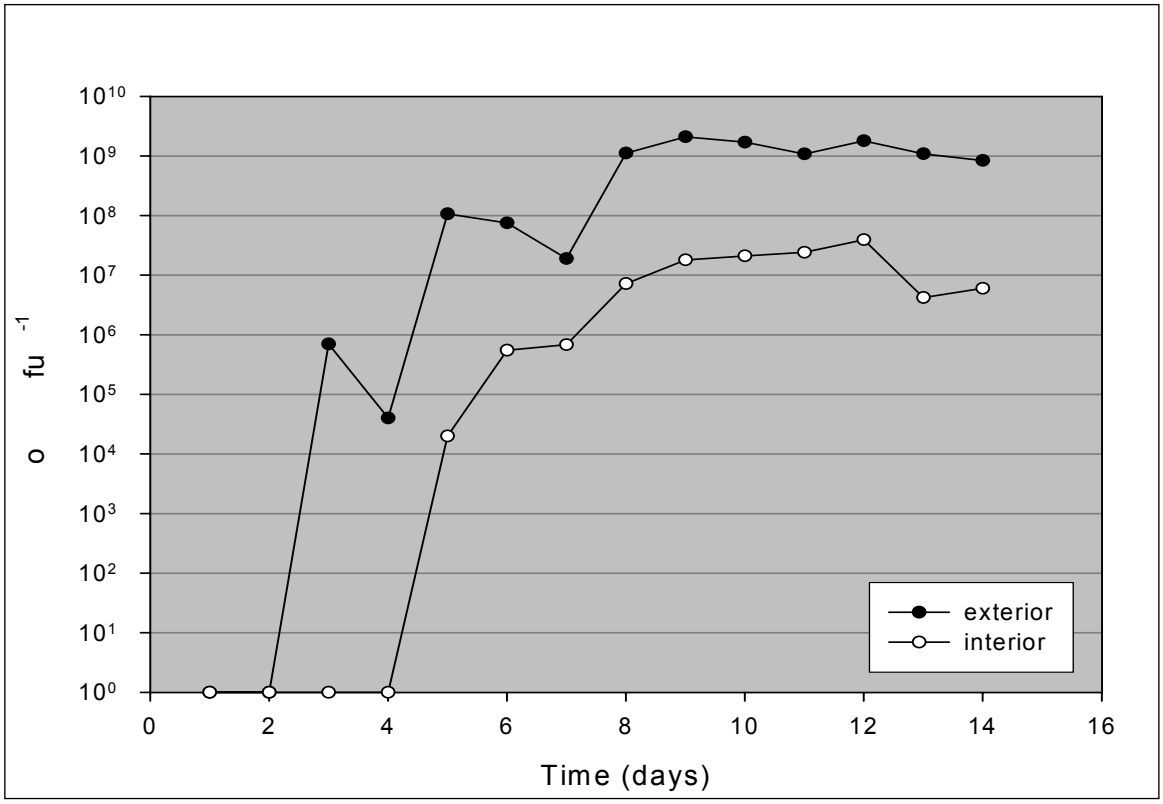

Fig. 3. Proliferation of coryneform bacteria during blue veined cheese ripening.

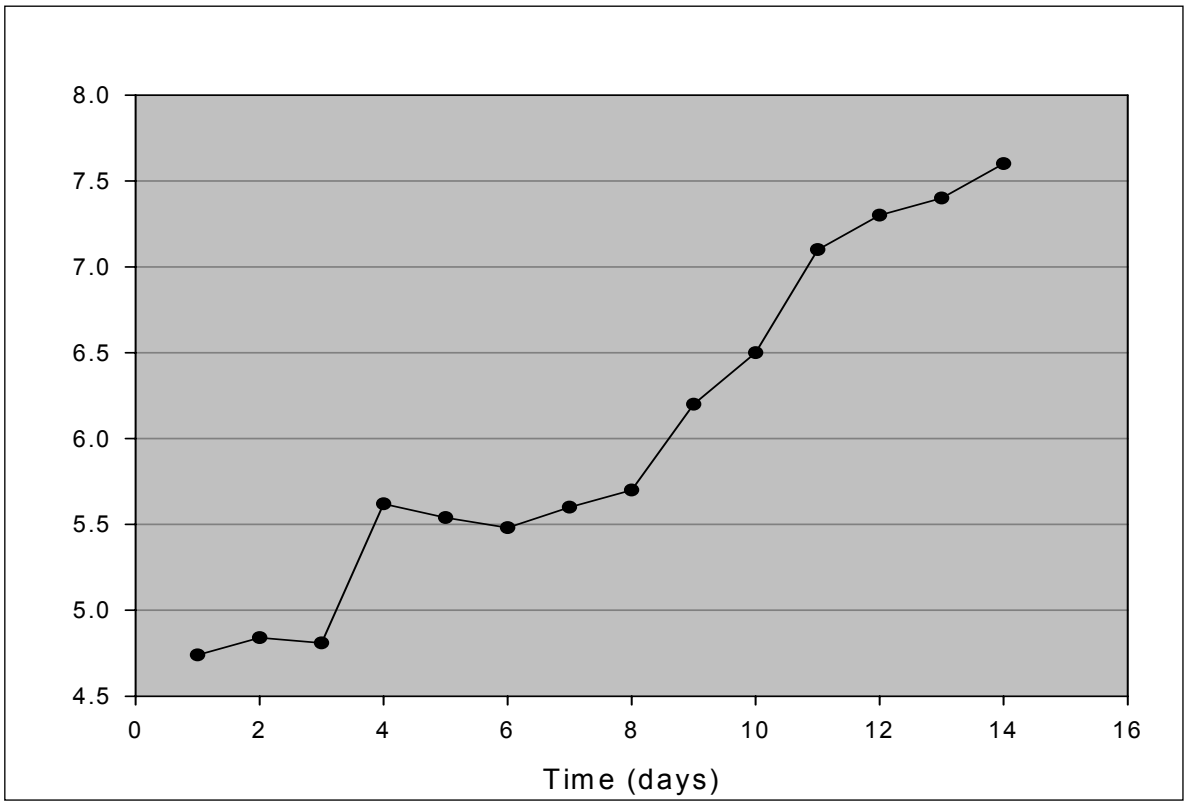

Fig. 4. Change in pH during blue veined cheese ripening.

CHAPTER 5

General discussion and conclusions

Blue veined cheese is a very characteristic cheese variety, produced throughout the world under varying conditions. Characteristic for these varieties are the growth of *Penicillium roqueforti*, the high salt content and a spicy, piquant flavour (Kosikowski, 1970). Some of the most well known blue veined cheese varieties in the world include Roquefort (France), Gorgonzola (Italy), Danish-blue (Denmark) and Stilton (England). All of these different types of blue veined cheese rely on the interactions between the different microbial groups that occur during ripening for the characteristic final flavour and aroma of the product. The exact role of the different microbial groups is not yet clearly understood. This is especially true for yeasts.

The occurrence of yeasts in dairy products and especially blue veined cheese varieties is not surprising as these products inherently possess environmental attributes which favour the growth of yeasts (Fleet, 1990). Many researchers have shown that yeasts occur in meaningful numbers ($>10^6$ cfu.g^{-1}) in most dairy products and blue veined cheeses (de Boer and Kuik, 1987; Besancon et al., 1992; Roostita and Fleet, 1996; van den Tempel and Jakobsen, 1998) which contributes to the outcome of the final product.

Most blue veined cheese varieties around the world are produced from pasteurised milk, except for certain Roquefort-style cheeses, due to increased awareness of public health and the possible health risk associated with unpasteurised dairy products (Purko et al., 1951). The methods for the production of industrial produced blue veined cheese were described in Chapter 1. The three main microbial groups and their growth and contribution to the production and ripening of blue veined cheese were also described. Yeasts as well as secondary bacteria develop as post-pasteurisation contaminants, as they are not added during the manufacture process. The physical as well as chemical factors influencing the growth of yeasts in dairy products are discussed in sections 1.5 and 1.6. During the ripening of blue veined cheese the different microbial groups participate in interactions and metabolic reactions contributing to the ripening of the cheese. The development of yeasts during the ripening period and the interactions between the yeasts and other microbial groups were discussed in sections 1.7 and 1.8 respectively. Although the exact nature of the contribution of yeasts to the ripening of cheese and especially blue veined cheese is not known, there can be no doubt that yeasts contribute positively to the ripening process (Choisy et al., 1986). Yeasts are generally not added as starter cultures during the manufacture process but become

established during the ripening period (Devoyod, 1990). It would be beneficial to replace these ‘wild’ yeasts with yeasts with known and favourable characteristics. The current state and future development of yeast starter cultures in the dairy industry was discussed in section 1.9.

Yeasts are one of the main microbial groups that occur during the ripening of blue veined cheese varieties. A lack in understanding their role and contribution persist and it is imperative that further studies are carried out to quantify their specific and important contribution to the ripening process of blue veined cheese. The selection of media designed for the enumeration of yeasts in the presence of high populations of moulds was therefore essential (Beuchat, 1992). It was important that a suitable medium should be selected and existing selective media evaluated and compared. This aided in the study of the establishment, growth and survival of yeasts in the ripening of blue veined cheese. It could also potentially be of help in the quality management programs of companies and enumeration and isolation of yeasts for routine processes in the industry as well as during research in the academic field. Several media are available but none of these proved truly satisfactory as they failed to support the unique characteristics of yeasts isolated from blue veined cheese varieties, as discussed in Chapter 2 (Welthagen and Viljoen, 1997). The main problem with enumerating yeasts in the presence of high numbers of moulds seemed to be the overgrowth of yeasts by the rapidly growing and spreading mould colonies (Addis et al., 1998). It is therefore necessary to add an anti-mycotic compound to the medium inhibitive towards the moulds but not the yeasts. In Chapter 2, ten different media were evaluated for their suitability to enumerate yeasts from blue veined cheese in the presence of high numbers of moulds. From the results obtained no statistical difference could be detected between any of the selective media.

It was clear however, based on qualitative results, that MEA + Ox and MEA + BP are far more superior.

Qualitatively OGGY, MEA + SP and molybdate containing media cannot be recommended for the enumeration of yeasts from blue veined cheese. Although MEA + NaCl proved to be satisfactory in terms of its quantitative results it cannot be recommended since this medium inhibited two of the five most frequently occurring

yeast species from blue veined cheese.

Yeasts play an important role during the ripening of blue veined cheese and further studies are needed to elicit their exact role during ripening and the specific yeast species that occur. Most milk intended for use in the production of blue veined cheese is pasteurised and yeasts usually become established during ripening. These yeasts originate as post-pasteurisation contaminants from the environment (Devoyod, 1990; Fleet, 1990). Possible sources include the brine, working surfaces, workers and equipment (Seiler and Busse, 1990; Welthagen and Viljoen, 1999). In Chapter 3 the development of yeasts and the diversity of species were studied. Environmental samples were collected from the air, equipment, walls and floors during the manufacture process in the production plant. Air samples revealed few yeasts whereas contact samples from equipment and the surroundings revealed high numbers of yeasts, implicating it as the major sources of post-pasteurisation contamination. Samples from the inner and outer core of the maturing cheeses had typical survival curves, while the number of yeasts on the outer core was about 100 fold more than those in the inner core. The most common yeast species isolated from samples were *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida versatilis* and *Cryptococcus albidus*. During and at the end of the ripening period, yeasts were present in high numbers making their occurrence meaningful.

All the major microbial groups occur during the ripening process of blue veined cheese. These groups participate in interactions contributing to the final flavour, aroma and acceptability of the product (Fleet, 1999; Addis et al., 2001). Limited knowledge is available regarding the interactions that occur between the yeasts, mould and secondary bacteria (Addis, 2002). The objective of Chapter 4 was therefore to study the proliferation of the yeasts, corynebacteria and micrococci during the ripening process and to examine the interactions that occur between the groups. All three microbial groups dominated throughout the ripening process with counts on the exterior exceeding those in the interior. The proliferation of yeast on the exterior corresponded with an increase in pH on the exterior and concomitantly with the multiplication of the secondary bacteria on the exterior of the cheese. The growth of the secondary bacteria was initiated only once the yeasts had sufficiently neutralised the surface of the cheese. Five of the seven yeast species isolated showed a neutral interaction towards

Penicllium roqueforti during the yeast-mould interaction studies. Only *Cryptococcus albidus* and *Saccharomyces kluyveri* exhibited negative interactions towards *Penicillium roqueforti*. The majority of yeast-bacterial interactions were neutral. *Debaryomyces hansenii* was the most stimulatory yeast species towards the micrococci and coryneform bacteria isolates, while *Torulospora delbrueckii* was the most antagonistic. More studies are needed to further elicit the nature of the interactions that occur between yeast species associated with blue veined cheese varieties and the secondary bacteria.

REFERENCES

Addis, E.P.H., Fleet, G.H. and Cox, J.M. (1998) Evaluation of methods for the isolation and enumeration of yeasts in mould-ripened cheese. In Yeasts in the dairy industry : positive and negative aspects **(**Eds M. Jakobsen, J. Narvhus and B.C. Viljoen) pp.109-116. Brussels. International Dairy Federation.

Addis, E.P.H., Fleet, G.H., Cox, J.M. and Leung, T. (2001) The growth, properties and interactions of yeasts and bacteria associated with the maturation of Camembert and blue-veined cheeses. *Int. J. Food Microbiol*. **69**, 25-36.

Addis, E.P.H. (2002) The microbial ecology of Australian Camembert and Blue-veined cheeses during the maturation process. PhD dissertation. University of New South Wales.

Besançon, X., Smet, C., Chabalier, C., Rivemale, M., Reverbel, J.P., Ratomahenina, R. and Galzy, P. (1992) Study of surface yeast flora of Roquefort cheese. *Int. J. Food Microbiol*. **17**, 9-18.

Beuchat, L.R. (1992) Media for detecting and enumerating yeasts and moulds. *Int. J. Food Microbiol*. **17**, 145-158.

Choisy C., Desmazeaud, M., Gripon, JC., Lamberet, G., Lenoir, J. and Tourneur, C. (1986) Microbiological and biochemical aspects of ripening. In Cheesemakning (Eds A. Eck) pp.62-89. New York. Lavoisier Publishing.

De Boer, E., Kuik, D. (1987) A survey of the microbiological quality of blue-veined cheeses. *Neth. Milk Dairy J*. **41**, 227-237.

Devoyod, J.J. (1990) Yeasts in cheese-making. In Yeast Technology (Eds JFT Spencer and D.M. Spencer) pp.228-240. Berlin. Springer-Verlag.

Fleet, GH. (1990) Yeasts in dairy products. *J. Appl. Bacteriol*. **68**, 199-211.

Fleet, GH. (1999) Microorganisms in food ecosystems. *Int. J. Food Microbiol*. **50**, 101-117.

Kosikowski, F.V. (1970). Cheese and fermented milk foods**.** pp. 255-278. New York, Kosikowski Publishers.

Purko, M., Nelson, W.O. and Wood, W.A. (1951) The associative action between certain yeasts and Bacterium linens. *J. Dairy Sci.* **34**, 699-706.

Roostita, R. and Fleet, G.H. (1996) The occurrence and growth of yeasts in Camembert and Blue-veined cheese. *Int. J. Food Microbiol*. **28**, 393-404.

Seiler, H. and Busse, M. (1990) The yeasts of cheese brines. *Int. J. Food Microbiol*. **11**, 289-304.

Van den Tempel, T. and Jakobsen, M. (1998) Yeasts associated with Danablu. *Int. Dairy J*. **8**, 25-31

Welthagen, JJ., Viljoen, BC. (1997) Comparison of ten media for the enumeration of yeasts in dairy products. *Food Research International* **30**, 207-211.

Welthagen, JJ. and Viljoen, BC. (1999) The isolation and identification of yeasts obtained during the manufacture and ripening of cheddar cheese. *Food Microbiol*. **16**, 63-73.

CHAPTER 6

Summary

Blue veined cheese is considered a speciality cheese and is not as popular as other cheese varieties, such as Cheddar or Gouda. Their popularity is however increasing worldwide making it important to study their microbiological, biochemical and technological properties. The aims of this study was consequently to elicit the establishment, growth and survival of yeasts during the ripening of blue veined cheese varieties and to study the interactions between yeasts and the different microbial groups which occur during the ripening process.

The enumeration of yeasts in foodstuffs with a high mould concentration proves to be difficult since yeasts are easily overgrown by the rapidly spreading moulds. This makes the enumeration, isolation and identification of yeasts difficult, hence also eliciting the ecological role of yeasts during the ripening of blue veined cheese varieties. Ten different selective media, containing anti-myctotic properties, were evaluated for their ability to suppress the growth and spreading of moulds and enhance the enumeration and recovery of yeasts from blue veined cheese. Quantitatively no statistical difference between the ten different media was detected. Qualitatively, however OGGY, MEA + SP and molybdate containing media could not be recommended. All other media performed satisfactory while MEA + Ox and MEA + BP gave superior results based on ease of isolation and enumeration. The usage of MEA + NaCl gave comparable results, but inhibited the growth of some of the yeast species most frequently found in ripening blue veined cheese varieties, when evaluated individually.

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. They continued to dominate until the end of the ripening period, in meaningful numbers. 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* once again predominated in the present study. This is 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 organisms to improve our understanding of the ripening process and the contribution each organism makes to the process. Interaction studies in Chapter 4 revealed that various interactions occurred between yeasts and *Penicillium roqueforti*, as well as yeasts and bacteria essential to the final outcome of the product. The majority of yeast-bacterial interactions were neutral, although some interactions were antagonistic and 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.

Key words: Blue cheese, Yeasts, Lactic acid bacteria, Moulds, Identification, Selective media, Interactions, Antagonistic, Stimulate, Secondary bacteria

HOOFSTUK 7

Opsomming

Blou kaas variante word as ‘n spesialiteits kaas beskou en is derhalwe nie so gewild soos ander variante, byvoorbeeld Gouda en Cheddar. Die gewildheid van die tipe kase neem egter wêreldwyd toe. Om dié rede is dit dus belangrik om die mikrobiologiese, biochemiese en tegnologiese aspekte van die kase te bestudeer. Die doel van die studie was om die ontstaan, groei en oorlewing van giste tydens die rypwordingsproses van blou kaas variante te bestudeer, asook die interaksies wat plaasvind tussen die verkillende mikrobiese groepe tydens rypwording.

Die optelling van giste in voedsel met ‘n hoë konsentrasie skimmels is moeilik aangesien die skimmels die giste oorgroei. Dit maak die optelling, isolasie en identifikasie van giste moeilik en gevolglik ook die bestudering van die ekologiese rol van giste tydens die rypwording van blou kaas variante. Tien verskillende selektiewe media wat anti-mikotiese middels bevat is getoets vir hul vermoë om die groei en verspreiding van skimmels te inhibeer. Hulle is ook getoets vir hul vermoë om te help met die optelling en isolasie van giste vanaf die blou kaas variante. Kwantitatief was daar geen statistiese verskil tussen die tien selektiewe media. Volgens kwalitatiewe data kon OGGY, MEA + SP en molibdaat bevatende media nie aanbeveel word nie. Al die ander media het bevredigend vertoon. MEA + Ox en MEA + BP het die beste resultate gelewer gebasseer op die feit dat isolasie en optelling vergemaklik was. MEA + NaCl het eenderse resultate getoon maar het die groei van sekere individuele gis spesies wat mees algemeen in blou kaas variante voorkom geinhibeer.

Giste vorm een van die hoof mikrobiese groepe wat voorkom tydens die rypwording van blou kaas variante en ontstaan as post-pasteurisasie kontaminante. In hoofstuk 3 is die ontstaan en ontwikkeling van giste uit die omgewing bestudeer. Gis spesies wat geisoleer en geidentifiseer is stem ooreen met die wat verkry is deur ander navorsers op soortgelyke blou kaas variante.

‘n Tien- to honderd-maal verskil in gis tellings tussen die buite- en binnekant van die kaas is gevind gedurende die rypwordingsproses. *Debaryomyces hansenii* was oorheersend gedurende die studie. Die redes hiervoor is die vermoë van die spesie om ekstra-sellulêre proteases en lipases te produseer asook ‘n toleransie ten opsigte van lae temperature en hoë sout konsentrasies.

Alhoewel giste een van die hoof groepe is wat voorkom tydens die rypwording van blou kaas variante is daar ook ander groepe, soos *Penicillium roqueforti* en verskeie bakteriële groepe. Al hierdie organismes dra gesamentlik by tot die finale geur en aroma van die kaas gebasseer op die interaksies wat hul ondergaan. Daarom is dit belangrik om hierdie verskillende interaksies te bestudeer om sodoende meer te wete te kom aangaande die rypwordings proses, asook die rol en bydrae van elke organisme. Interaksie studies wat uitgevoer is in hoofstuk 4 het aangedui dat verskeie interaksies bestaan tussen giste en *Penicillium roqueforti*, asook tussen giste en bakterieë, wat essensieël is vir die finale produk. Die meerderheid gis-bakterië interaksies was neutraal alhoewel sommige ook antagonisties en stimuleerend was. *Debaryomyces hansenii* was die mees stimulerende gis spesie ten opsigte van bakterieë en *Torulaspora delbrueckii* was die mees antagonisties. Al drie mikrobiese groepe wat bestudeer is het gedurende die rypwordings proses voorgekom en was teenwoordig in betekenisvolle getalle aan die einde van die rypwordingsproses.