Yeast diversity in white mould-ripened cheeses.

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Chapter 1

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1.1. Introduction

Surface mould-ripened cheeses represent a small proportion of world cheese production. However, these cheeses are becoming increasingly popular with the consumer, as there is an increasing demand for it (Gripon, 1987).

Brie and Camembert are typical white surface mould-ripened cheeses that are among the specialty cheeses and therefore receive high consumer interest. Surface mould-ripened cheeses are intrinsically thought to be Camembert, Roquefort and Blue cheese. However, these do not represent all the mould-ripened cheeses, nor are the moulds the single responsible microorganisms for the ripening of these cheeses. The high bacterial populations in the interior of these cheeses and the yeasts and bacterial populations on the surface are also important during maturation (Kosikowski, 1997).

The objective of this study was to investigate the integrated roles of moulds, yeasts and bacteria involved in the production and ripening of white surface mould-ripened cheeses and the assistance of the diverse microflora in the development of the characteristic taste, texture and the distinctive aroma of mould-ripened cheeses.

1.2. Historical background

The discovery of the highly esteemed French cheese, Camembert, is attributed to a certain Madam Marie Harel, who lived in Camembert, Normandy during the 18th century. She most likely mastered an age-old receipe and chronicled the method (Pike, 1982). In contrast, according to Mocquot (1955), an earlier related French cheese was referred to as far back as the 13th century in the famous volume of that period, Roman de la Rose. Brie, is a soft rennet cheese, ripened by moulds similar to those used for the ripening of Camembert cheese. It originated in France where it has been produced for over five hundred years (Lampert, 1975).

The moulds, *Penicillium caseicolum* and *P. candidum*, are currently used to manufacture both Brie and Camembert cheeses (Kosikowski, 1997), although the closely related *Penicillium camemberti* was applied in the production of traditional Camembert cheese (Thom and Fisk, 1918). The species most commonly used in Europe is *P. caseicolum* because it renders a perfect snow-white mould surface, while *P. camemberti* gives a grayish appearance, although its mycelia are white, its spores are gray. Besides the colour, a notable difference in cheese flavour exists between the two mould species, with *Penicillium caseicolum* being the preferred species (Kosikowski, 1997).

The production of Camembert, a white surface-mould ripened cheese, was limited to France for a long time but during the last few decades, many countries have tried to develop the production of such cheeses. The presence of moulds on the surface of the cheese renders these cheeses a unique appearance whereas the high biochemical activity of the mould produces a very typical aroma and taste. *Penicillium camemberti* leads to more complex ripening than other cheese varieties with a simpler flora (Gripon, 1987).

Several soft cheeses are produced in France of which Camembert and Brie is the most notable. These are full fat soft cheeses that contain 45-40% FDM and a maximum moisture content of 55%. They are mainly made from cow's milk, although ewe's or goat's milks are used for certain varieties. These cheeses are manufactured from whole milk, rennet and lactic acid bacteria starter cultures are added to produce a mildly acidic and soft curd. The curd is then transferred to suitable perforated shaping moulds and drainers. Adequate drainage is obtained by regular turning in the shaping moulds. This is essential for ripening and gives the cheese a sufficient dry surface for moulds and yeasts to develop.

Surface mould-ripened cheeses are made in small sizes to ensure the maximum surface for mould growth and to optimize the diffusion distance of their enzymes. Camembert is the most important French soft cheese and it originated in the province, Normandy, where it is extensively produced in both small and large dairies. Brie, another French cheese, is basically the same as Camembert. It is a thin, cylindrical and triangular surface mould-ripened soft cheese, but it is produced in many diameters that are larger than the circular shaped Camembert. These cheeses are produced from milk of varying fat contents and therefore differ in the degree of ripeness when consumed. The moisture content of the fresh curd and matured Brie is higher than that of Camembert, consequently the sequence of ripening occurs more rapidly. As the reddish coloured and aerobic bacterium, *Brevibacterium linens* develops, the mould frequently becomes reddish with the characteristic soft texture, smooth body and fine flavour (Chapman and Sharpe, 1981).

Some varieties of mould-ripened cheese, for example, true French Brie are made with *Brevibacterium linens*. The mould and this aerobic bacterium occur on the rind to produce a characteristic type of cheese. The surface growth of the bacteria supports several mould species to render a unique cheese with desirable flavours (Kosikowski, 1997). During the later stages of maturation, the mould is overgrown by *B. linens* and related coryneforms to produce the yellow

rind, and the distinct sulphury flavours and the characteristic soft textures of mature Brie and Camembert cheese (Karahadian et al., 1985).

1. 3. The Cheese Making Process

The making of cheese is basically a means of preserving milk for a lengthened period, with the primary characteristics being the decreasing of pH and water activity (Shaw, 1986). According to Davis (1965), cheese making can be summarized into three stages:

1) The coagulation of milk by rennet and lactic acid.
2) Breaking the curd and removal of most of the whey.
3) Ripening of the partly dried curd.

Rennet and other proteolytic enzymes have the ability to convert liquid milk into a very weak jelly. This solidification is due to a slight change in the structure of casein, the major milk protein and later precipitation by soluble calcium salts. According to Shaw (1986), surface mould-ripened soft cheeses are characterized as cheese that has been subjected to other fermentations as well as lactic fermentation. The curd is not scalded and the cheese is not pressed but they are ripened.

According to Kosikowski (1970), the making of Camembert cheese is different to that of many soft curd cheeses, because it begins with well acid-ripened milk of approximately 0,22 % titratable acidity. This elevated acidity aids whey drainage and inhibits the growth of contaminating microorganisms. However, excess acid in the milk which is always pasteurized, results in a curdy, pasty and short-grained body. The firm cheese develops rapidly and is dipped from the vat almost immediately. Adequate drainage is important in order to obtain a good cheese.

Several different methods of producing soft mould-ripened cheeses have evolved over the years. There are two types of manufacture i.e. the traditional and

industrial. Figure (Fig.) 1 illustrates the typical steps for the production of Camembert according to industrial methods.

Time/temperature		*Operation*
63°C/15s overnight, 10-14°C 72-76°C/15-20s	**RAW MILK**	±standardisation ±thermisation ±starter, 0,1-0,2% Pasteurization
32-35°C 20-25min 32-35°C/1 h to 2 h 30min from renneting	**'BASSINES' OR VATS**	± mould spores Starter culture,1,5 - 3,0 % ± calcium chloride, 0,006 % Ripening to 0,18 – 20 % lactic acid Coagulent: 22-30ml/100 litres
25 –30 min	**COAGULUM**	Cut, 30mm cube Settle Partial whey extraction
18-24h 5 h @ 27°C 15 h @ 23°C RH: 90-95%	**MOULD FILLING**	Mould turning, every 5 h
10-15°C/30-60min	**WHEY DRAINAGE**	Brine salting, saturated or mechanised dry salter to 1,5-1,8 % salt in cheese ± mould spores
7 – 14 days, 12-15°C RH: 90-95%	**CHEESE**	Ripening/maturation
	PACKAGING	

Fig. 1. Steps in Camembert manufacture (industrial), (Shaw, 1986).

During the production of traditional Camembert, raw milk is used and acidification results from the natural lactic acid bacterial populations. The raw milk is filled into tanks or 'bassines' and calcium chloride may be added to assist coagulum formation. Acid development continues and rennet is added. The curd is not cut but is ladled manually from the bassine to a series of moulds which are open-ended cylinders with whey drainage holes, placed on drainage mats in trays that permit turning of the filled moulds. The moulds are turned regularly to assist the drainage of the whey. The cheeses are removed from the moulds and dry salt is sprinkled over the surfaces. The cheese is matured at a temperature of 11-13 °C with relative humidity of 90-95 % for a period ranging from three weeks to one month. After ripening, the matured cheese is packaged and stored at 4 to 8°C before it is distributed.

The typical coat of white mould found on Camembert and other soft ripened cheeses was traditionally formed by a naturally occurring mould strain found in cheese ripening rooms. In contrast, surface mould growth is currently restricted by utilizing pure cultures of *Penicillium candidum,* a strain that has the capacity to form a pure white coat and is characterized by its high salt tolerance and aerobic nature. Ripening of the cheeses is initiated by the action of both lactic acid bacteria and *P. candidum.* Camembert normally matures from the outer surface as *Penicillium candidum* secretes proteolytic enzymes. As the hydrolysis proceeds casein is broken down into ammonia, the body becomes smooth and the hydrolysis of fat produces typical flavours. The central white and pasty layer slowly diminishes as maturation occurs and the cheese will eventually become over-ripe and liquefy with a distinctive aroma of ammonia. The ripening of traditional Camembert is different to that of industrial varieties. This is due to a difference in bacterial populations present in the cheese (Shaw, 1986).

In South Africa, Camembert and Brie cheeses are produced according to the following method. Milk specially selected for Camembert and Brie is passed through heat exchangers and pasteurized for 15 seconds at 73°C.

The pasteurized milk is pumped into a cheese vat and the lactic acid bacteria starter cultures are added. The starter contains selected strains of *Leuconostoc cremoris*, *Streptococcus diacetylactis*, *Streptococcus cremoris* and *Streptococcus lactis*. The starter consists of several different strains of each of the four mentioned species and possesses high phage resistance. Acidification, coagulation and the separation of the curd from the whey follows.

The fresh curd is transferred into small forms and hoops and allowed to drain. The hoops are then removed and the curd is sliced into circles and triangles for Camembert and Brie, respectively.

The sliced curd is salted in 18% NaCl concentrated brine for approximately 10 to 15 minutes. The fresh curd is inoculated with spores of *Penicillium candidum*. The cheese is incubated in a dark and moist room at 14°C for a 5 to 7 day period. After incubation the cheese is wrapped in foil, placed into cardboard boxes and distributed to retailers. Brie and Camembert are perishable cheeses and must be refrigerated. The shelf life of South African Camembert and Brie are eight weeks. During this time, the cheese matures and acquires a soft body, sharp odour and surface growth of *Penicillium candidum* that contributes to the characteristic flavour.

1.4. The Microflora Associated with the Production of Surface Mould-Ripened Cheese

The three main steps involved in the production of mould-ripened cheeses are:

Production of a curd by fermentation of milk with lactic acid bacteria and by the addition of proteolytic enzymes, further processing of the curd by heating, addition of salt and inoculation of the curd with certain fungi and finally, ripening of the curd by storage at a low temperature (Marth, 1987; Gripon, 1987).

There are three groups of microorganisms involved in the ripening process, namely filamentous fungi, yeasts and bacteria.

1.4.1. The Fungi

According to Lenoir (1984), there are only two moulds involved, namely *Penicillium camemberti* and *Geotrichum candidum*. *P. camemberti* is capable of the following.

It can break down the lactic acid in the curd and this is important in the deacidification of the cheese.

It possesses the ability to synthesize proteolytic enzymes. Two endopeptidases have been identified, metalo-proteases (is the principle component in cultures at pH 6,5) and aspartyl protease (is the major fraction of the proteolytic system of cultures at pH fraction of the proteolytic system of cultures at pH 4,0).

The mould also possesses amino and carboxyl-peptidase activities and has a rather high capacity for lipolysis.

The mycelium of the mould is capable of oxidative degradation of fatty acids (Lamberet et al., 1980).

1.4.1.1. The Role of Moulds Involved in the Maturation of Cheese

The role of the moulds during maturation is linked with their biochemical activities. The moulds neutralize the surface of the cheese. This affects the appearance; the texture becomes "supple" and homogenous due to the protein/water and protein/mineral "liasons", enzymatic activities which include proteolysis, lipolysis and fatty acid oxidation, as well as the establishment of an acid-sensitive bacterial flora, including micrococci and corynebacteria, that contribute to the formation of the aroma and taste of cheeses.

The degradation of caseins in Camembert is mainly due to the action of *Penicillium camemberti*. This predominant action is evidenced by the differences in the changes in nitrogenous matter of the outer and inner regions of the cheese during maturation (Lenoir, 1962). Similarly, the growth of the mycelia and the changes in proteolytic activity at the surface in comparison with the centre of the cheeses, where this activity remains very low (Lenoir, 1970), is also an indication of the activity of *P. camemberti*.

The hydrolysis of triglycerides is strong in Camembert cheese, especially in the rind. *Penicillium* is thought to be the principal lipolytic agent of Camembert on the basis of the changes that occur during cheese development, as well as the characteristics of this hydrolysis (Lamberet and Lopez, 1982). Oxidative degradation of fatty acids is obvious during ripening. Methyl ketones and their reduction products, the secondary alcohols, occur in large proportions in mould-ripened cheeses and they form the major aroma compounds in this type of cheese (Dumont et al., 1974).

The mould *Geotrichum candidum* is present in a variety of soft cheeses, including Camembert, Pont l'Eveque, Limburg, Ribbola, Taleggio, etc. The biochemical activities of *G. candidum* that are similar to those of *P. camemberti*, are mainly, lactic acid degradation, proteolysis and lipolysis. Guegen et al. (1975), identified two main biotypes of this mould, one that grows rapidly, has a strong proteolytic activity and forms true mycelium and has an alkaline action on culture media. Another that grows poorly is only slightly proteolytic, has a yeast like appearance and an acidifying action. The role of *G. candidum* during cheese development is more difficult to determine than that of *Penicillium*. It was found that in Camembert and Pont L'eveque, the proportions of free oleic acid that are higher than those bound to triglycerides, probably results from the actions of *G. candidum*. Controlled growth of this mould during the production of Camembert from pasteurized milk, results in cheeses that have a more distinct and characteristic taste and aroma.

In experimental cheeses containing only *Penicillium* as a control, another with only *Geotrichum* and one with both *Penicillium* and *Geotrichum*. The cheese with only *Geotrichum*, resulted in slower maturation, less protolysis and a slower increase in pH resulting in incomplete ripening. While the cheese that contained the *Penicillium-Geotrichum* association, matured more rapidly and had a quicker and stronger proteolytic activity and more ammonia was produced. The latter cheese was considered to be improved in comparison with the control and contained a characteristic Camembert taste that was "strong" and more typical. It is therefore obvious that *Geotrichum* contributes to cheese ripening through its proteolytic and lipolytic activities and its action on amino acids (Greenberg and Ledford, 1979).

1.4.2. The Yeasts

The presence of yeasts in cheese is expected because of the low pH, low moisture content, increased salt concentration and refrigerated storage. In some

cheeses, yeasts aid in the development of flavour during the maturation stages, while they may contribute to spoilage in others (Fleet, 1990). Certain yeast species are capable of growth under these environmental conditions reaching populations exceeding 10^7 colony forming units per gram ($cfu.g^{-1}$). Yeasts can grow in conditions that are unfavourable to several bacteria and play an important role in spoilage of dairy products (Fleet and Mian, 1987; Seiler and Busse, 1990). Yeasts are also natural contaminants of the cheese making process.

Yeasts effect cheese quality as they produce lipolytic and proteolytic enzymes, ferment residual lactose and utilize lactic acid (Choisy et al., 1987 a,b; Devoyod, 1990; Fleet, 1990). In semi-soft cheeses with surface films like Limburger and Tilsit and mould-ripened cheeses that include Camembert and Roquefort, the yeasts utilize the lactic acid and thereby increase the pH resulting in enhanced bacterial growth, leading to the second maturation phase.

Certain proteolytic and lipolytic enzymes derived from yeasts contribute directly to the ripening process (Devoyod and Sponem, 1970; Law, 1978; Marth, 1982; Noomen, 1983; Schmidt et al., 1979). In the study of microbial succession of Bethlehem St. Nectaire cheese that is similar to Camembert, Marcellina and Benson (1992), reported that on the second day of ripening the surface of the cheese was densely covered with budding yeasts, primarily *Debaryomyces* and *Candida* which were embedded in the cheese rind. A study conducted by Sable et al. (1997) on soft raw goat's milk cheese, (similar to Camembert), the viable yeast counts remained at a high level in the rind but were significantly less in the core of the cheese during ripening.

Yeasts have a special relationship with mould-ripened cheeses (Fleet, 1992). Retail samples of Camembert and Brie have a very high occurrence of yeasts, with most samples exhibiting counts exceeding 10^6 cells per gram (de Boer and Kuik, 1987; Nooitgedagt and Hartog, 1988). After the curd of Camembert and

Brie has been inoculated with spores of *P. camemberti*, mould growth is accompanied by extensive growth of natural yeast species during cheese development. A mixed population of oxidative and fermentative species grows in the centre and on the surface of mould-ripened soft cheeses. The yeasts species include *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Candida famata*, *Debaryomyces hansenii*, *Pichia membranaefaciens*, *Pichia fermentans*, *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* (de Boer and Kuik, 1987; Galzin et al., 1970; Lenoir, 1984; Nooitgedagt and Hartog, 1988; Nunez et al., 1981; Olson, 1969; Schmidt and Lenoir, 1978, 1980; Tzanetakis et al., 1987; Vergeade et al., 1976).

The growth kinetics differs depending on the location in the curd, type of yeast species and the specific type of cheese. The salt concentration used in the production of these cheeses varies between 5 to 10%, which affects the profile of yeast growth (Fleet, 1992). *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Candida famata*, *Yarrowia lipolytica*, *Pichia membranaefaciens*, *Pichia fermentans* and other *Candida* species are the dominant yeast species present during the ripening of soft cheeses. The same species were observed at high populations in retail samples of these cheeses (de Boer and Kuik, 1987; Nooitgedagt and Hartog, 1988; Tzanetakis et al., 1987).

The growth of yeasts affects the development and final quality of cheese due to the following.

Fermentation of residual lactose within the curd by species such as *Kluyveromyces marixanus* results in the formation of secondary (flavour) metabolites, as well as carbon dioxide that opens up the texture of the curd.

Lactic acid utilization is a major consequence of yeast growth in cheeses. This activity decreases the acidity of the curd, which alone may be a favourable sensory attribute and causes an increase in pH that stimulates the growth of

ripening bacteria. The change in pH also affects the activity of lipases and proteases produced by the *Penicillium* species.

Extra cellular protease and lipase activity produced by certain yeasts such as *Yarrowia lipolytica*, could change the flavour and texture of the curd.

Yeasts release autolytic products that may influence the flavour of the cheese as well as bacterial growth (Devoyod, 1990; Lenoir, 1984).

One must realize that the yeast flora that develop during cheese ripening are a rather uncontrolled mixture of wild species. Certain species may contribute to cheese quality, while others may have a detrimental effect (Fleet, 1992).

1.4.2.1. Yeasts in Dairy Products

Yeasts are the most important microorganisms exploited by man from and economical and traditional perspective. Yeasts are used in the production of bread, beer, wine and other alcoholic beverages as well as a vast range of other products, including the production of ethanol for fuel, yeast extracts, pigments, probiotics and several other substances for foods and feeds (Jakobsen and Narvhus, 1996).

Yeasts are essential in the dairy industry as they are involved in the production of certain fermented products and in the ripening of certain cheese varieties, cause spoilage of milk and dairy products and are used to ferment whey which is a major by-product of cheese making (Marth, 1987).

Milk is a nutritious substrate and supports the growth of many microorganisms including yeasts. Fresh or raw milk contains varying amounts of yeasts depending on the milking conditions. Psychrotrophic strains multiply in raw milk stored at refrigeration temperatures. The pasteurization of milk eliminates the

majority of microorganisms except for thermoduric bacteria. The occurrence of yeasts in pasteurized market milk is therefore due to secondary contamination. Yeasts are mostly beneficial in the development of cheeses as they metabolize lactic acid and cause an increase in pH that allows growth of the proteolytic bacteria. The actions of yeasts are important in both the exterior and interior of soft cheeses (Deak, 1991).

According to Jakobsen and Narvhus (1996), the role of yeasts in microbial interactions in dairy products has been reported on several examinations of blue cheese, white mould cheese, bacterial surface-ripened cheeses and fermented milk products such as kefir. Yeasts in dairy products may interact with other microorganisms in three different ways. They may suppress or eliminate undesirable microorganisms that cause quality defects or may contain potential pathogenic properties, they may inhibit the starter culture and may contribute to the fermentation or the ripening process by supporting the role of the starter culture. With regard to the suppression or elimination of undesired microorganisms, Deiana et al. (1984) speculated that *Debaryomyces* hansenii inhibits the germination of *Clostridium butyricum* and *Clostridium tyrobutyricum*, possibly due to the depletion of organic acids such as lactic and acetic acids in the cheese.

Siewert (1986), reported on the suppression of *Mucor* growth on the rind of Camembert and Brie cheeses by the dominating yeast species. Inhibition of the moulds *Penicillium roqueforti* and *P. camemberti* by yeasts has not yet been observed. Although, it may occur and should be kept in mind in cases of slow development of mould cultures and especially if yeasts are to be added as part of the starter cultures in cheeses (Jakobsen and Narvhus, 1996).

The beneficial contribution of yeasts to the ripening and production of aromatic compounds in Camembert has been suggested on several occasions (Anderson and Day, 1966; Baroiller and Schmidt, 1990; Gripon, 1993; Rousseau, 1984;

Schmidt and Lenoir, 1978, 1980 a, b; Schmidt et al., 1979; Schmidt and Daudin, 1983; Siewert, 1986). These experiments also proved that the high populations of yeasts present on the surface might be more important than the lower populations of yeasts found in the interior of the cheese.

The desired properties of yeasts used as starter cultures for cheeses are due to their lipolytic and proteolytic properties, ability to form aroma compounds, fermentation and assimilation of lactose, positive interactions with the primary starter cultures, *Penicillium* starters, as well as with *Brevibacterium brevis*, osmotolerance and other physiological characteristics (Siewert, 1986; Baroiller and Schmidt, 1990; Fleet, 1990). Due to their proteolytic and lipolytic activities, yeasts may also become a part of the overall enzymatic activity in the cheese (Fox and Law, 1991).

Yeasts, however, may also contaminate different cheeses. Excessive growth of yeasts may cause unfavourable organoleptic changes and may result in softening. Under unhygienic conditions the opportunistic pathogenic yeast, *Candida albicans* may appear in cheese (El-Bassiony et al., 1980). Excessive growth of *Geotrichum candidum* caused an undesirable flavour in a German fresh cheese type (Engel, 1986,b).

1.4.2.2. Yeasts as Spoilage Organisms

The occurrence of microorganisms in foods is important to the human community because the microorganisms may be pathogenic and pose a risk to public health, they may cause undesirable changes of the product to the extent that it is considered spoiled and unacceptable and certain microorganisms are beneficial as they contribute to favourable changes, as in the production of fermented foods and beverages (Fleet, 1992).

Yeasts are capable of spoiling foods and beverages due to their physiological properties, i.e. their ability to grow at low temperatures, metabolic activities and their resistance to physico-chemical stresses and are also important in food preservation. But the role of yeasts as spoilage organisms in dairy products is related to their nutritional requirements, certain enzymatic activities and the ability to grow at low temperatures, low pH values, low water activities and increased salt concentrations (Jakobsen and Narvhus, 1996). The enzymatic activities of yeasts in foods result in physical, chemical and sensory changes that contribute to the spoilage of food. Yeasts have extremely diverse metabolic capabilities as they can utilize a wide variety of food substrates under varying environmental conditions (Deak and Beuchat, 1996).

The occurrence of yeasts in cheeses has been reported since the beginning of the previous century (Fleet, 1990; Devoyod, 1990). However, yeasts are not regarded as a significant component of the microflora of several cheeses. Yeasts arise as natural contaminants in the curd during the ripening process and are less important at the beginning of cheese making. As the cheese matures at a low temperature, it develops an atmosphere of an acidic pH, low moisture content and higher salt concentration. These are desirable and selective conditions for the growth of yeasts.

The spoilage symptoms caused by yeasts result in fruity, bitter or yeasty-off flavours and a gassy open texture with semi-hard or hard cheeses. Determination of cheese spoilage is complicated by personal opinions as to whether the actions of yeasts during ripening and retailing are detrimental or beneficial to the quality of the product. During maturation and retailing, continued lactose fermentation results in elevated acidity, gassiness and fruit flavours whereas continued fat and protein digestion softens the texture of the product and bitter and rancid flavours develop (Fleet, 1992).

The detrimental effect of yeasts is still considered a problem. But this negative attribute can most significantly be solved by improved sanitation and hygiene, procurement of raw materials, fruit bases of sufficient microbiological quality and in certain cases, by pasteurization or other treatments (Jakobsen and Narvhus, 1996).

1.4.3. The Bacterial Flora

1.4.3.1. Lactic acid bacteria (starter cultures)

Microorganisms play a role in the production of most dairy products. Lactic acid bacteria are fundamental in the making of all fermented milk products, cheese and butter. These harmless microorganisms are called dairy starters and they impart certain characteristics to a variety of dairy products. The reasons for utilizing starter cultures can be summarized as follows.

- They degrade lactose to lactic acid. The lactic acid coagulates the milk in fermented products and since the coagulation time by rennet is decreased by the elevation in the acidity of the milk, it assists the enzymatic coagulation of the milk during cheese making.

The rapid development of lactic acid during the production process suppresses the growth of undesirable bacteria. Insufficient lactic acid production may lead to gassy, bitter and an unclean flavoured cheese due to strayed fermentation. During cheese making the acid production assists the action of rennet and subsequently coagulum formation (Rosenthal, 1991; Shaw, 1986). Lactic acid promotes the separation of the whey from the curd and when a languid starter is utilized, the cheese often contains a high moisture content.

The acid-producing lactic acid bacteria also excrete proteolytic enzymes that assist in the hydrolysis of cheese proteins. Starter cultures influence the flavour, body and texture of the final product.

Several years ago, the naturally occurring lactic acid bacteria were responsible for the deliberate souring of milk. However, the modern dairy industry uses commercially available starters formulated as singular or mixtures of strains and species that perform best in symbiotic relationships. When high quality starters are utilized in the dairy industry they behave in a reproducible, consistent and predictable manner resulting in better product uniformity (Rosenthal, 1991).

In the dairy industry, contaminating microorganisms such as bacteria, yeasts, moulds or a combination of these are involved during lactic acid fermentation. Lactic acid bacteria are by far the most significant group of bacteria used as starter cultures that includes the genera *Streptococcus*, *Lactobacillus* and *Leuconostoc*. Starter cultures are frequently used in the dairy industry since these organisms are responsible for acidifying milk rapidly and contribute to product uniformity, particularly in fermented milk products and cheeses.

Starter cultures ferment lactose and produce lactic acid that is important during coagulation and texturing of the curd during cheese making (Tamine, 1981). The non-starter lactic acid bacteria present within the cheese originating from the milk or environment increase the diversity of Camembert-type cheese and may also play a role in producing the typical organoleptic properties of cheese during ripening. In soft-ripened cheese, lactococci are initially involved in the production of lactic acid that lowers the pH (Corroler et al., 1998). The acidic condition in the cheese prevents the growth of pathogens as well as several spoilage organisms. Starter cultures are also involved in proteolysis that may contribute to the ripening of these cheeses.

Research performed by Sable et al. (1997), showed that the mesophilic Gram-positive bacteria are the predominant microbial group during cheese making at the early ripening stages. The rapid decline in lactose concentration results due to the drainage of whey and its fermentation to lactate by this group of bacteria with a corresponding decline in pH. Lactic acid bacteria are the main components of the microbial flora in the interior during ripening, whereas their significance on the surface decreases as other microorganisms develop. The mesophilic Gram positive bacteria present in raw milk consist of *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Micrococcus*, whereas only *Micrococcus* and *Lactococcus* are present in the rind.

1.4.3.2. Other bacteria

The surface of Camembert-type cheese consists mainly of two groups of bacteria, micrococci and corynebacteria, which share certain physiological and biochemical characteristics. These two groups grow mainly on the surface of cheese because of their aerobic nature. Their numbers on the surface increase during ripening, while remaining constant in the centre (Lenoir, 1984). Richard and Zadi (1983) reported that numbers of corynebacteria in the surface bacterial flora vary according to the type of cheese and for a given type like Camembert, according to the batch and degree of ripening. The surface bacterial flora is capable of biochemical activities that include proteolysis, lipolysis, esterification and the break down of amino acids (Boyaval and Desmazeaud, 1983). Amino acid degradation plays an important role in the production of aromatic components in a variety of cheeses (Hemme et al., 1982).

1.5. The Cheese Ripening Process

According to Kosikowski (1997), a cheese is ripened, cured or matured by placing it in a temperature-controlled room at a selected optimum relative humidity for 2 to 48 months. The temperature of such rooms varies from 2 to 16°C. The ripening of cheese allows the microorganisms and enzymes in the cheese the opportunity to hydrolyze fat, protein and other compounds. The hydrolysis renders a softer, more pliable body, and a more aromatic flavour, as the rigid, insoluble protein changes to soluble nitrogenous forms and the neutral fat breaks down partially into free fatty acids and glycerol.

During maturation, the available oxygen is soon consumed by bacteria and the centre of the cheese changes rapidly from an aerobic to an anaerobic state. Lactose is converted to other compounds within the second week of ripening resulting in the remainder of only trace amounts of sugars. The maturation of normal cheese releases carbon dioxide as an end product, but at a slow and steady pace. The gas arises during the decarboxylation of six selected amino acids, which are released during ripening. For Camembert and Brie, the production of carbon dioxide may be accompanied by free ammonia resulting from the enzymatic deamination of certain amino acids. The rate of free ammonia elevates with the usual increase in pH of the cheeses during the ripening process. Cheese ripening also catalyzes the production of several water-soluble aromatic compounds such as peptides, amino acids, amines, fatty acids and carbonyls. In properly balanced proportions, these compounds form the typical flavour of a ripened cheese.

Mould surface-ripened cheeses produced from raw milk have a more complex composition and evolution of microorganisms than cheeses made from pasteurized milk. The latter contains mostly microorganisms added as starter cultures, i.e. mesophilic streptococci and *Penicillium camemberti* resulting in a taste and aroma that is less accentuated and more neutral (Gripon, 1987). The

microflora of cheeses produced from raw milk consist of the starters which are primarily mesophilic lactic streptococci, *Streptococcus lactis* and *Streptococcus cremoris* (Lenoir, 1963). After the curd has been formed, salt tolerant yeasts accumulate on the surface, (Lenoir, 1963; Schmidt and Lenoir, 1978, 1980), whereas the salting limits the growth of the mould *Geotrichum candidum*. *P. camemberti* forms a white felt that covers the entire surface of the cheese. The aerophilic and acid-sensitive bacterial flora, which consists of micrococci and coryneform bacteria, mostly *Brevibacterium linens*, only grow on the surface after *P. camemberti* has consumed the lactic acid and thereby increase the pH of the surface (Lenoir, 1963; Richard and Zadi, 1983; Richard, 1984).

Chapman and Sharpe (1981) stated that ripening involves changes in the chemical and physical properties of cheese that is accompanied by the development of a characteristic flavour. Fresh or young cheese contains varying proportions of mainly protein moisture and fat, together with lower amounts of salt, lactic acid, lactose, whey proteins and minerals. Enzymes then slowly hydrolyze the curd and the mature cheese possesses either a firm, plastic or soft body, which is characteristic of the particular cheese involved. The chemical changes which occur during cheese ripening comprise, the fermentation of lactose to lactic acid, the production of small amounts of acetic and propionic acid, carbon dioxide and diacetyl. Proteolysis and lipolysis also occur. These changes are due to the enzymatic actions of either the lactic acid bacteria starter culture, non-starter bacteria in the milk, the rennet used during coagulation of the milk, the milk itself or other contaminating microorganisms present in the interior or on the exterior of the cheese.

1.6. Compounds Involved in the Flavour and Aroma of Surface Mould-Ripened Cheeses

Cheese flavour is obtained through a series of chemical changes that occur in the curd during cheese ripening. The breakdown of lipids yields free fatty acids, which serve as substrates for further reactions. The peptides and amino acids originating from proteolysis, also produce aromatic compounds through enzymatic and chemical reactions (Molimard and Spinnler, 1996).

The moulds, *Penicillium camemberti* and *Geotrichum candidum,* are the most important species involved in the production of mould-ripened cheeses. Yeasts and *Brevibacterium linens* however, are also important since they actively contribute to the production of aromatic compounds. There are three major metabolic pathways responsible for the synthesis of aromatic compounds in cheese, i.e. the lactose, lipid and protein catabolisms. The endogenous enzymes of milk, clotting enzymes, manufacturing and ripening microbial enzymes, activate these pathways. Essentially, fatty acids, ketones, methyl ketones, alcohols, lactones, sulphur compounds, aldehydes, amines and pyrazines are the compounds derived from these metabolic pathways (Molimard and Spinnler, 1996). Table 1 summarizes the composition of the volatile compounds found in Camembert cheese.

1.6.1. Fatty Acids

The fatty acids are important contributors to the aroma of mould-ripened cheeses, as they by themselves, are aromatic products. Fatty acids also serve as precursors of alcohols, methyl ketones, lactones and esters. The hydrolysis of fat is important in soft cheeses, especially Camembert and Blue cheeses, as it plays a role in cheese ripening (Molimard and Spinnler, 1996). The fat in milk consists of 98% of glyceride neutral lipids characterized by the wide variety of

fatty acids of which they are composed. Cheese produced form raw milk free fatty acids are synthesized during the hydrolysis of glycerides by the natural lipases present in milk as is illustrated in Fig. 2 (Choisy et al., 1984).

Most of the free fatty acids containing between 4 and 20 carbon atoms are derived from the lipolysis of triglycerides by moulds. The degradation of lactose and amino acids results in a lower proportion of free fatty acids, which generally contains between 2 and 6 carbon atoms. The smaller free fatty acids can also be synthesized from the oxidation of aldehydes, ketones and esters. Free fatty acids in cheese, made from raw milk, are derived from the hydrolysis of glycerides by lipases, which break down triglycerides to form diglycerides, monoglycerides and free fatty acids (Molimard and Spinnler, 1996).

1.6.2. Methyl ketones and Ketones

Methyl ketones and their corresponding secondary alcohols are of the most abundant and important aromatic compounds in surface mould-ripened cheeses (Dumont et al., 1974; Shwartz and Parks, 1963). Methyl ketones in Camembert and Brie cheeses are present from the 8^{th} day of ripening and onward, but seemed to disappear during maturation. The moulds *Penicillium camemberti* and *Geotrichum candidum* are important in the synthesis of methyl ketones. Fatty acids serve as the precursors of methyl ketones, which is related to the β-oxidation pathways of the moulds. These moulds possess the enzymatic activity that allows a deviation from the usual β-oxidation pathway. The moulds utilize this pathway for the detoxification of the fatty acids in the media.

1.6.3. Alcohols

Primary and secondary alcohols as well as ketones are thought to be the most important aroma compounds of mould-ripened cheeses. Adda et al. (1973),

reported that oct-1-en-3-ol produces the typical mushroom note on the characteristic flavour of Camembert cheese. However, when the oct-1-en-3-ol level is extensively high, the aroma becomes faulty. Phenyl-2-ethanol is one of the alcohols encountered in Camembert after the 7th day of ripening but oct-1-en-3-ol is by far one of the key products in the general aromatic note of Camembert. The latter is produced by the *Penicillium camemberti* metabolism and is present only at the end of ripening.

Many metabolic pathways are responsible for the formation of alcohol in cheeses. The lactose metabolism results in the formation of ethanol, and butan-2,3-diol originates via the pentose phosphate and mixed acids pathways (Adda, 1984; Choisy et al., 1984). Methyl ketones can be degraded to their corresponding secondary alcohols by reductases. The alcohols are present immediately after the methyl ketones are synthesized and this also appears to be a detoxifying pathway to protect the microorganisms (Kinsella and Hwang, 1976).

In the amino acid metabolism, peptides can be degraded to amino acids by the aminopeptidases and carboxylases from *P. camemberti, G. candidum* and yeasts. The amino acids can be converted to α-ketoacids and then to aldehydes by a decarboxylase, through oxidative deamination (Fig. 3). The aldehydes can then either be reduced to their corresponding primary alcohols or oxidized to acids. Oxidative deamination occurs due to oxidoreductase, which are either dehydrogenases or oxidases and these enzymes are found in *G. candidum*, which has a deaminative action on glutamic and aspartic acids as well as leucine, phenylalanine and methionine (Greenberg and Ledford, 1979). Phenylethanol is produced from phenylalanine and yeasts are essentially involved in this conversion (Lee and Richard, 1984). Valine is catabolized to 2-methylpropanol and leucine to 3-methylbutanol by *P. camemberti* (Karahadian et al., 1985).

Linoleic and oleic acids are precursors of several aroma components. The presence of oct-1-en-3-ol in Camembert and Brie, is due to the *Penicillium*

metabolism (Dumont et al. 1974; Karahadian, 1985). Lipoxygenase and hydroperoxide lyase are the mould enzymes involved in the synthesis of this alcohol (Chen and Wu, 1984).

1.6.4. Lactones

The lactones found in Camembert cheese were γ-decalactone, δ-decalatone, γ-dodecalactone and δ-dodecalactone and are characterized by their very pronounced fruity notes (peach, apricot and coconut). Hydroxylated fatty acids are the precursors for lactone synthesis and arise from the normal fatty acid catabolism and can be synthesized by lipoxygenases or hydratases (Duffose et al. 1994).

1.6.5. Esters

In Camembert cheese the esters correspond to the acids and alcohols and most esters found in cheese have a fruity or floral note. 2-Phenylacetate and 2-phenylethyl propanoate are of qualitative importance in Camembert. 2-Phenylacetate was found to be the main aromatic compound on the 7^{th} day of ripening. Methylcinnamate might be of particular significance in obtaining the characteristic aroma of Camembert cheese. Esters are formed due to reactions between short to medium chain fatty acids and alcohols produced during lactose fermentation or during the amino acid catabolism. Most of the microorganisms present during cheese ripening possess esterification enzymes such as carboxylesterases and arylesterases but, yeasts are mainly involved in ester formation (Molimard and Spinnler, 1996).

1.6.6. Sulphur Compounds

Sulphur products play a particular role because they produce the garlic odour that occurs in traditional Camembert cheese. Methylsulphide, methyldisulphide

and 3-methylthiopropanol present in other cheese varieties produce a basic cheesy note, while dithiapentane, 2,4,5-trihihexane and 3-methylthio-2,4-dithiapentane are found in typical Camembert (Adda, 1984). Coryneform bacteria are thought to be the main contributors to the production of sulphur products in surface mould-ripened cheeses. In Brie cheese, Karahadian and co-workers (1985), found dimethyldisulphide, dimethyltrisulphide and methionol in matured cheeses that had undergone secondary fermentation by *Brevibacterium linens* as well as other corynebacteria.

Sulphur compounds are synthesized mainly through methionine degradation due to the cleavage of a carbon-sulphur bond by the enzyme methionine-demethiolase (Collin and Law, 1989; Hemme et al. 1982).

1.6.7. Amines

Amino acid deamination results in the formation of ammonia, which is one of the important elements of the aroma of traditional Camembert. *P. camemberti, G. candidum* and *B. linens* play an important role in the production of ammonia (Karahadian et al. 1987). The decarboxylation of amino acids results in carbon dioxide and free amines, and this can be transferred to oxidative deamination that results in aldehydes.

1.6.8. Aldehydes

Aldehydes are present mainly in trace amounts and appear during the 1st week of ripening in Brie and Camembert cheeses. They are synthesized from amino acids by transamination that results in an imide that can then be decarboxylated. Aldehydes function as transitory products in cheese because they are rapidly converted to alcohols or their corresponding acids (Dunn and Lindsay, 1985; Kinsella and Hwang, 1976; Lees and Jago, 1978).

Table 1 **Volatile compounds isolated from Camembert cheese**	
1-Alkanols	C 2, 3, 4, 6, 2-methylbutanol, oct-1-en-3-ol, 2-phenylathanol
2-Alkanols	C 4, 5, 6, 7, 9, 11
Methyl ketones	C 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15
Aldehydes	C 6, 7, 9, 2 & 3-methylbutanal
Esters	C 2, 4, 6, 8, 10-ethyl, 2-phenylethylacetate
Phenols	Phenol, p-cresol
Lactones	C_9, C_{10}, C_{12}
Sulphur compounds	H_2S, methyl sulphide, methyldisulphide, methanethiol, 2,4-dithiapentane, 3,4-dithiahexane, 2,4,5-trithiahexane, 3-methylthio 2,4-dithiapentane, 3-methylthiopropanol
Anisoles	Anisole, 4-methylanisole, 2,4-dimethylanisole
Amines	Phenylethylamine, $C_{2,3,4}$, diethylamine, isobutylamine, 3-methylbutylamine
Miscellaneous	Dimethoxybenzene, isobutylacetamide

(Adda, 1984)

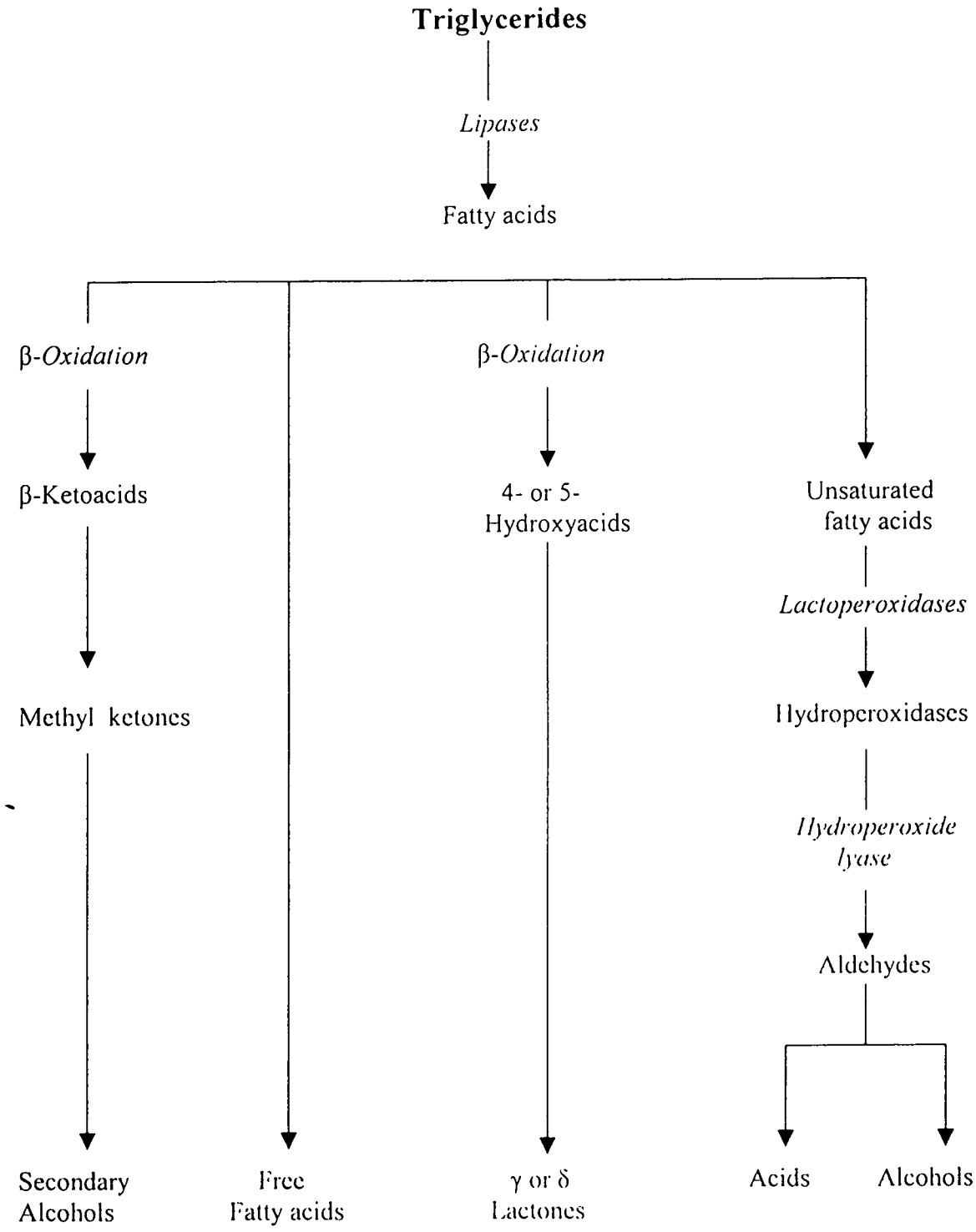

Fig. 2. Formation of flavour compounds from lipids (Dumont and Adda, 1978).

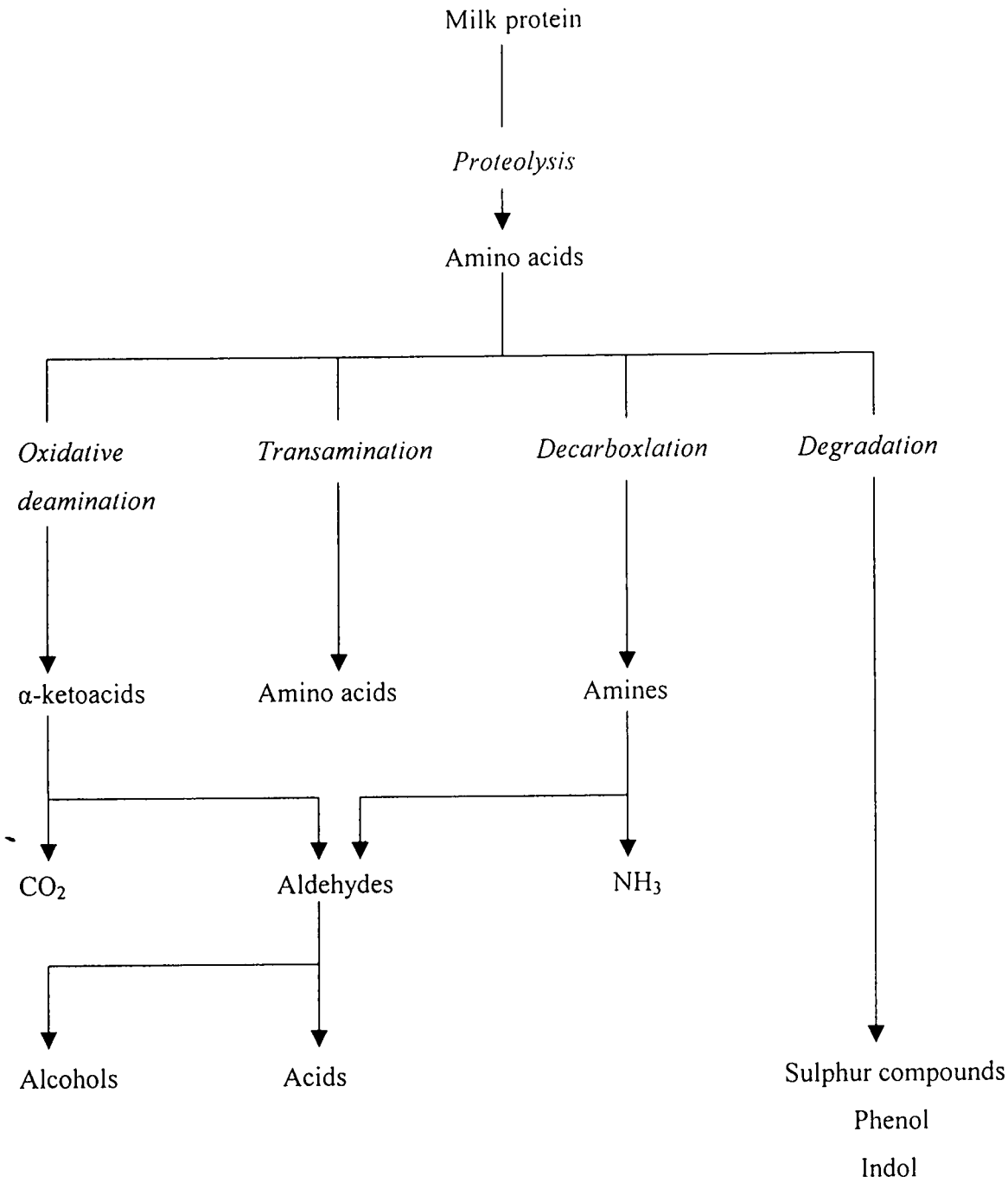

Fig. 3. Microbiological catabolysis of amino acids during cheese ripening (Choisy et al., 1984).

1.7. Changes in Texture

The generation of texture and flavour in surface mould-ripened cheeses depends on the biochemical activity of the microbial populations that develop on the surface and in the interior of the cheese during the ripening process (Gripon, 1987).

A study performed on St. Nectaire cheese which is similar to Camembert and Brie, showed that the texture, appearance and taste of the cheese change considerably during maturation, especially during the earlier stages. In the beginning, when the cheese is removed from the cheese press, the surface has a moist and pale yellow appearance, with no apparent microbial growth. A well-defined rind has not yet developed at this stage. The curd of the cheese has a white appearance with a uniform rubber consistency. A white crust is visible on the surface by the 4^{th} day of ripening. In the interior, the curd is light yellow with a much softer consistency than at the beginning of ripening. From approximately day 30 until the end of the 60-day ripening process, the rind becomes increasingly drier, while the curd becomes softer and creamier (Marcellino and Benson, 1992).

In cheeses produced with an initial low pH, such as Camembert and Brie, softening usually begins at the outside and slowly extends into the interior of the cheese. The change in texture is due to the proteolytic enzymes produced by the microbial populations on the surface that migrate into the cheese and cause protein hydrolysis (Noomen, 1983). According to Karahadian and Lindsay (1987), the firm texture of fresh cheese is due to the low solubility of casein at pH 4,9 to 5,1 and calcium-based solubility effects. When lactic acid is metabolized, and the pH increases above the isoelectric point that results in higher solubility of casein leading to softer textures. The initial softening of maturing mould-ripened cheese produce a "salve-like" or "pasty" body and texture rather than a smooth, "gel-like" texture of fully matured cheese.

The outer region of Camembert undergoes a considerable change in texture. The initial brittle and firm curd softens towards the centre as ripening proceeds (Gripon, 1987). Camembert has a high water content of approximately 55% and if it is too high, the outer part tends to flow when the cheese is cut. *Penicillium camemberti* creates a high level of proteolysis that leads to these changes in texture (Knoop and Peters, 1971, 1972). Due to lactic acid consumption and the production of ammonia by *P. camemberti* and the surface flora, a pH gradient is formed that extends from the exterior to the interior. This pH gradient can be induced by incubating young Camembert (i.e. 3 days of ripening without inoculating with *Penicillium*), in an ammoniacal environment. The ammonia dissolves in the curd and after equilibration, cheese softening occurs as a result of the formation of a pH gradient. This process is more evident closer to the surface where there is an elevated pH.

An increase in pH is therefore important in cheese softening since the increase in pH augments the net charge on casein and modifies the protein-protein interactions and subsequently the water sorption capacity of the caseins (Ruegg and Blanc, 1976). The physico-chemical conditions such as water content and pH in Camembert are not the only factors that contribute to cheese softening. It is argued that rennet plays a similar role. When experimental cheeses with the exclusion of rennet were incubated in an ammoniacal environment, the cheeses did not soften but had a hard and springy texture. In contrast, cheese containing rennet became soft (Noomen, 1983). Thus, softening of Camembert is due to firstly, α_{s1}-casein hydroylsis by rennet and secondly, an increase in pH caused by the surface flora.

1.8. Spoilage of Surface Mould-Ripened Cheese

Several defects in mould-ripened cheeses are due to an incorrect curd composition, or incorrect environmental conditions during maturation. Cheeses that contain a high initial moisture content or those that are exposed to high temperatures in the incubation room, develop excessive proteolysis and a strong flavour. However, cheeses with a dry curd or dry surfaces due to humidity, will not allow normal mould growth. Over salting or under-salting may also interfere with proper surface growth. Excessive growth of *Brevibacterium linens* on cheeses with wet surfaces may result in cheeses with a surface smear instead of a surface mould. Early gas formation is sometimes formed during the draining of the curd, especially if the cheese is produced from raw milk. The high acid and salt content of the curd prevent the growth of clostridia and subsequently late gas production seldomly occurs. Excessive yeast growth will cause the rind to become soft, resulting in poor development of the mould *Penicillium caseicolum* on Camembert cheese. Proper hygienic precautions are important in the factory as contamination by wild moulds such as *Penicillium glaucum*, *P. roqueforti* and *P. bruneoviolaceum* should be prevented. Prolonged ripening or failure to maintain ripened cheeses at low temperatures, may lead to rapid deterioration of the cheese (Chapman and Sharpe, 1981; Seiler and Busse, 1990).

The utilization of milk that is contaminated with psychtrophic bacteria for the production of mould-ripened cheese, results in organoleptic defects. Dumont et al., (1977), reported that the lipase activity of these bacteria is evidenced by increased lipolysis, a rancid taste and bitterness in the cheese. The coliform bacteria of Camembert cheese are difficult to control and even a low level of coliform contamination of milk results in a high growth rate during ripening.

Acidification eliminates the majority of this flora, but when the pH increases, the bacteria multiply again and this sometimes leads to high populations of coliforms in cheese.

Acidification plays an important role by controlling syneresis and the degree of mineralization of surface mould cheeses. When acidification is too high, the curd of Camembert is too dry and brittle, and the enzyme activities are limited. In contrast, insufficient acidification results in a cheese that is moist at the end of maturation. Research performed by Pelissier et al. (1974), showed that mould-ripened cheeses are more sensitive to bitterness than other cheese varieties, and the intensity of this defect may cause significant damage. A too abundant growth of the mycelium of *P. camemberti* may result in bitterness and therefore this mould has a crucial role in the production of bitterness in cheese. Bitterness occurs when high populations of lactic acid bacteria are present in the curd, whereas this defect does not occur if these populations are decreased (Martley, 1975). This defect might not result directly from high populations of lactic acid bacteria, but could also be caused by the growth and protease production of *Penicillium*, which might be higher in very acidic conditions (Gripon, 1987).

1. 9. Conclusion

The ripening of Camembert and Brie cheeses results from an integrated pattern of development of bacteria and fungi, leading to the modification of the curd and thickening of the rind over time. The surface microflora that develop on the cheese rind during maturation, impart a distinctive aroma and flavour to surface mould-ripened cheeses. Certain attributes of *Penicillium camemberti* are expressed in surface-ripened cheeses, giving it the characteristic appearance and typical taste. However, the secondary flora is responsible for obtaining traditional quality products. Due to the increasing consumer demand for white mould cheeses, the storage life of these cheeses should be improved in order to make the distribution easier and to develop its production.

While several studies on traditional mould-ripened cheeses produced from raw milk have been performed, it is necessary to perform research on white mould-ripened cheeses produced from pasteurized milk, as the latter cheeses possess a milder flavour, which the majority of South African consumers prefer. The occurrence of a wide diversity of microbial populations in these cheese varieties makes it necessary to perform further research to ensure good quality products, as well as to improve product uniformity.

From section 1.4.2. it is clear that yeasts are an important component of the microflora of surface mould-ripened cheeses. Future research should be performed to determine whether yeasts are beneficial i.e. contribute to cheese flavour or if yeasts have a negative effect by causing bitter and yeasty-off flavours. And if so, the contributing yeasts species should be identified and then they may be added as part of the starter cultures in cheese.

1. 10. References

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Chapter 2

Statistical comparison of ten media for the enumeration of yeasts from white-mould cheeses.

Food Microbiology

Abstract

The isolation and enumeration of yeasts in white mould-ripened cheeses, such as Camembert cheese, are complicated, as yeast colonies are often overgrown by mould spreaders. The ideal medium for the enumeration of yeasts should inhibit bacteria and moulds but supports growth of all the yeasts present. Due to the fact that this ideal medium does not exist, ten selective mycological media were evaluated and compared statistically for their suitability to enumerate yeasts, and simultaneously suppress moulds, in white-mould cheeses. Malt extract agar (MEA) supplemented with 0.5% sodium propionate (SP), proved to be the only medium that totally suppressed mould growth. However, the medium also restricted yeast development, as the mean yeast counts obtained on this medium differed significantly ($p<0.5$) compared to the other media. The most suitable media were also compared for supporting the growth of the six most predominant yeasts associated with white-mould cheeses. MEA-SP totally inhibited the growth of *Zygosaccharomyces rouxii*, making it an inappropriate medium for the enumeration of yeasts from white-mould cheeses. The mean yeast counts obtained on MEA-SP and modified molybdate agar differed statistically from that obtained on the other media. Dichloran Rose Bengal agar (DRBC) and Oxytetracyline Gentamycin Glucose Yeast Extract agar (OGGYA) retarded mould growth and supported the growth of the six most dominant yeast species recovered from white-mould cheeses, making these media suitable for the isolation and enumeration of yeasts in the presence of moulds.

Keywords: selective media, yeasts, Camembert cheese, enumeration.

2.1. Introduction

Varieties of white-mould ripened cheeses manufactured in South Africa include Camembert and Brie, or a combination of the two cheeses. These cheeses are produced from pasteurized cows' milk, to which lactic acid bacteria starter cultures are added. Spores of the mould *Penicillium candidum* are sprayed onto the surface of the fresh curd and maturation occurs in incubation rooms at 14°C. During the ripening period of eight weeks, several non-starter microorganisms, such as bacteria and yeasts develop on the surface and in the centre of these cheeses. Yeasts have frequently been recovered from mould-ripened cheeses at populations higher than 10^6 cfu.g^{-1} (Addis et al., 2001; Devoyod et al., 1968; Lenoir, 1984; Roostita and Fleet, 1996; Schmidt and Lenoir, 1978).

Yeasts play an important role in the ripening of Camembert cheese. They are part of a complex microflora that develops on the surface and contributes to ripening by producing proteases and lipases, as well as metabolizing lactic acid (Lenoir, 1984). Moulds develop rapidly into large colonies on agar media, usually overgrowing and covering yeast colonies making the isolation and enumeration of yeasts an impossible and difficult process. The inhibition of moulds during yeast isolation is complex because both are generally sensitive to the same fungistatic chemicals (Addis et al., 1998).

Several culture media are available for the isolation of yeasts for standard, differential, or selective purposes (Rale and Vakil, 1984). The typical medium for the enumeration of yeasts, should totally inhibit the growth of moulds and bacteria, and be nutritionally adequate to support the growth of all yeasts present (Beuchat, 1993; Jakobsen and Narvhus, 1996). Although a medium with these ideal properties does not exist, several satisfactory media are available and most yeasts of interest in the dairy industry are easily enumerated by standard methods (Baroiller and Schmidt, 1990; Beuchat, 1993; Fleet and Mian, 1987; Rohm et al., 1992).

Media typically used are potato dextrose agar (Baroiller and Schmidt, 1990), malt extract agar (Fleet and Mian, 1987), yeast extract glucose agar (Nooitgedagt and Hartog, 1988; Rohm et al., 1992), yeast nitrogen base (Besancon et al., 1992) and similar media supplemented with various antibiotics like oxytetracylcine, chlortetracycline, chloramphenicol, gentamycin and streptomycin (Beuchat, 1993). Acidified media have been used for many years for isolating fungi from dairy products, soils and foods with the H-ion concentration limiting growth of bacteria (Beuchat, 1979; Koburger, 1971; Koburger and Farhat, 1975; Nelson, 1972). The disadvantage of acidified amended media is the inability of stressed fungi to tolerate the low pH of the media (Henson et al., 1982).

Media designed to restrict the spreading growth of moulds contain inhibitors such as dichloran (2,6-Dichloro-4-Nitro-Aniline) or rose bengal (Hocking and Pitt, 1980; Jarvis, 1973; King et al., 1979). Dichloran-Rose Bengal Chlortetracycline medium (DRBC) was developed to restrict the spreading of *Rhizopus, Mucor* and other genera of rapidly growing fungi during the enumeration of fungi from foods (King et al., 1979). DRBC is recommended for the isolation of yeasts in the presence of moulds (Beuchat, 1993; Mislivec et al., 1992; Pitt et al., 1992). Dichloran-Glycerol agar (DG18) contains dichloran to reduce spread of moulds and 18% (w/v) glycerol to lower the water activity (a_w) to 0.95. Originally recommended for the enumeration of xerophilic fungi from low-moisture foods (Hocking and Pitt, 1980), DG18 is also accepted as a general-purpose medium for the isolation of moulds and yeasts (Pitt et al., 1992).

Mislivec and Bruce (1988), reported the suppression of spreading moulds isolated from a number of different food types, by the addition of 7.5% sodium chloride into an agar medium. The incorporation of sodium chloride to the diluent and the growth media for the isolation of yeasts, to simulate the cheese surroundings, could increase the recovery of yeasts, by reducing osmotic shock and also meeting the requirements of moderate halophiles (Baross and Lenovich, 1992; Jakobsen and Narvhus, 1996; Jarvis and Williams, 1987).

There is still a need for a selective medium to ensure reliable quantitative enumeration of yeast colonies from white-mould cheeses. In this study, we compared ten different media for the isolation of yeast colonies from Camembert cheese. From the ten media, only three media that suppressed spreading moulds were selected to ascertain whether the media suppressed yeasts that frequently occur and predominate in white-mould cheeses. The aim of this study was to select the most suitable medium for the isolation and enumeration of all yeasts from white-mould cheeses.

2.2. Materials and methods

2.2.1. Camembert cheese manufacture

Camembert and Brie cheeses were manufactured at a commercial cheese factory in the North West Province region of South Africa. The procedure for cheese making was carried out as described by Kosikowski (1997).

2.2.2. Enumeration media

Rose-bengal chloramphenicol agar (RBCA) (Merck, C107, Darmstadt, Germany – pH 7.2), Dichloran Rose-bengal chloramphenicol agar (DRBC) (Oxoid, CM 727, Basingstoke, England – pH 5.6), Dichloran 18% Glycerol agar (DG18) (Oxoid, CM 729 – pH 5.6) and oxytetracycline-gentamycin-glucose-yeast extract agar (OGGYA) (Merck, 10877 – pH 6.6) were prepared according to the manufacturer's instructions. In order to temper OGYYA medium, $0.1g^{-1}$ oxytetracycline (filter sterilized) and $0.05\ g^{-1}$ gentamycin (filter sterilized), were added aseptically. Malt extract agar (MEA) (Merck, C10 - pH 5.4) supplemented with biphenyl (BDH Chemicals Ltd, Poole, England, 28240) (MEA-BP), was prepared according to the method described by Addis et al. (1998). Molybdate agar was prepared according to the method described by Atlas (1993), while modified molybate agar supplemented with 5 ml of a 10% sodium propionate (SP), added aseptically just before use, was also prepared. Stock solutions of sodium propionate were sterilized at 108°C for 10 min. MEA (Merck, C10 - pH 5.4) supplemented with 0.5 % sodium propionate (MEA-SP) (to inhibit moulds) was prepared, and 2ml of a 10% lactic acid solution (filter sterilized) were added to every 100ml of media to inhibit bacterial growth. Potato Dextrose agar (PDA) (Merck, C100 - pH 5.6) supplemented with 7.5% NaCl (Merck, 6400) was prepared as described by Mislivec and Bruce (1988). 0.2% Ox-bile (Oxoid, L50) were incorporated into MEA (Merck, C10 - pH 5.4) (MEA-OX), before autoclaving, to suppress mould growth. Media (15-20 ml) were poured into 90-mm Petri dishes and held 1-2 days at room temperature to facilitate removal of excess surface water.

2.2.3. *Sampling methods*

A 10g sample of Camembert cheese was aseptically weighed into 90 ml sterile peptone in a sterile plastic bag (Whirl-pak, Nasco) and homogenized in a stomacher (Colworth 400) for 2 mins. Further decimal dilutions were carried out in triplicate as required for microbiological assays in 9ml sterile peptone water and 0.1 ml aliquots were spread plated on each of the media described above. Plates were incubated in an upright position in the dark, without being disturbed, at 25°C and counted after 5 days. This process was repeated five times.

2.2.4. *Yeast strains*

Six yeast strains were used: *Debaryomyces hansenii* UOFS Y – 0219, *Yarrowia lipolytica* UOFS Y – 1138, *Torulaspora delbrueckii* UOFS Y – 0227, *Kluyveromyces marxianus* UOFS Y – 0866, *Saccharomyces cerevisiae* UOFS Y – 2169 and *Zygosaccharomyces rouxii* UOFS Y – 0699).

2.2.5. *Preparation of inoculum and application to agar media*

Yeasts were grown in YM broth (Wickerham, 1951) at 25°C for 48h, then serially diluted in 9ml sterile peptone water and spread plated (0.1 ml in triplicate) on DRBC agar, OGGYA and MEA-SP for their performance in supporting colony development by test yeasts. Plates were incubated in an upright position in the dark at 25°C for 5 days. Plates on which 15 – 150 colonies developed were selected for enumeration. Observations on differences in size, colour, general appearance and ease of counting colonies on the test media were recorded. This process was repeated five times and plated in triplicate.

2.2.6. *Statistical analysis*

All results were tabulated and statistically analyzed employing the Analysis of Variance (ANOVA). Means were compared by applying the Student-

Neumann-Keuls test. A significant F-value of $p < 0.05$ was employed (Schefler, 1979).

2. 3. Results and discussion

Table 1 shows the means for the colony counts of yeasts and moulds recovered from Camembert cheese, utilizing ten different selective media. The incorporation of 0.5% sodium propionate into MEA, totally inhibited mould growth, and concurrently retarded the development of yeasts, as the mean yeast count obtained on MEA-SP differed statistically from the data obtained on all the other media, except for molybdate and modified molybdate agars (Table 1). Compared to most of the other media, yeasts colonies appeared off-white, and were smaller on MEA-SP. Bacterial growth was totally inhibited by the addition of 10% lactic acid. In contrast, mould growth occurred on both molybdate and modified molybdate agars, while yeast development did occur, but the colony sizes were reduced. The enumeration of yeasts and moulds on both molybdate and modified molybdate agar, was a difficult task as the yeasts were overgrown by moulds. The agar, being dark blue, made it difficult to distinguish separate mould colonies, and the yeasts appeared in small and navy colonies.

Smith et al. (1974), also encountered fungal growth on molybdate agar from clinical samples. Contradictory results were obtained by Rale and Vakil (1974), who reported that molybdate agar was very effective in isolating yeasts from a variety of sources such as fruits, food preserves, dairy wastes etc. Research performed at a later stage by the same authors revealed a 100% recovery of yeasts on molybdate agar supplemented with 0.125% calcium propionate, when challenged with artificially prepared samples containing known numbers of viable yeasts heavily covered with moulds and actinomycetes. The researchers obtained similar results with natural samples (Rale and Vakil, 1984).

Propionic acid has been an accustomed food additive (Rale and Vakil, 1984), and a suitable medium supplement for yeast selectivity (Deak and Beuchat, 1996; Rale and Vakil, 1984). However, Etchells et al. (1954), reported a prominent inhibition of yeasts at 0.35% concentration of sodium propionate. In

order to suppress moulds in the presence of yeasts, compounds such as sodium, potassium or calcium propionate (0.1 - 0.2%) can be effective (Beuchat, 1993; Beech and Carr, 1955; Bowen and Beech, 1967; Goto and Yokotsuka, 1977). Addis et al. (1998), compared the use of sodium propionate and biphenyl in media for the isolation of yeasts, and determined biphenyl as the favoured inhibitor. Mould growth was not significantly suppressed by the addition of 0.05% biphenyl, however from our experience, mould colonies were slightly restricted and yeasts were easy to enumerate. This is in agreement with research performed by Addis et al. (1998) who reported that mould growth was restricted by the incorporation of 0.05% biphenyl into MEA, permitting yeasts to be counted after 3 - 4 days incubation.

The mean mould counts obtained on OGGYA and MEA-SP were considerably lower and differed significantly from those obtained on the other media (Table1). OGGYA totally inhibited bacterial growth. According to Beuchat (1993) and Mossel et al. (1975) a combination of two antibiotics at concentrations in the order of 10-100 $\mu g.ml^{-1}$ is used to effectively inhibit bacterial growth. Beuchat and Nail (1985) revealed that the presence of oxytetracylcine played a more effective role by inhibiting bacteria compared to the addition of chloramphenicol and chlortetracycline. The addition of ox-bile to MEA did not retard the development of moulds making the enumeration of yeasts, a tedious process. However, it was easy to enumerate yeasts in the presence of moulds on DG18 agar and on PDA-NaCl, as the media suppressed the mould colonies from spreading. Mislivec and Bruce (1988) added NaCl to lower the water activity of PDA, in order to prevent seed germination, and reported that NaCl effectively inhibited spreader moulds.

DG 18 agar was developed for the detection of moderately xerophilic fungi (Hocking and Pitt, 1980) but is currently used as a general purpose medium for the enumeration of both yeasts and moulds (Deak et al., 2001). King (1986) reported that the recovery of yeasts on DG 18 agar was similar to that of media such as DRBC agar, oxytetracycline glucose yeast extract, acidified or chloramphenicol – supplemented PDA, or tryptone glucose yeast extract (TGY) agar. Other researchers have noted that DG 18 agar might support the

development of lower numbers of yeast colonies than DRBC or TGY agars, depending on the composition of the food being analyzed and the profile of yeasts present (Deak, 1992; Nunez et al., 1996).

Although DRBC agar did not suppress mould growth significantly (Table 1), moulds were prevented from spreading, and the colony sizes were much smaller, in comparison with those detected on the other media. The enumeration of yeasts on DRBC agar was effortless and yeasts were not suppressed by this medium as the colony sizes were large (Table 2), making it a suitable and recommended medium for the enumeration if yeasts in the presence of moulds. This is in agreement with research performed by others (Beuchat, 1993; Mislivec et al., 1992; Pitt et al., 1992). Hocking and Pitt (1992) found DRBC agar to be a consistent medium giving unvarying mould and yeast counts. Even though no statistical difference was found between RBCA and DRBC agar, the latter medium restricted moulds from spreading much better than RBCA.

Smith and Dawson (1944) discovered an inhibitory effect of rose bengal on the growth of fungi isolated from soil. Mossel et al. (1975) reported that media containing rose bengal reduces the size and height of many mould colonies. The reliability of media containing rose bengal for the enumeration of yeasts from foods is questionable (Mossel et al., 1975) and the low pH increases the inhibition of yeasts by rose bengal (King et al., 1979). Photodegradation of rose bengal produces breakdown products that are toxic or inhibitory to yeasts and other microorganisms (Banks et al., 1985; Banks and Board, 1987; Jarvis et al., 1983). Therefore it is important to incubate media containing rose bengal in the dark.

From the above and the statistical analysis in Table 1, only DRBC agar, MEA-SP and OGGYA were selected for further evaluation. The yeasts listed in Table 2 were tested on the three media in order to assess whether the media suppressed or supported the development of the six most dominant yeasts present in white-mould cheeses. Differences in mean populations of all test yeasts recovered on the three enumeration media were minimal. Only

Zygosaccharomyces rouxii was suppressed by MEA-SP (Table 2). There was also a statistical difference between the growth of this yeast on DRBC agar and OGGYA (Table 2). The other yeasts colonies were largest on DRBC agar and OGGYA, while MEA-SP exhibited smaller colony sizes.

Based on the results obtained in this study, all ten of the selective media proved to retard bacteria and mould growth to a certain extent. However, it is generally accepted that antibiotic-supplemented fungal enumeration media are superior to acidified media for the enumeration of fungi in foods (Beuchat, 1979; Koburger, 1971; Koburger and Farhat, 1975; Nelson, 1972).

2. 4. Conclusion

Statistical differences for the enumeration of yeasts from Camembert cheese on MEA-SP and modified molybdate agar were obtained. Modified molybdate agar is not practical being dark in colour, making yeast enumeration difficult. Yeasts were inhibited by both the molybdate and sodium propionate added to this medium. The combination of the antibiotics, oxytetracycline and gentamycin in OGGYA, proved to be effective, the antibiotics totally suppressed bacterial growth and partially retarded mould growth, as the mean mould count obtained on this medium was lower and statistically different to that of the other media (Table 1).

OGGYA and DRBC agar supported the growth of yeasts present in Camembert cheese, as the highest mean yeast counts were obtained from these media. In addition, these two media supported the growth of the most dominant yeast species occurring in white-mould cheeses (Table 2). However, a lower mean count of *Zygosaccharomyces rouxii* was obtained on DRBC agar than on OGGYA. No mould growth occurred on MEA supplemented with 0.5% sodium propionate, which makes this compound an appropriate mould inhibitor. However, the mean yeast counts obtained on this medium were lower and significantly different to that of the other media (Table 1). MEA-SP also supported the growth of the majority dominant yeast species, as no significant differences in the mean yeast counts were obtained (Table 2), except that *Zygosaccharomyces rouxii* was totally inhibited by 0.5% sodium propionate. This characteristic may be of taxanomical importance. Although the need for an ideal medium for the isolation and enumeration of yeasts in the presence of moulds still exists, DRBC agar and OGGYA are the most appropriate media for this purpose.

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Table 1.

Mean populations of yeasts and moulds recovered from Camembert cheese on ten media.

Media	Mean[a] yeast counts (log cfu.g^{-1})	Mean[a] mould counts (log cfu.g^{-1})
DG 18	6.28	6.98
S^b	0.49^{ab}	0.31^b
DRBC	6.56	6.67
S^b	0.28^b	0.44^b
MEA-BP	6.40	6.92
S^b	0.44^b	0.30^b
MEA-SP	5.83	0
S^b	0.56^a	0^a
Molybdate-SP	5.86	6.80
S^b	0.39^a	0.42^b
Molybdate agar	6.17	6.99
S^b	0.46^{ab}	0.23^b
OGGYA	6.55	5.30
S^b	0.31^b	2.74^a
MEA-OX	6.51	6.82
S^b	0.37^b	0.33^b
PDA-NaCl	6.57	6.76
S^b	0.33^b	0.27^b
RBCA	6.52	6.87
S^b	0.29^b	0.32^b

[a] Mean values of yeasts and moulds recovered on each media in triplicate, from five separate trials; means with different superscripts in the same row differ significantly ($P<0.05$)

[b]Standard error of the mean

DG18 = Dichloran-Glycerol agar; DRBC = Dichloran Rose Bengal Chloramphenicol agar; MEA-BP = Malt Extract agar + biphenyl; MEA-SP = Malt Extract agar + sodium propionate; Molybdate-SP = Molybdate agar + sodium propionate; OGGYA = Oxytetracycline Gentamycin Glucose Yeast agar; MEA-Ox = Malt Extract agar + Oxbile; PDA-NaCl = Potato Dextrose Agar + sodium chloride; RBCA = Rose Bengal Chloramphenicol agar.

Table 2.
Mean populations of yeasts recovered on three media

Yeast	Mean populations[a] (log cfu.g^{-1}) Recovery medium			
	DRBC	MEA-SP	OGGYA	P[•]
Yarrowia lipolytica	7.94	7.88	7.90	0.8560
S[b]	0.23	0.25	0.34	
Debaryomyces hansenii	8.44	8.56	8.56	0.1982
S[b]	0.18	0.22	0.22	
Torulaspora delbrueckii	8.46	8.42	8.48	0.2188
S[b]	0.08	0.10	0.10	
Zygosaccharomyces rouxii	7.81	0	8.03	0.0004
S[b]	0.18[a]	0	0.10[b]	
Kluyveromyces marxianus	8.22	8.18	8.26	0.4850
S[b]	0.17	0.17	0.15	
Saccharomyces cerevisiae	7.50	7.43	7.42	0.5720
S[b]	0.17	0.30	0.17	

[a]Mean populations of each yeast recovered on the three media; means with different superscripts in the same row differ significantly ($P<0.05$)

[b]Standard error of the mean

[•]$P>0.05$ not significant

DRBC = Dichloran Glycerol agar; MEA-SP = Malt Extract agar; OGGYA = Oxytetracycline Gentamycin Glucose Yeast agar.

Chapter 3

Seasonal diversity of yeasts associated with white-surface mould-ripened cheeses.

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Abstract

The yeasts present in Camembert and Brie cheeses during processing were monitored in a single cheese factory during summer and winter, to determine the seasonal diversity of yeasts over a ripening period of 56 days. Despite the predominance of lactic acid bacteria during the making of Camembert and Brie, yeasts play a significant role in the ripening process reaching counts as high as 10^6 cfu.g^{-1} at the later stages of ripening. The sources of yeast contamination that may lead to contamination of the curd were also determined. The starter culture preparation, whey, brine, air and equipment surfaces were responsible for the highest yield of contaminating yeasts. A diverse variety of 20 yeast species representing 10 genera were present during the winter period associated with the factory environment, during processing and ripening, whereas only seven yeast species representing six genera were isolated during summer. Samples were taken at critical control points during the manufacturing process and the yeast populations enumerated after incubation at 25°C for 96h. Although a broad spectrum of yeasts were isolated from Camembert and Brie cheeses, *Debaryomyces hansenii* was the most abundant yeast isolated. Other species encountered were *Yarrowia lipolytica*, *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa*, *Rhodotorula minuta*, and various species of *Candida*.

Keywords: Cheese; Enumeration; Camembert; Brie; Identification; Yeasts

3. 1. Introduction

Reports on the presence of yeasts in cheeses date back to the early part of the previous century and ample literature on the subject have since accumulated (Devoyod, 1990; Fleet and Mian, 1987; Fleet, 1990; Walker, 1988). However, it is not generally accepted that the occurrence of yeasts is an important constituent of the microflora of many cheese varieties (Fleet, 1990; Viljoen and Greyling, 1995; Viljoen 2001; Welthagen and Viljoen, 1998).

In certain cheeses, especially in semi-soft cheeses with surface films, e.g. Limburger and Tilsit and mould-ripened cheeses such as Camembert, Brie and Roquefort, yeasts contribute to the ripening process (Eliskases-Lechner and Ginzinger, 1995; Nunez, 1978; Seiler and Busse, 1990; Szumski and Cone, 1962, Viljoen, 2001). Yeasts utilize the lactic acid present derived from the breakdown of lactose by the starter cultures which increases the pH and consequently stimulates bacterial growth and initiates the second phase of ripening. In addition, certain proteolytic and lipolytic enzymes produced by some yeast strains, directly influence the maturation process (Devoyod and Spoonem, 1970; Law, 1978; Marth, 1982; Noomen, 1983; Schmidt et al., 1979). In some cases, yeasts contribute positively to the fermentation and ripening process of cheeses by suppressing the growth of undesired microorganisms present (Devoyod et al., 1968; Kaminarides and Laskos, 1992), as well as supporting the role of the bacterial starter culture (Kalle et al., 1976). Yeasts also excrete growth factors (Jakobsen and Narvhus, 1996) and produce gasses contributing to curd openness (Coghill, 1979).

The occurrence of yeasts in retail cheeses has been reported on several occasions (Fleet, 1990; Roostita and Fleet, 1996; Schlesser et al., 1992), where counts were often $10^5 - 10^6$ cfu.g^{-1} and in certain cheese varieties, as high as $10^7 - 10^8$ cfu.g^{-1} (de Boer and Kuik, 1987; Eliskases-Lechner and Ginzinger, 1995; Fleet, 1990; Lenoir, 1984; Nooitgedagt and Hartog, 1988; Roostita and Fleet,

1996). The importance of the presence of yeasts depends on the specific type of cheese. Yeasts may contribute to spoilage in some cheeses, or aid in the development of a characteristic flavour during the ripening process of other cheeses (Fleet, 1990; Fleet and Mian, 1987). Numerous studies have identified the diversity of yeast species that contribute to the flavour development, interactions and secondary flora and these are referred to in reviews by Fleet (1990), Addis et al. (2001), Stanley (1998 a, b), Jakobsen and Narvhus (1996), and Corsetti et al. (2001). These studies also indicated on the yeast profiles during maturation (Addis et al., 2001), the key properties that affected the quality, and attempts to identify species that might be developed as starter cultures (Martin et al. 1999; Wyder and Puhan, 1999). None of these studies, however, gave any indication of how the diversity may change due to seasonal variations. Therefore, a need exists to examine the yeast profiles during seasonal changes since an altering yeast profile might also affect product quality.

The objectives of this study were to detect the sources of yeast contamination within a white mould cheese factory, to identify the yeast strains present and to report on the frequency and seasonal diversity of the occurrences of yeasts.

3. 2. Materials and methods

3.2.1. Camembert and Brie cheese manufacture

Camembert and Brie cheeses were manufactured on four different occasions during the winter and summer at a commercial cheese factory in the North West Province region of South Africa. The procedure for cheese making was carried out as described by Kosikowski (1997).

3.2.2. Sampling methods and selection of isolates

On all four occasions, the surfaces (Table1) were sampled using cotton swabs and dipped into 9ml sterile standard peptone water. Portions (0,5ml) of the suspensions were spread inoculated onto 90mm Petri dishes containing Rose Bengal Chloramphenicol agar (RBCA) (Merck, C98, Darmstadt, Germany – pH 6.6) for the isolation of yeasts and De Mann Rugosa and Sharpe agar (MRS) (Merck, C86 - pH 6.5) for the isolation of lactic acid bacteria.

Air was sampled using settle plates (standard 90mm Petri dishes) containing RBCA with an exposure time of 5min.

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

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 the highest dilutions on plates containing Rose Bengal Chloramphenicol agar. The yeast isolates were subcultured on Malt Extract agar (MEA) (Merck, C10 - pH 5.4) for 48h at 25°C and checked for purity by colony morphology and microscopy. The pure cultures were stored at 4°C on Yeast Malt Extract agar (YM) (Wickerham, 1951) slants during the period of investigation, until characterization.

3.2.3. Sampling during ripening

Camembert (eight) and Brie (eight) cheeses from the same batch were kept on each occasion under controlled conditions (4°C) and sampled directly after processing at consecutive intervals on a weekly basis during ripening over a 56 day period. Cheese samples were prepared for microbiological analysis by opening the cheese aseptically with a sterile trier. For each sample, surface and centre, 10g were aseptically weighed into 90ml sterile peptone water in a sterile plastic bag (Whirl-pak, Nasco) and homogenized in a stomacher (Colworth 400) for 2min. Further decimal dilutions were carried out in duplicate as required for microbiological assays in 9ml sterile peptone water and spread plated on the media described above. Similar incubation and duration procedures were applied as described earlier.

3.2.4. Sample analysis

All plates containing between 15 and 150 colony forming units (cfu) from the highest dilution (or the highest number if below 15), 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.

3.2.5. *Yeast Identification*

Individual yeast isolates were identified by conducting physiological, sporulation and morphological tests as described by Kurtzman and Fell, (1998). Data were interpreted using the keys of Kurtzman and Fell, (1998) and the computer program of Barnett et al. (1987). The tests performed included cellular morphology, sporulation, carbohydrate fermentation, carbohydrate assimilation, nitrogen assimilation, growth in vitamin free medium, urea hydrolysis and cycloheximide resistance.

3. 3. Results and discussion

3.3.1. Microbial enumeration during processing

Microbial analyses were performed during the processing and maturation of white-mould cheeses in the summer and winter. Lactic acid bacteria, as expected, were the major component of the microflora during processing and maturation (Table 1 and Figs. 1 - 4). The lactic acid bacteria starter cultures were present at high populations during processing, reaching counts as high as 10^9 cfu.g^{-1}, but were lower (10^8 cfu.g^{-1}) after submersion into the brine. It is interesting to note that higher numbers of lactic acid bacteria occurred in the cheese curd before submersion into the brine, during the winter, despite a lower initial count in the starter culture preparation, compared to the cheese manufactured during the summer (Table 1). Similar bacterial counts, however, were observed after submersion into the brine and after four days at 14°C incubation.

Yeasts on the other hand, increased from 4.08 log cfu.g^{-1} to 6.48 log cfu.g^{-1} during processing in the winter, and from 3.67 log cfu.g^{-}1 to 4.70 log cfu.g^{-1} in the summer. According to the data obtained (Table1), the brine had little or no affect on the viability of the yeasts. No yeasts were isolated from the milk starter culture or any of the ingredients added during processing which corresponded with results obtained by Welthagen and Viljoen (1998, 1999), Fleet (1990) and others who indicated that yeast populations originated as post-pasteurization contaminants (Baroiller and Schmidt, 1990; Vadillo et al., 1987).

Sources of yeast contamination were the air, floors, walls and shelves, equipment and the brine (Table1). The brine was responsible for the highest yield of yeast contamination exceeding 3 log units. High yeasts numbers in the brine was also encountered by Welthagen and Viljoen (1998, 1999), Viljoen and Greyling (1995), Seiler and Busse (1990), in Gouda and Feta cheeses. According to Viljoen

(2001), the immediate ecosystems of yeasts itself, the environmental conditions prevailing, and the pasteurization of raw milk contribute towards the selection of a uniform and well-defined yeast domain (Jakobsen and Narvhus, 1996). Similar yeast counts, however, were obtained from environmental samples during winter and summer (Table 1), although some differences were observed on the equipment, in the air and the brine. Variations in yeast levels, however may be due to various environmental attributes like salt concentration in the brine (Seiler and Busse, 1990), temperature (Davenport, 1980), accidental occurrences of contaminating yeasts (Fleet, 1990; Deak and Beuchat, 1996), or the standards of hygiene during cheese making and the efficiency of pasteurization the day of sampling (Fleet and Mian, 1987; Welthagen and Viljoen, 1998). Consequently, the yeast numbers present may vary between dairy plants and even between consecutive days in the same plant.

3.3.2. Microbial enumeration during maturation

Figs. 1 – 4 show the quantitative evolution of the lactic acid bacteria and yeasts in the centre and surface of Camembert and Brie cheeses. Lactic acid bacteria were the major component of the microflora during ripening in the centre and surface of both Camembert and Brie cheeses (Figs. 1 – 4). As seen in Table 1, the milk was inoculated with 8.7 log cfu.ml^{-1} of starter lactic acid bacteria in summer and 7.9 log cfu.ml^{-1} in winter. During both seasons the bacteria grew rapidly during processing reaching optimums of 8.9 log cfu.g^{-1} in summer and 9.2 log units.g^{-1} in winter. These initial increases in both cheeses during both seasons, were followed by gradual decreases on the surfaces within the first two weeks after the beginning of maturation. The general tendency of viability of the lactic acid bacteria on the surfaces after two weeks was to remain constant or to stabilize (Figs. 1 – 4).

The viability of the lactic acid bacteria populations, however, was substantially lower in the summer on the surfaces of both cheeses, whereas similar counts were present in the centre of Camembert and Brie cheeses during both seasons. Despite favourable environmental conditions which prevailed in the maturation rooms, i.e. lower temperatures and higher relative humidities, it is interesting to note the stabilization of lactic acid bacteria after week 2 and the corresponding increase in yeast numbers on the rind of Camembert and Brie cheeses in winter and summer (Figs. 1 and 3). This is in agreement with results obtained by Welthagen and Viljoen (1998), who attributed the apparent accelerated increase of yeasts after the stabilization of lactic acid bacteria, due to the utilization of organic acids produced by the lactic acid bacteria, which consequently lead to an increase in yeasts.

Substantial differences in the number of yeasts present during the ripening of the cheeses in the winter and summer were observed. In winter, yeasts tended to increase steadily during maturation and generally higher yields were obtained. The yeasts on the surfaces of both cheeses increased from 10^6 cfu.g^{-1} to populations as high as 10^8 cfu.g^{-1} during the winter (Figs. 1 and 3). Roostita and Fleet (1996), Addis et al. (2001) and Lenoir (1984), also reported high numbers of yeasts on the surfaces of mould-ripened cheeses. The number of yeasts in the core of Camembert, decreased from 10^4 cfu.g^{-1} during the first two weeks of maturation, to 10^3 cfu.g^{-1} at week 3. They increased steadily to 10^5 cfu.g^{-1} at week 4, then remained at populations between 10^4 cfu.g^{-1} and 10^5 cfu.g^{-1} until the end of the ripening period (Fig. 2). The yeast populations in the core of Brie cheese were high at week one (10^6 cfu.g^{-1}), and stabilized at 10^5 cfu.g^{-1} after week 2 until the end of ripening (Fig. 4).

During summer, yeast numbers on the surface of Camembert cheese, increased from 10^5 cfu.g^{-1} to 10^6cfu.g^{-1} from week 2 until week 3, then decreased slightly after week 3 and stabilized at 10^5 cfu.g^{-1} from week 4 until week 7, and increased to 10^6 cfu.g^{-1} during the final week of maturation (Fig. 1). However, the yeast

populations on the surface of Brie cheese increased from 10^6 cfu.g^{-1} to 10^7 cfu.g^{-1} from week 2 until week 3, then decreased slightly to 10^5 cfu.g^{-1} at week 4, and remained between 10^5 cfu.g^{-1} and 10^6 cfu.g^{-1} until the end of ripening (Fig. 3). The number of yeasts in the core of Camembert cheese increased from 10^2 cfu.g^{-1} to 10^5 cfu.g^{-1} from week 2 until week 4, and then decreased and stabilized at 10^2 cfu.g^{-1} until the end of ripening, in summer. In contrast, the yeast populations in the centre of Brie cheese increased steadily from 10^2 cfu.g^{-1} to 10^6 cfu.g^{-1} from week 1 until week 2, and then remained between 10^4 cfu.g^{-1} and 10^5 cfu.g^{-1} from week 3, until week 7, and were absent during the final week of maturation (Fig. 4). The number of yeasts on the surface of both cheeses was much higher than in the centre, during both seasons. Whereas, the yeasts encountered on the surface and in the centre of both cheeses, occurred between one to two log cycles higher, in winter than in summer.

3.3.3. Yeast identification

Since yeasts are ubiquitous in agricultural environments, a broad spectrum of yeasts was, as expected, found during cheese manufacture (Tables 2, 3 and 4). A total of 93 yeast strains consisting of 10 genera, with 20 different representing species, were isolated from environmental sources and during the processing and ripening of Camembert and Brie cheeses in winter, while only 76 yeasts strains consisting of only six genera with seven representing species were obtained during the summer. From Tables 2, 3 and 4, it is clear that there is a significant difference in the variety of yeast species isolated during summer and winter, i.e. a higher diversity of yeasts was obtained during the winter.

Sources of yeast contamination representing the environmental samples were the air, floor, walls and equipment surfaces, while those representing samples from the processing line were the starter culture preparation, whey, curd before brine, brine bath and cheese before ripening (Table 2). During winter, nine different yeast species were isolated from the environmental samples, while the

processing line yielded eight different yeast species. Twelve different yeast species were encountered during the ripening of Camembert cheese (Table 3), while only nine different yeast species were isolated during the ripening of Brie cheese (Table 4).

During summer, only four different yeast species were isolated from the environmental samples, while the processing line yielded five different yeasts species (Table 2). Five different yeast species were obtained during the ripening of Camembert cheese (Table 3), and six different yeast species were isolated during the ripening of Brie cheese (Table 4).

The equipment surfaces, floor and the brine bath were mainly responsible for the purveyance of various contaminating yeast species. Since the curd before brine and whey is inherently part of the cheese processing, various yeast species were obviously also isolated from the whey and curd before brine.

The most predominant species isolated form the environmental samples and during processing and ripening of Camembert and Brie cheeses, were *Debaryomyces hansenii* and *Yarrowia lipolyica* and various species of *Candida*. The predominance of *Debaryomyces hansenii* strains in the present study is consistent with results presented elsewhere: Deak and Beuchat (1996), reported on the frequent occurrence of the species in dairy products, Seiler (1991) and Viljoen and Greyling (1995) indicated the dominance of *Debaryomyces* strains in cheese brines, and the dominance in cheeses (Roostita and Fleet, 1996). Strong growth in the presence of salt, growth at low temperatures and the ability to utilize lactic and citric acids, are considered as key determinants that encourage the predominance of *Debaryomyces hansenii* in cheeses (Besancon et al., 1992; Roostita and Fleet, 1996). In this study *Debaryomyces* strains were present in the environmental samples, in the brine and almost every occasion in samples obtained during the ripening process. During the winter, *Debaryomyces hansenii* was the most dominant species isolated in the centre and on the surface

of both Camembert and Brie cheeses during ripening, with *Yarrowia lipolytica* occurring only during the later stages of maturation (Tables 3 and 4). However, *Yarrowia lipolytica* was dominant during the summer, in the centre and on the surfaces of Camembert and Brie cheeses, throughout the ripening process. *Debaryomyces hansenii*, in contrast, was isolated on fewer occasions from the surface and in the centre of both cheeses, during the summer (Tables 3 and 4).

The predominance *Yarrowia lipolytica* during the later stages of maturation may be due to their lipolytic and proteolytic activies. The occurrence of *Yarrowia lipolytica* in cheeses is in agreement with its strong extracellular lipolytic and proteolytic properties (Weltagen and Viljoen, 1998). This species was isolated from the environmental samples, during processing and maturation. Alford and Pierce (1961), reported that *Yarrowia lipolytica* is characterized by strong lipolytic activities below 0°C. Viljoen and Greyling (1995), isolated *Yarrowia lipolytica* from the brine and equipment surfaces during the making of Gouda and Cheddar cheeses. According to Addis et al. (2001), *Debaryomyces hansenii* was the dominant species encountered during the ripening of Australian Camembert cheeses, while *Yarrowia lipolytica* was inconsistently isolated at much lower populations, and was less prominent as it only occurred at the later stages of maturation.

Corsetti et al. (2001), stated that the most significant yeast species isolated from Camembert (Lenoir, 1984) and Tilsiter (Rohm et al., 1992; Eliskases-Lechner and Ginzinger, 1995) are those that can be considered as contributing towards the maturation of other surface-ripened cheeses; namely *Kluyveromyces lactis*, *Debaryomyces hansenii* and their imperfect forms, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Candida catenulata* and *Yarrowia lipolytica*.

The yeast species most frequently isolated from mould-ripened soft cheeses include: *Kluyveromyces marxianus, Debaryomyces hansenii, Candida famata, Yarrowia lipolytica, Pichia membranaefaciens, Pichia fermentans,*

Saccharomyces cerevisiae and *Zygosaccharomyces rouxii* (de Boer and Kuik, 1987; Galzin et al., 1970; Lenoir, 1984; Nooitgedagt and Hartog, 1988; Nunez et al., 1981; Olson, 1969; Schmidt and Lenoir, 1978, 1980; Tzanetakis et al., 1987; Vergeade et al., 1976). Deak (1991), reported that *Kluyveromyces marxianus* and *K. lactis* were the most dominant yeasts isolated from young Camembert cheese, while *Debaryomyces hansenii, Saccharomyces cerevisiae* and *Candida versatilis* were also well represented. However, only the lactose-fermenting yeasts, *K. lactis* and *K. marxianus* prevailed after maturation. However, of the yeast species most often isolated in Camembert and Brie cheeses, *Debaryomyces hansenii* and *Yarrowia lipolytica* were the dominating species isolated during this study. This is in agreement with research performed by Roostita and Fleet (1996), who reported that the most predominant yeast species isolated from Australian Camembert cheese were *Debaryomyces hansenii* and *Yarrowia lipolytica*. We failed to detect strains of *Kluyveromyces lactis* or *K. marixanus* and this may be attributed to a higher salt concentration found in South African produced Camembert and Brie cheeses.

Rhodotorula minuta, *Rhodotorula mucilaginosa* and *Sporobolomyces roseus* were isolated from the environmental samples from the processing line and during ripening (Tables 2, 3 and 4). The occurrence of these yeast strains in cheese has been reported previously (Deak and Beuchat, 1996; Fleet and Mian, 1987; Viljoen and Greyling, 1995). Roostita and Fleet (1996) and Viljoen and Greyling (1995) previously referred to the species as common air contaminants or natural contaminants in the cheese before they were stored, although *Rhodotorula* species are able to grow at sub-zero temperatures (Davenport, 1980).

According to the present results obtained from a single cheese factory, we demonstrated the frequent occurrence of yeasts associated with the making of Camembert and Brie cheeses. The species recovered from the different sources are more or less the same as those normally isolated from raw milk that

corresponds with the results presented by other workers (Walker and Aryes, 1970). *Debaryomyces hansenii* and *Yarrowia lipolytica* species predominated in the present study that could be related to the species' tolerance to low temperatures and high salt concentrations.

The presence of a large number of yeasts during the later stages of maturation suggests that these yeasts may play and important role in the ripening of cheese. *Debaryomyces hansenii* and *Yarrowia lipolytica* were the most proliferating and resistant yeasts found in this study. The other yeast species encountered only occurred sporadically. This suggests that these two species are an important component of the microflora of Camembert and Brie cheeses, as they were not included in the starter culture preparation.

3. 4. Conclusion

From the research presented in this report, it is apparent that there is a significant difference in the diversity of the yeast species isolated during different seasons. In winter, a more diverse variety of yeast species were obtained. Of the 93 yeast species isolated, 10 different genera and 20 different species were identified, whereas in the summer, only 76 yeast species were isolated ranging over only 6 genera containing 7 species.

Definite differences in the occurrence of *Yarrowia lipolytica* and *Debaryomyces hansenii* were observed during winter and summer, which might contribute to the final qualitiy. Further research to assess whether *Debaryomyces hansenii* and *Yarrowia lipolytica* affect the flavour, aromatic compounds and organoleptic characteristics of the cheeses, under different simulated environmental conditions (pH, A_w, temperature and salt concentration), seems very promising.

3. 5. References

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Table 1.

Environmental samples of yeast colonies during Camembert and Brie processing in the winter and summer. (Results are the means of four replications done in duplicate)

Sample	**Yeast colonies per 16 cm^2**	
Equipment	**Winter**	**Summer**
Silo	1.78	0
Balance tank	0	>3.78
Cheese vat	0	>3.78
Draining vat	>3.78	2.00
Camembert filler	1.30	>3.78
Brie filler	1.30	2.87
Camembert mould	1.90	2.15
Brie mould	>3.78	3.18
Mould draining vat	2.90	3.73
Floor	>3.78	>3.78
Wall	1.30	0
Incubation room - shelf	>3.78	2.70
Incubation room - floor	>3.78	1.60
Incubation room - wall	>3.78	>3.78
Air Samples (Yeast colonies on 90mm Petri dish)		
Cheese-making room	0	0
Incubation room	48	157
Processing (Log counts g^{-1}ml^{-1})		

	Yeasts	**LAB**	**Yeasts**	**LAB**
Pasteurised milk	0	0	0	0
Starter culture preparation	0	7.86	0	8.68
Whey	5.18	8.38	4.83	8.07
Before brine bath	4.08	9.20	3.67	8.93
Brine	3.25	5.48	2.30	5.98
After brine bath	3.72	8.67	4.81	8.52
During incubation (4 days at 14°C)	6.48	8.54	4.70	8.23

Table 2
Yeast isolates obtained from the cheese making equipment and processing during winter and summer

Cheese making equipment	**Winter**	**Summer**
Silo (before pasteurisation)	*Candida sake*	
Balance tank		*Debaryomyces hansenii*
Cheese vat		*Debaryomyces hansenii*
Draining vat	*Debaryomyces hansenii* *Rhodotorula minuta*	
Camembert filler	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Torulaspora delbrueckii*
Brie filler	*Candida sake* *Torulospora delbrueckii*	*Debaryomyces hansenii*
Camembert shaping mould	*Pichia farinosa*	
Brie shaping mould	*Debaryomyces hansenii*	*Debaryomyces hansenii* *Torulaspora delbrueckii*
Mould draining vat	*Debaryomyces hansenii* *Yarrowia lipolytica*	*Torulospora delbrueckii*
Floor	*Rhodotorula mucilaginosa* *Candida silvae* *Candida versatilis*	*Debaryomyces hansenii* *Candida versatilis*
Incubation room (air sample)	*Rhodotorula minuta*	*Debaryomyces hansenii* *Yarrowia lipolytica*
Processing		
Whey	*Rhodotorula minuta*	*Torulaspora delbrueckii*
Before brine	*Candida sake* *Kluyveromyces thermotolerans* *Rhodotorula mucilaginosa* *Yarrowia lipolytica*	*Debaryomyces hansenii* *Yarrowia lipolytica*
Brine	*Debaryomyces hansenii* *Rhodotorula minuta* *Sporobolomyces roseus* *Torulaspora delbrueckii*	*Debaryomyces hansenii*
After brine		*Candida rugosa* *Debaryomyces hansenii* *Yarrowia lipolytica*
During incubation (4 days)		*Candida rugosa* *Debaryomyces hansenii* *Yarrowia lipolytica*

Table 3 Yeast strains isolated from the centre and surface of Camembert cheese during ripening at selected time intervals in winter and summer.

Isolate	Winter Weeks																Summer Weeks													
	1		2		3		4		5		6		7		8		1		2		3		4		5		6		7		8	
	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
Candida																																
C. intermedia			+											+																	
C. rugosa																					+											
C. versatilis											+			+								+									
C. zeylanoides												+																				
Debaryomyces																																
D. hansenii	+		+	+	+		+	+	+	+		+	+	+	+	+	+			+			+	+	+	+		+				
Kluyveromyces																																
K. thermotolerans				+																												
Rhodotorula																																
R. minuta															+																	
Saccharomyces																																
S. cerevisiae													+																			
Torulaspora																																
T. delbrueckii																	+								+							
Yarrowia																																
Y. lipolytica	+				+	+	+	+			+		+	+	+		+	+		+	+	+	+	+	+	+		+	+	+		+
Zygosaccharomyces																																
Z. florentinus								+																								
Z. mellis																+																
Z. rouxii									+																						.	

C - centre; S - surface; + indicating the presence of the strain at the time of sampling.

Table 4 Yeast strains isolated from the centre and surface of Brie cheese during ripening at selected time intervals in winter and summer.

Isolate	Winter Weeks																Summer Weeks													
	1		2		3		4		5		6		7		8		1		2		3		4		5		6		7		8	
	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
Candida																																
C. catenulata							+																									
C. rugosa																	+			+												
C. sake				+																												
C. versatilis																				+					+			+		+		+
C. zeylanoides									+	+					+																	
Debaryomyces																																
D. hansenii	+	+		+			+	+		+	+	+	+		+	+		+	+		+		+	+		+	+		+			
Pichia																																
P. haplophila				+										+																		
Rhodotorula																																
R. mucilaginosa								+																								
Saccharomyces																																
S. cerevisiae														+						+												
Torulaspora																																
T. delbrueckii			+																		+	+										
Yarrowia																																
Y. lipolytica						+		+			+	+	+			+		+	+		+	+		+	+		+	+	+	+		+

C - centre; S - surface; + indicating the presence of the strain at the time of sampling.

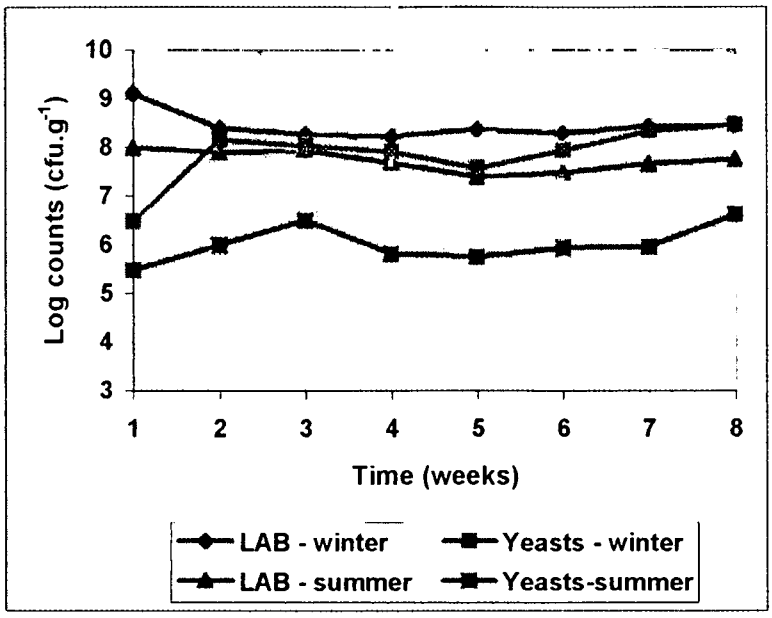

Fig.1. Yeast and Lactic acid bacteria counts on the surface of Camembert cheese during ripening in winter and summer.

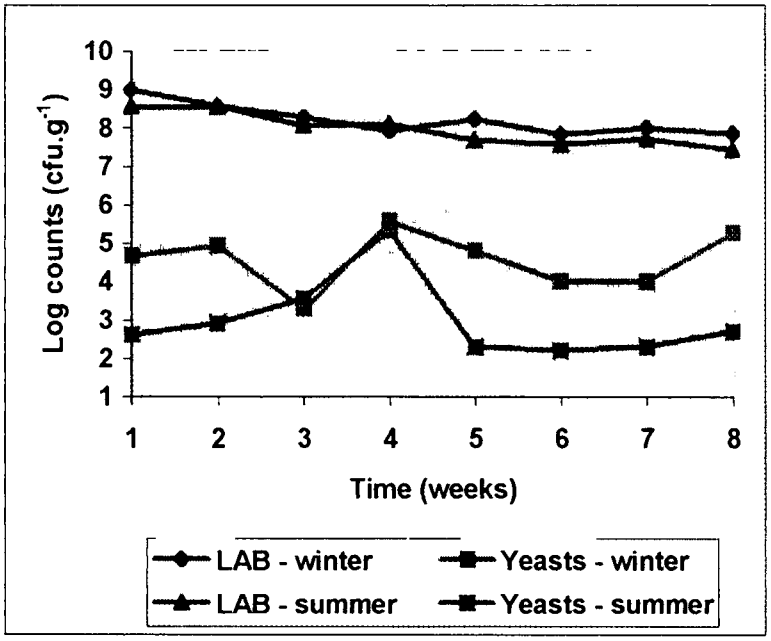

Fig. 2. Yeast and Lactic acid bacteria counts in the centre of Camembert cheese during ripening in winter and summer.

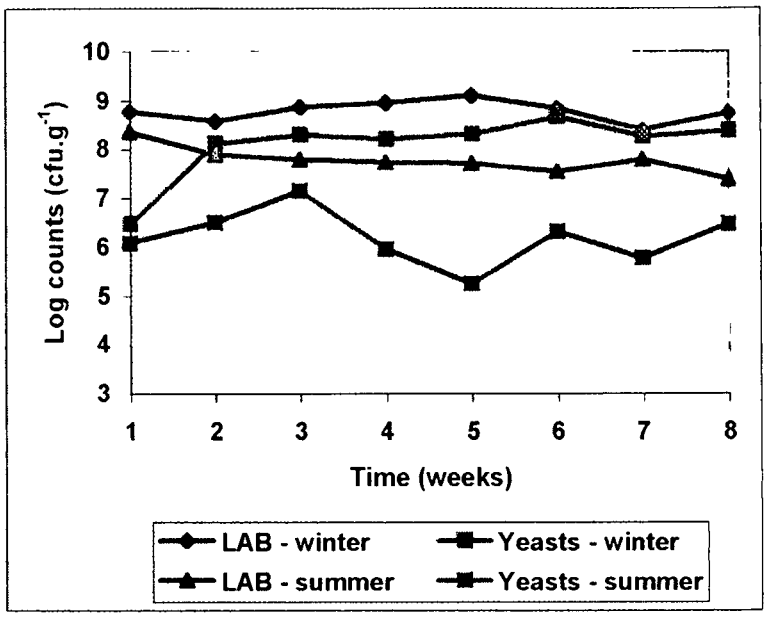

Fig. 3. Yeast and Lactic acid bacteria counts on the surface of Brie cheese during ripening in winter and summer.

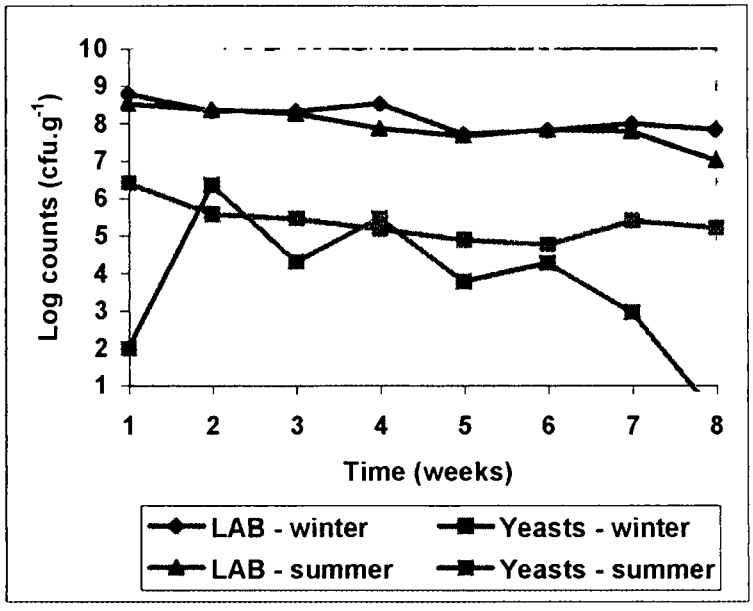

Fig. 4. Yeast and Lactic acid bacteria counts in the centre of Brie cheese during ripening in winter and summer.

Chapter 4

Variations in microbial profiles during white-mould cheese manufacturing.

Abstract

Camembert and Brie cheeses were manufactured from pasteurized cows' milk in order to determine the quantitative evolution of the microbial populations. The yeasts, moulds, lactic acid and coliform bacteria present in Camembert and Brie cheeses during processing and maturation were monitored in a single cheese factory during a 56 day ripening period. The lactic acid bacteria counts were high in the cheese throughout processing and ripening, both on the surface as well as in the centre of the curd. Despite the predominance of lactic acid bacteria during the making of Camembert and Brie, yeasts and moulds play a significant role in the ripening process reaching counts as high as 10^{6} $cfu.g^{-1}$ at the later stages of ripening. The sources of microbial contamination that may lead to contamination of the curd were also determined. The whey, brine and equipment surfaces were responsible for the highest yield of contaminating yeasts and coliforms. Samples were taken at critical control points during the manufacturing process and the microbial populations enumerated after incubation at 25°C for 96h. Samples taken during manufacturing and ripening were also analyzed for organic acid (lactic, acetic, succinic and iso-butyric acids) and sugar (glucose, fructose, galactose and lactose) content using HPLC.

Keywords: Cheese; Enumeration; Camembert; Brie; Evolution

4. 1. Introduction

Surface mould-ripened cheeses represent a small proportion of world cheese production. However, these cheeses are becoming increasingly popular with the consumer, as there is an increasing demand for them (Gripon, 1987).

Brie and Camembert are typical white surface mould-ripened cheeses that are among the specialty cheeses and therefore receive high consumer interest (Karhadian et al., 1985). Mould-ripened cheeses are intrinsically thought to be Camembert, Roquefort and Blue cheese. However, these do not represent all the mould-ripened cheeses, nor are the moulds the single responsible microorganisms for the ripening of these cheeses. The high bacterial populations in the interior of these cheeses, as well as the yeasts and bacterial populations on the surface, are also important during the maturation process (Kosikowski, 1997).

A large variety of cheeses are characterized by the surface development of a specific microflora composed of moulds, yeasts and specific groups of aerobic bacteria. This microflora represents a major component in the "personality" of the cheese (Lenoir, 1984).

The presence of the mould, *Penicillium camemberti* on the surface of Camembert and Brie cheeses, renders these cheeses a unique appearance, whereas the high biochemical activities of the mould produce a very typical aroma and taste. The mould also leads to more complex ripening than in other types of cheeses with a simple flora (Gripon, 1987).

Yeasts have a special relationship with mould-ripened cheeses (Fleet, 1992). Retail samples of Camembert and Brie have a very high occurrence of yeasts, with most samples exhibiting counts exceeding 10^6 cells/g (Tolle et al., 1980; Schimdt and Lamberet, 1981; de Boer and Kuik, 1987; Nooitgedagt and Hartog, 1988; Fleet, 1990; Roostita and Fleet, 1996; Addis et al., 2001). After the curd of Camembert and Brie has been inoculated with *Penicillium*

camemberti, mould growth is accompanied by extensive growth of natural yeast species during cheese development. One must realize that the yeast flora that develops during cheese ripening is a rather uncontrolled mixture of wild species. Certain species may contribute to cheese quality, while others may have a detrimental effect (Fleet, 1992).

Some varieties of mould-ripened cheeses, for example, true French Brie, are made with the reddish bacterium, *Brevibacterium linens*. The mould and this aerobic bacterium occur on the rind to produce a characteristic type of cheese. The surface growth of the bacteria supports several mould species to render a unique cheese with desirable flavours (Kosikowski, 1997). During the later stages of maturation, the mould is overgrown by *B. linens* and related coryneforms, to produce the yellow rind, with distinct sulphury flavours and the characteristic soft textures of mature Brie and Camembert cheese (Hammer, 1948; Law, 1982; Olson, 1969; Rousseau, 1984).

The objective of this study was to report on the quantitative evolution of the microbial groups during the ripening of Camembert and Brie cheeses; to assess the possible areas of contamination during the processing of these cheeses and to perform chemical analysis (organic acids, sugars and pH) of the cheeses during manufacturing and throughout the ripening period.

4. 2. Materials and methods

4.2.1. Camembert and Brie cheese manufacture

Camembert and Brie cheeses were manufactured at a commercial cheese factory in the North West Province region of South Africa. The procedure for cheese making was carried out as described by Kosikowski (1997).

4.2.2. Sampling methods and selection of isolates

All surfaces (Table1) were sampled using cotton swabs and dipped into 9ml sterile standard peptone water. Portions (1ml), of the suspensions were spread inoculated onto 90mm Petri dishes containing Rose Bengal Chloramphenicol Agar (Merck, C98, Darmstadt, Germany – pH 6.6) for the enumeration of yeasts; Malt Extract Agar (Merck, C10 – pH 5.4) for the enumeration of moulds; De Mann Rugosa and Sharpe Agar (MRS) (Merck, C86 – pH 5.4) for the enumeration of lactobacilli and MacConkey Agar (Merck, C2 - pH 7.1) for the enumeration of coliform bacteria.

Air was sampled using settle plates (standard 90mm Petri dishes) containing the same media with an exposure time of 5min.

Samples were also taken during the manufacturing of the Camembert and Brie cheeses at selected points as indicated in Table 1. Liquid samples (1ml) were diluted in 9ml sterile peptone water. For all solid samples, 10g portions immersed into 90ml sterile peptone water, were homogenized in a Colworth 400 stomacher (London, UK) for 2 min and the liquid portion diluted.

Further decimal dilutions of the suspensions were performed as required. Aliquots (0.1ml) of the dilutions were spread inoculated over the surface of plates of media. The plates for the yeast and mould counts were incubated at 25°C for 96h, lactic acid bacteria at 25°C for 48h and coliform bacteria at 37°C for 24h. Yeast colonies were isolated from the highest dilutions on plates

containing Rose Bengal Chloramphenicol Agar after incubation at 25°C for 5 days. The yeast isolates were subcultured on Malt Extract Agar for 48h at 25°C and checked for purity by colony morphology and microscopy. The pure cultures were stored at 4°C on Yeast Malt Extract Agar (Wickerham, 1951) slants during the period of investigation, until characterization. The plates for enumeration of lactobacilli were aerobically incubated at 25°C for 48h; for coliforms at 37°C for 24h and moulds at 25°C for 48h.

4.2.3. Sampling during ripening

Camembert (eight) and Brie (eight) cheeses from the same batch were kept under controlled conditions (4°C) and sampled directly after processing at consecutive intervals on a weekly basis during ripening over a 56 day period. Cheese samples were prepared for microbiological analysis by opening the cheese aseptically with a sterile trier. For each sample, surface and centre, 10g was aseptically weighed into 90ml sterile peptone water in a sterile plastic bag (Whirl-pak, Nasco) and homogenized in a stomacher (Colworth 400) for 2min. Further decimal dilutions were carried out in duplicate as required for microbiological assays in 9ml sterile peptone water and spread plated on the media described above. Similar incubation and duration procedures were applied as described earlier.

4.2.4. Sample analysis

All plates containing between 15 and 150 colony forming units (cfu) from the highest dilution (or the highest number if below 15), 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.

4.2.5. Chemical analysis

During sampling as described for the microbial samples, an additional 10g of cheese were weighed into 10ml of distilled water in sterile plastic bags (Whirl-

pak, Nasco) and homogenized in a stomacher (Colworth 400) for 2 min, for chemical analysis.

Sugar contents were measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector (Molnar-Perl and Morvai, 1992), whereas the organic acid contents were measured by means of a HPLC system equipped with a variable wavelength detector set at 220nm. A biorad-aminex 87H column with a 0.01N H_2SO_4 at 0.6ml/min. eluent was used (Molnar-Perl and Morvai, 1992). The pH was measured at 24°C with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1970).

4. 3. Results and discussion

4.3.1. Microbial enumeration during processing

Microbial analyses were performed during the processing and maturation of Camembert and Brie cheeses. Except for the coliform bacteria, lactic acid bacteria, yeasts and moulds did not occur in the milk after pasteurization (Table 1).

The lactic acid bacteria starter cultures, as expected, was the major component of the microflora during manufacturing and ripening (Table 1 and Figs. 1 – 4). The lactic acid bacteria starter cultures were present at high populations during processing, reaching counts as high as 10^9 cfu.g^{-1}. Thereafter the tendency was to stabilize to 10^8 cfu.g^{-1} during the later manufacturing stages.

Sources of microbial contamination were the air, walls, floors and shelves in the incubation room, the cheese-making equipment surfaces, as well as the wall and floors in the cheese factory. The results in Table 1 show that the pasteurized milk contained high numbers of coliform bacteria. In contrast, coliform bacteria were not present in the starter culture preparation.

The numbers of yeasts increased from 4.08 log cfu.g^{-1} to 6.48 log cfu.g^{-1} during cheese making. According to the data obtained in Table 1, the brine had no affect on the viability of the yeasts. No yeasts was isolated from the milk, starter culture or any of the ingredients added during processing which corresponded with results obtained by Welthagen and Viljoen (1998, 1999), Fleet (1992) and others who indicated that yeast populations originated as post-pasteurization contaminants (Baroiller and Schmidt, 1990; Vadillo et al., 1987). After the curd was inoculated with the mould, *Penicillium candidum*, mould growth was accompanied by extensive growth of yeast species, during maturation. This is in agreement with results reported by Fleet, (1992).

The brine and the vicinity of the brine baths were responsible for the highest yield of microbial contamination of the cheese during incubation. If one compares the cheese before incubation to the cheese during incubation, it is clear that the air, wall, floor and shelves of the incubation room were responsible for high populations of microbial contamination.

It is interesting to note the high initial numbers of coliform bacteria enumerated on MacConkey agar from the pasteurized milk. In contrast, no yeasts, moulds and lactic acid bacteria were isolated from the milk (Table1). Since yeasts were not added as part of the starter culture, the results are in agreement with those reported by Fleet (1990), who indicated that yeasts present in the milk after pasteurization, originate as post-pasteurization contaminants.

4.3.2. Microbial enumeration during maturation

Figures 1 – 4 show the quantitative evolution of the lactic acid bacteria, yeasts, moulds and coliform bacteria during ripening. The values at each stage of ripening for the lactic acid bacteria in the rind were slightly higher than in the core, for both Camembert and Brie cheeses. In contrast, the other microflora, being oxidative, grew significantly faster in the rind due to the presence of oxygen.

Yeasts on the other hand, tended to increase steadily during maturation. The yeasts on the surface of both cheeses increased from 10^5 cfu.g^{-1} to populations as high as 10^8 cfu.g^{-1} (Figs. 2 and 4). Yeast numbers in the core were much lower, but also increased slightly from 10^4 cfu.g^{-1} at the beginning to 10^5 cfu. g^{-1} during the ripening period (Figs. 1 and 3). The values for yeasts in the core of Brie cheese were high during the first week of ripening, but stabilized after the second week.

However, the values for the yeasts in the core of Camembert were low until week 4. They increased steadily from week 4 to week 5, stabilized from week 6 to week 7, and increased again during the final week of maturation. From

the beginning until the end of ripening the yeasts in the rind and core remained at a high level. However, the level of yeasts was significantly lower in the core and remained constant.

Despite favourable environmental conditions which prevailed in the maturation rooms, i.e. lower temperatures and higher relative humidities, it is interesting to note the stabilization of lactic acid bacteria after week 2 and the corresponding increase in yeast numbers on the rind of Camembert and Brie cheeses (Figs. 3 and 4). This is in agreement with results obtained by Welthagen and Viljoen (1998), who attributed the apparent accelerated increase of yeasts after the stabilization of lactic acid bacteria, due to the utilization of organic acids produced by the lactic acid bacteria, which consequently lead to an increase in yeasts. Research performed in our laboratory proved that there is a definite synergistic relationship between lactic acid bacteria and yeasts (Viljoen, 2001). Roostita and Fleet (1996), Lenoir (1984), Nooitgedagt and Hartog (1988), de Boer and Kuik (1987), Fleet (1990) and Addis et al. (2001), also reported high numbers of yeasts in surface mould-ripened cheeses.

Yeasts may contribute positively to the maturation process by supporting the function of the starter culture. The role of the positive interactions between yeasts and starter cultures, is well documented for surface-ripened cheeses. Several yeasts could assist the starter cultures in cheeses by their proteolytic and lipolytic activities, and could possibly participate in maturation, including the formation of flavour compounds (Jakobsen and Narvhus, 1996).

The moulds were present in significant quantities from the first week of ripening in the rind samples. According to Lenoir (1984), there are only two moulds involved in the production of surface mould-ripened cheeses, i.e. *Penicillium camemberti* and *Geotrichum candidum*. *P. camemberti*, forms the white film on the surface of Camembert and Brie. Moulds appeared after the 3rd day of incubation (Table 1). Moulds were also present in the core of Brie cheese throughout ripening, but in Camembert, only from week 2 until week 4. As expected, the moulds occurred in higher numbers on the surface of both

cheeses than in the centre. The role of *P. camemberti* during ripening is linked with its biochemical activities. The mould neutralizes the surface of the cheese and this affects the appearance, texture, enzymatic activities (which are weak at a pH lower than 5,0) and the development of an acid sensitive bacterial flora that plays a major role in the formation of taste and aroma of cheeses. *P. camemberti* is also responsible for the degradation of caseins, the hydrolysis of fatty acids and oxidative degradation of fatty acids (Lenoir, 1984).

The values at each stage of ripening, for the coliform bacteria in the rind were higher than in the core, in both cheeses. The coliforms only appeared after week 4 and stabilized until the end of ripening in the core of Camembert cheese (Fig. 1). The coliforms increased their numbers after week 4, and remained in high populations, due to an increase in pH. In contrast, these bacteria were present in the core of Brie cheese throughout the ripening period. Brie and Camembert cheeses are highly perishable foods. Rapid growth of undesirable microorganisms is supported by high pH and A_w values (relatively low salt concentrations) and the availability of nutrients in maturing cheeses (Nooitgedagt and Hartog, 1988). The results showed that these cheeses are highly contaminated with undesirable bacteria, especially *Escherichia coli*. These facts indicate the failure of systems for good manufacture and distribution practice, which is unacceptable from a public health point of view. Gripon (1987) reported that it was difficult to control the coliform bacteria of Camembert. Even a low level of coliform contamination of the milk can result in a high multiplication rate later. At the beginning of cheese making, there was already some development before the pH had declined enough. Acidification destroyed a large part of this flora, but when the pH increased, the bacteria multiplied again, resulting in high numbers of coliforms in the cheese. To avoid this in pasteurized milk cheese, coliform bacteria should be less than 100 cells.g^{-1} three days after curd making. This may be achieved by taking the necessary hygienic precautions. The high numbers of coliforms may be attributed to unsuccessful pasteurization or post-pasteurization contamination.

From the results presented in Fig. 1, it is evident that the lactic acid bacteria occurred at high populations at the beginning of ripening, directly after being inoculated as starter cultures, then stabilized during the middle period of maturation and retained their numbers until the end of ripening. At the same time, yeast numbers increased, due to the utilization of organic acids produced by the lactic acid bacteria. The yeasts in return, produced valuable vitamins and growth factors, supporting the growth of the lactic acid bacteria. Consequently, an equilibrium typical synergistic relationship between the yeasts and lactic acid bacteria numbers developed. This may be attributed to a possible depletion of the available nutrients and an increase in pH. It is interesting to note that the moulds in the centre disappeared after week 4. This may be attributed to an increased pH, and to a more closed texture in which less oxygen is available for mould growth. From the above it is evident that the increase in pH lead to higher numbers of coliform bacteria and the disappearance of moulds.

Based on the results obtained from this study, we clearly demonstrated the frequent occurrence of yeasts and lactic acid bacteria in high numbers during the making of Camembert and Brie cheeses in this factory. However, high populations of undesirable coliform bacteria were also reported during processing and ripening.

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

4.3.3. Chemical analysis

4.3.3.1. Changes in pH during ripening

The mean pH values for the interior and exterior of Camembert cheese was, 5.78 and 7.76, respectively, and for the interior and exterior of Brie cheese,

6.59 and 7.47, respectively. These data agree with published values (Gripon, 1987; Choisy et al. 1987a; Roostita and Fleet, 1996). According to Roostita and Fleet (1996), the pH of the cheese sample at the time of analysis depends on its age as well as the activity of the ripening flora in causing proteolysis and utilization of organic acids during processing.

The pH increased during the first two weeks of ripening and a neutral pH was reached on the surfaces of both Camembert and Brie cheeses by day 22. The pH then increased further from 7.73 on day 28, to 8.77 on day 49, and then decreased slightly during the final week of ripening. A pH of 5 was obtained during the first week of maturation, in the centre of both cheeses. The values increased gradually during each week of ripening, until a neutral pH was obtained on day 42. The pH then increased further to a value of 7.79 in the centre of Camembert cheese (Fig. 8), but decreased from 7.81 to 7.49 in the core of Brie cheese during the final week of ripening.

4.3.3.2. Changes in sugar and organic acids percentages during ripening

According to Roostita and Fleet (1996), mould-ripened cheeses have unique physical and chemical properties. These properties include high concentrations of fat (23 - 30% w / w) and protein (19 - 24% w / w), residual unfermented lactose (0.02 - 0.5% w / w), high concentration of lactic acid (1 - 1.6% w / w), significant concentrations of salt and small amounts of citric and acetic acids (Choisy et al., 1987b).

The lactose content (Fig. 5), of Camembert and Brie cheeses at the beginning of processing was 2.4 %, and then decreased to 1.75 % after the milk was pasteurized, but then increased to 2.68 % before cheese making, after all the ingredients had been added to the pasteurized milk. The lactose content steadily decreased to a value of 0.33 % before and after salting of the curd, and was depleted after the curd was incubated for three days at 14°C. The decrease in lactose is due to the utilization of lactose by the lactic acid bacteria starter cultures, as well as the growth of yeasts, as the number of yeasts increase at the end of processing (Table 1). Most dairy yeasts possess

the ability to ferment or assimilate lactose and lactic acid, and to produce protease and lipase enzymes which enables them to hydrolyze milk casein and fat (Roostita and Fleet, 1996). The apparent increase of yeasts on the surface and in the centre of the cheeses during the first week of maturation (Figs. 1 – 4), after lactose was depleted, may be attributed to the possible utilization of organic acids produced by the lactic acid bacteria, which consequently lead to and increase in pH.

The assimilation and fermentation of lactose by yeasts influence the aroma of the cheese in various ways: by the formation of alcohol (ethanol and acetaldehyde), which limits acidification due to the utilization of lactic acid, and thus affecting the texture of the cheese and the production of CO_2 (Deak and Beuchat, 1996; Roostita and Fleet, 1996). There is however, a risk of too much "openness" and the development of a yeast flavour if the yeast populations in the core exceed counts of 10^6 cfu.g^{-1} (Lenoir, 1984). Lactose is a disaccharide that is broken down into glucose and galactose. There was no accumulation and subsequent depletion of galactose in the core of the cheeses during the initial ripening phases, which is very unusual.

During ripening, yeasts grow in the outer part of the cheeses (Lenoir, 1963), where the oxygen supply is favourable. The implementation of yeasts as part of the starter culture mixture for the production of mould-ripened cheeses, is still the exception rather than the rule (Nooitgedagt and Hartog, 1988). However, yeast contamination can easily occur during the cheese making process. Yeasts developing on the rind, increase the pH, by metabolizing lactic acid, thus rendering the surface fit for growth of the moulds, *Geotrichum candidum* and *Penicillium camemberti* (Rousseau, 1984). Lactose fermenting yeasts also contribute to the flavour of the cheese.

Acetic acid was the major organic acid detected in the curd during processing of Camembert and Brie cheeses. The other organic acids i.e. succinic, lactic and iso-butyric acids occurred at much lower percentages ranging from 0,02% to 0,08%. Only trace amounts of acetic and lactic acids were present in the

brine (Fig. 6). The acetic acid content increased after salting from 0,11% to 1,34% during the incubation of the young cheese.

Similarly, acetic acid occurred at the highest percentage, and much lower percentages of the other organic acids were measured in the cheeses during the ripening process (Figs. 7-10). From figs. 2 and 4, it is clear that the yeasts develop particularly well during the first three weeks of maturation, due to their tolerance of curds that are acidic and rich in salt. Yeasts metabolize the lactic acid produced by the lactic acid bacteria. Subsequently, the consumption of lactic acid and the formation of alkaline metabolites during this period resulted in an increase in the cheese surface pH (Figs. 7 and 9).

The iso-butyric acid values remained stable on the surface of Camembert cheese during ripening, but the percentage succinic acid increased after the first week, and then decreased gradually thereafter to 0.011%cduring the fifth week and was depleted by week six. As the acetic acid content decreased from 1.37%, during week six, to 0.71% at week seven, the pH subsequently increased from 8.51 to 8.80 and then decreased to 8.20, once the acetic acid content increased to1.16% during the final week of ripening (Fig. 7).

The acetic acid percentage decreased significantly from 1.20% to 0.23% from week one to week two, then increased gradually from week two to week four, decreased again during week four, and then finally increased and stabilized at 0.40% until the end of maturation. The iso-butyric content increased during the initial stages of ripening, and the values remained at 0.18% until the end of maturation. Succinic acid was depleted by week four, and lactic acid occurred at very low percentages (Fig. 8).

As the acetic and iso-butyric acid percentages increased after the fourth week (Fig. 9), the pH decreased and increased again from weeks five to seven, when the organic acid contents decreased on the surface of Brie cheese. The succinic and lactic acid percentages were very low during ripening. The percentage acetic acid decreased from 0.82% to 0.29% after the first week of maturation in the core of Brie cheese, and stabilized at 0.28% up until week

five. Both acetic and iso-butyric acids decreased at week six and increased during the final two weeks of ripening. The lactic and succinic acid contents were again very low (Fig. 10).

4. 4. Conclusion

The ripening of Camembert and Brie cheeses results from an integrated pattern of development of bacteria, filamentous fungi and yeasts, leading to the modification of the curd and thickening of the rind over time. The surface microflora that develop on the cheese rind during maturation, impart a distinctive aroma and flavour to surface mould-ripened cheeses.

Due to the increasing demand for surface mould-ripened soft cheeses, the storage life of these cheeses should be improved in order to make the distribution easier and to develop its production. The cheeses exhibited decreased sensory appeal on storage, with the rind becoming brittle and the core softer in texture and developing a less attractive appearance and odour. The occurrence of a wide diversity of microorganisms in these types of cheeses makes it necessary to perform further research, to ensure good quality products and to improve product uniformity.

The results of the quality control of the cheese factory showed that a better sanitation program should be implemented to ensure the proper hygienic conditions for cheese making. The milk to be used for the production of soft cheeses should be adequately pasteurized to eliminate the high populations of coliform bacteria that cause defects in the cheeses. Future research to improve product uniformity and to prevent wild moulds from contaminating the cheeses should also be performed.

4. 5. References

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Table 1

Surface and air samples of microbial colonies in log counts (cfu.ml^{-1}) during Camembert and Brie cheese processing (results as indicated are the means of duplicate samples)

Sample	Yeast colonies per 16^{2}cm	Mould colonies per 16^{2}cm	Coliforms per 16^{2}cm
	RBCA	MEA	MA
Silo (before pasteurization)	1.78	0	0
Balance tank (before pasteurization)	0	0	0
Cheese vat	0	0	0
Draining vat	>3.78	0	0
Camembert filler	1.30	0	0
Brie filler	1.30	0	0
Camembert mould	1.90	0	0
Brie mould	>3.78	0	0
Mould draining vat	2.90	1.78	0
Floor	>3.78	>3.78	>3.78
Wall	1.30	1.90	0
Incubation room -shelf	>3.78	>3.78	>3.78
Incubation room - floor	>3 78	>3.78	>3.78
Incubation room - wall	>3.78	>3.78	1.90
Air Samples (Colonies per 90mm Petri dish)			
Cheese vat	0	0	0
Incubation room	48	2	0

Processing (Log counts g^{-1}ml^{-1})				
	RBCA	MEA	MRS	MA
Pasteurized milk	0	0	0	3.33
Starter culture preparation	0	0	7.86	0
Whey	5.18	0	8.38	5.48
Before brine bath	4.08	0	9.20	3.26
Brine	3.25	2.15	5.48	0
After brine bath	4.72	0	8.67	4.23
During incubation (4days at 4°C)	6.48	4.48	8.54	4.44

RBCA, Rose Bengal Chloramphenicol Agar, MEA, Malt Extract Agar; MRS, for the isolation of lactobacilli; MA, MacConkey Agar

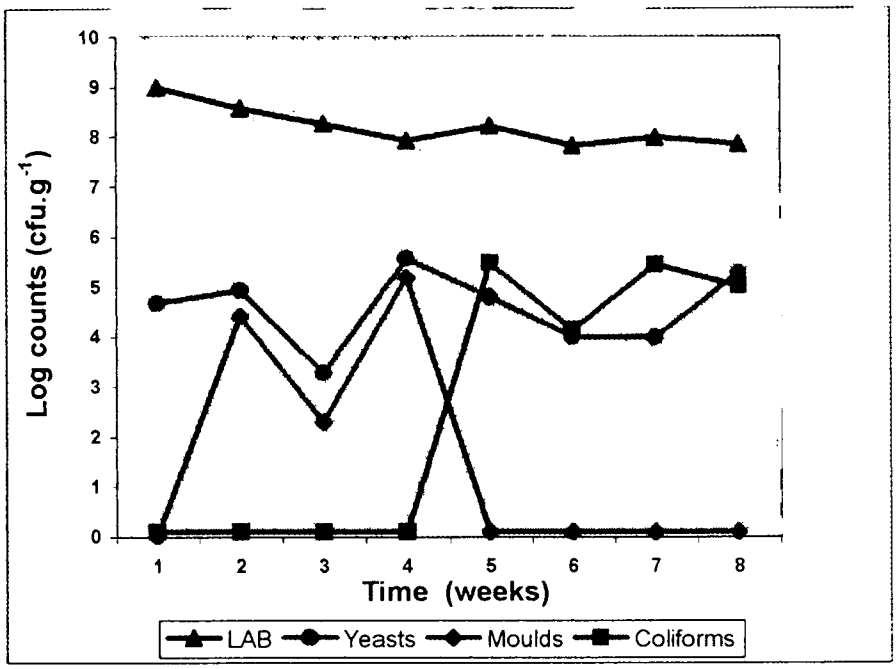

Fig.1. Log counts per cfu.g^{-1} from the centre of Camembert cheese during ripening.

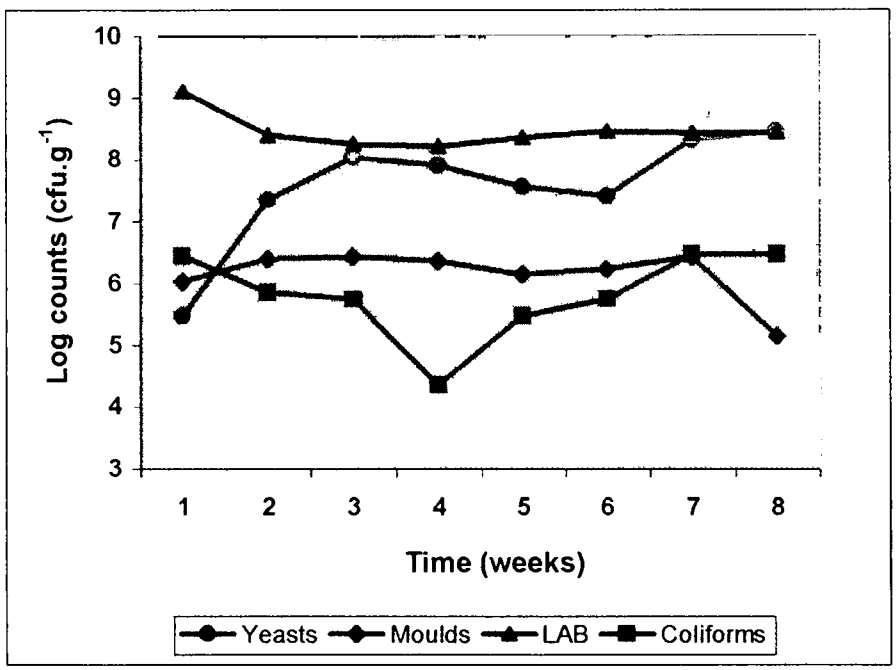

Fig. 2. Log counts per cfu.g^{-1} from the surface of Camembert cheese during ripening.

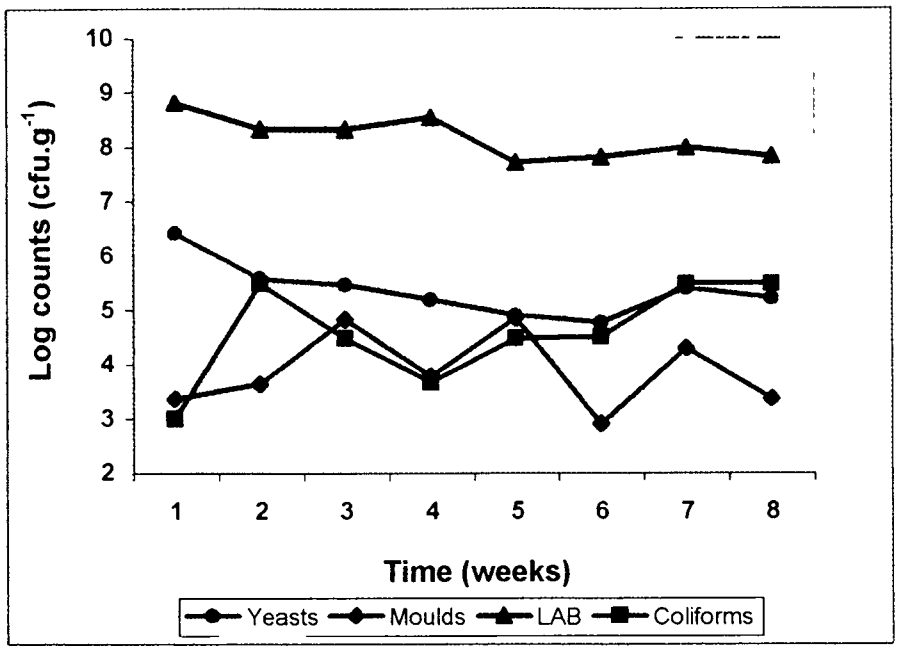

Fig. 3. Log counts per cfu.g^{-1} from the centre of Brie cheese during ripening.

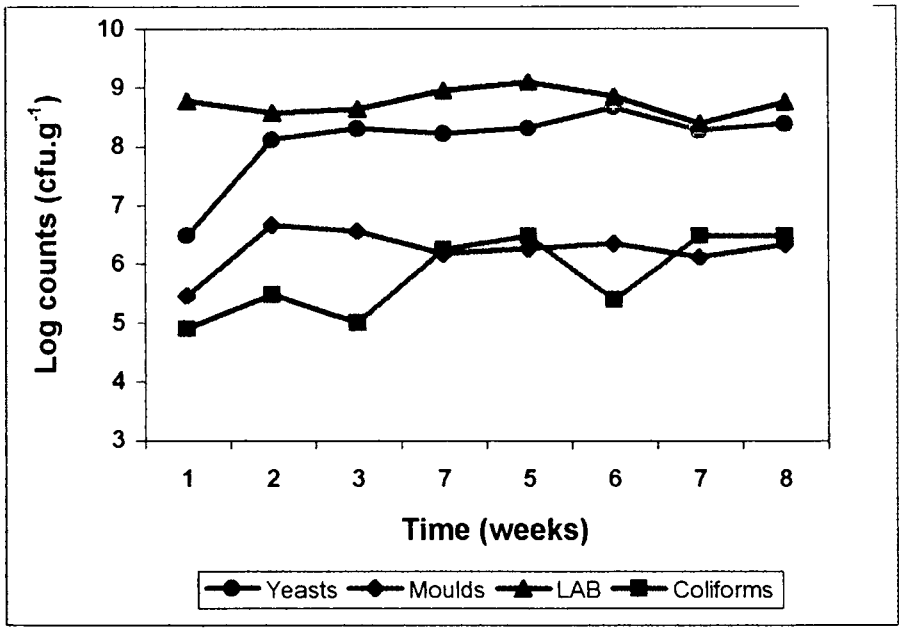

Fig. 4. Log counts per cfu.g^{-1} from the surface of Brie cheese during ripening.

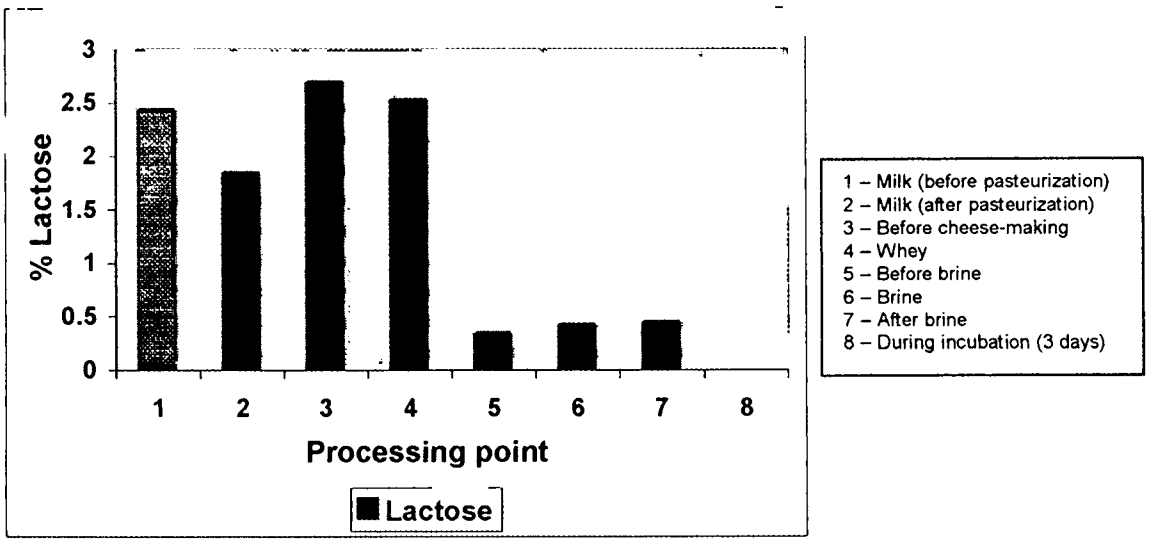

Fig. 5. Lactose present in the curd during processing

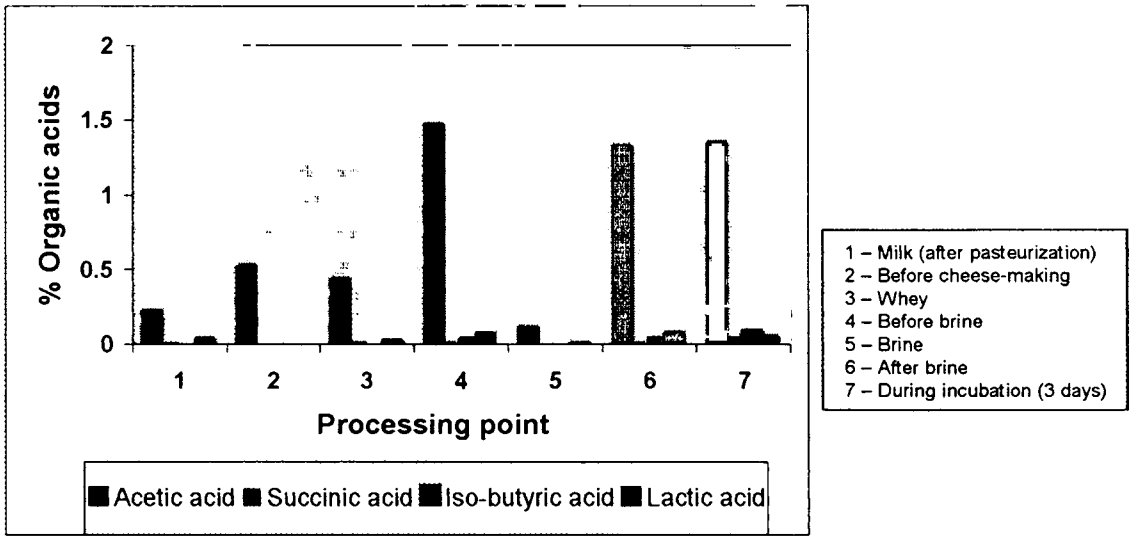

Fig. 6. Organic acids present in the curd during processing

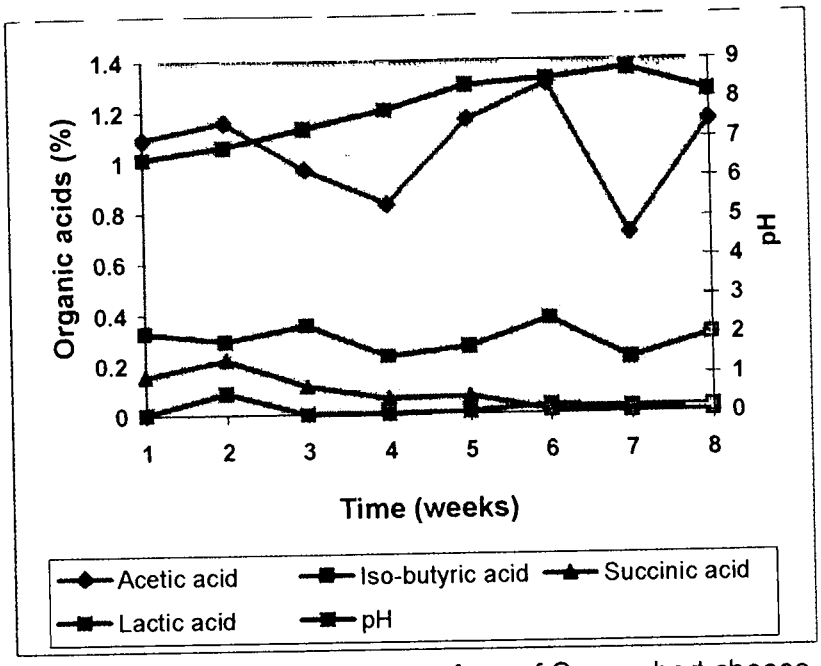

Fig. 7. Organic acids present in the surface of Camembert cheese during ripening.

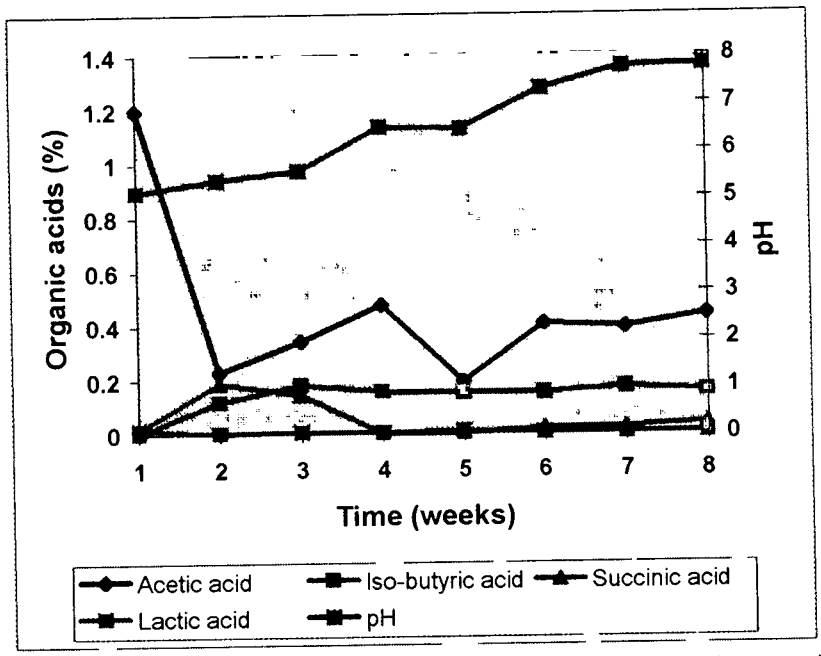

Fig. 8. Organic acids present in the centre of Camembert cheese during ripening.

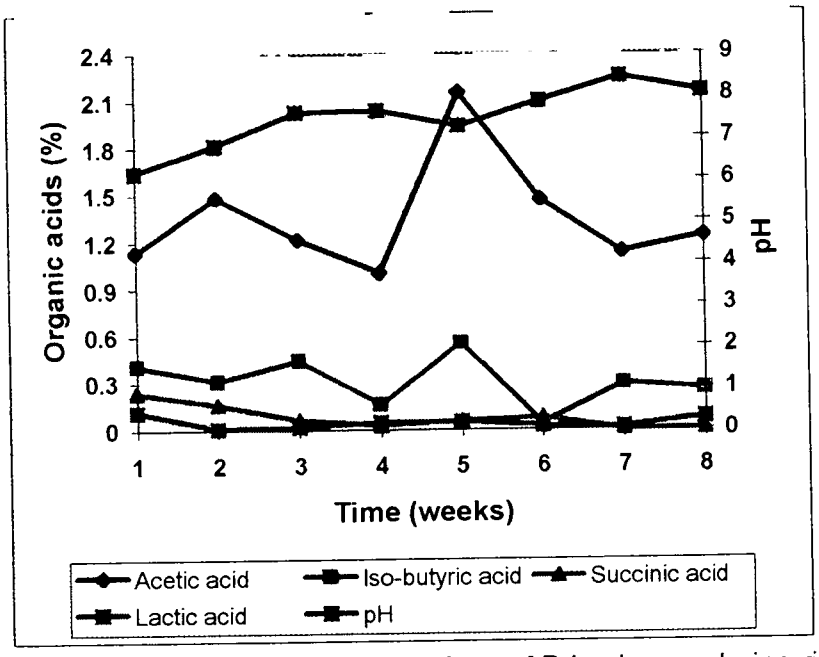

Fig. 9. Organic acids present in the surface of Brie cheese during ripening.

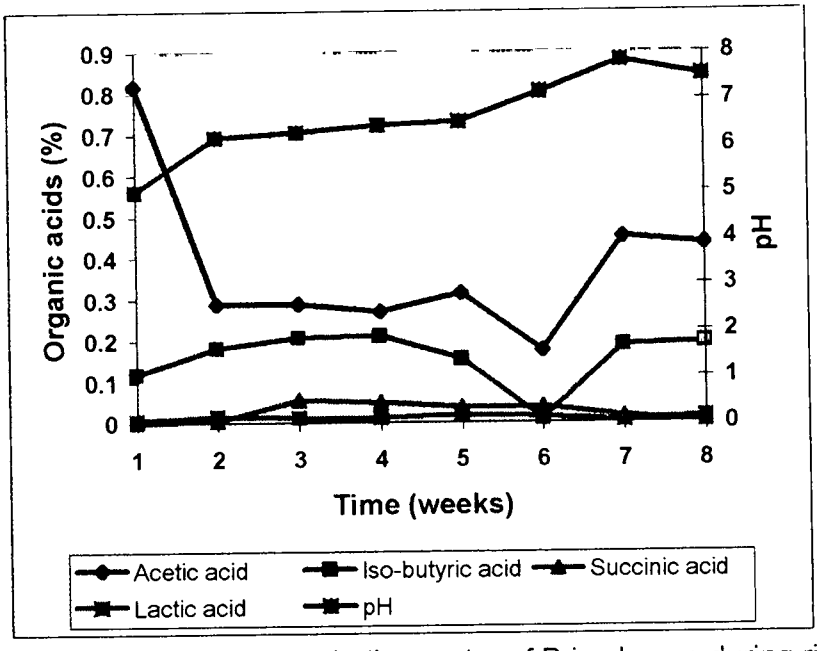

Fig. 10. Organic acids present in the centre of Brie cheese during ripening

Chapter 5

General discussion and conclusions

Cheeses such as Camembert and Brie are called white surface mould-ripened cheeses. Their ripening is characterized by the development of a specific microflora that usually consists of yeasts, moulds, micrococci and coryneform bacteria. These cheeses ripen rapidly due to their high moisture content and the fast growth of the surface mould, *Penicillium camemberti* (Schlesser et al., 1992). Bacteria usually cause fermentation of milk and are therefore considered to be of major importance during cheese making (Cousin, 1982).

Yeasts, however, possess the ability to grow under conditions unfavourable to many bacteria and play a significant role in the spoilage of dairy products as well as the ripening of certain cheese varieties (Fleet and Mian, 1987; Seiler and Busse, 1990). The occurrence of yeasts in cheeses is not unusual due to its tolerance towards low pH and the lack of moisture, as well as the elevated salt concentrations and low storage temperatures (Fleet, 1990). Yeasts have a special relationship with mould-ripened cheeses (Fleet, 1992). Retail samples of Camembert and Brie have a very high occurrence of yeasts, with most samples exhibiting counts exceeding 10^6 cfu.g^{-1} (de Boer and Kuik, 1987; Nooitgedagt and Hartog, 1988). However, the yeast flora that develops during the maturation of these cheeses is an uncontrolled mixture of wild species. Certain species may contribute to cheese quality while others may have a detrimental effect (Fleet, 1992).

The integrated roles of moulds, yeasts and bacteria involved in the production of white-mould cheeses and the assistance of the diverse microflora in the development of the characteristic taste, texture and aroma of mould-ripened cheeses were described in Chapter 1. Several methods of producing soft mould-ripened cheeses have evolved over the years. There are two types of manufacture, i.e. the traditional method where raw milk is used and acidification results from the natural lactic acid bacterial populations. Pasteurized milk with commercial lactic acid bacteria starter cultures and mould spores are used in the industrial method. The three groups of microorganisms involved in the ripening of

white-mould cheeses are described in section 1.4. with the emphasis on filamentous fungi, the role of moulds during ripening, yeasts (in ripening, dairy industry, and as spoilage organisms) and the bacterial flora. Cheese ripening involves changes in the chemical and physical properties of cheese that is accompanied by the development of a typical flavour. In addition, a characteristic flavour is obtained through a series of chemical changes that occur in the curd during maturation. The generation of texture in mould-ripened cheeses depends on the biochemical activity of the microflora that develops on the rind and in the centre of the cheese during ripening. Various defects in mould-ripened cheeses were also discussed in section 1.8. and this may be attributed to an incorrect curd composition, or incorrect environmental conditions during maturation. Due to the occurrence of a wide diversity of microbial populations in these cheese varieties, it is necessary to perform further research to ensure good quality products and to improve product uniformity.

Yeasts are an integrated part of the microbial flora (Lenoir, 1984), present in white mould-ripened cheeses and therefore it is imperative to include them during an ecological study. Moulds develop rapidly into large colonies on agar media, usually overgrowing and covering yeast colonies making the isolation and enumeration of yeasts an impossible and difficult process. The typical medium for the enumeration of yeasts, should totally inhibit the growth of moulds and bacteria, and be nutritionally adequate to support the growth of all yeasts present (Beuchat, 1993; Jakobsen and Narvhus, 1996). Since this ideal medium does not exist, there is still a need for a selective medium to ensure reliable quantitative enumeration of yeast colonies from white-mould cheeses. In Chapter 2, ten different media for the isolation of yeast colonies from Camembert cheese were compared. Statistical differences for the enumeration of yeasts from Camembert cheese on Malt Extract agar supplemented with 0.5 % sodium propionate (MEA-SP) and modified molybdate agar were obtained. Oxytetracycline Gentamycin Glucose Yeast agar (OGGYA) and Dichloran Rose-bengal Chloramphenicol agar (DRBC) agar supported the growth of yeasts

present in Camembert cheese, as the highest mean yeast counts were obtained from these media. No mould growth occurred on MEA supplemented with 0.5% sodium propionate, which makes this compound an appropriate mould inhibitor. However, the mean yeast counts obtained on this medium were lower and significantly different to that of the other media. DRBC agar and OGGYA are the most appropriate media for the isolation and enumeration of yeasts from white mould-ripened cheeses.

Numerous studies have identified the diversity of yeast species that contribute to the flavour development, interactions and secondary flora and these are referred to in reviews by Fleet (1990), Addis et al. (2001), Stanley (1998 a, b), Jakobsen and Narvhus (1996), and Corsetti et al. (2001). These studies also indicated on the yeast profiles during maturation (Addis et al., 2001), the key properties that affected the quality, and attempts to identify species that might be developed as starter cultures (Martin et al. 1999; Wyder and Puhan, 1999). None of these studies, however, gave any indication of how the diversity may change due to seasonal variations. Therefore, a need existed to examine the yeast profiles during seasonal changes, since an altering yeast profile might also affect product quality.

The objectives of Chapter 3 were to detect the sources of yeast contamination within a white mould cheese factory, to identify the yeast strains present and to report on the frequency and seasonal diversity of the occurrences of yeasts. From the data presented in Chapter 3, it is apparent that there was a significant difference in the diversity of the yeast species isolated during different seasons. In winter, a more diverse variety of yeast species were obtained. Of the 93 yeast species isolated, ten different genera and 20 different species were identified, whereas in the summer, only 76 yeast species were isolated ranging over only six genera containing seven species.

Definite differences in the occurrence of *Yarrowia lipolytica* and *Debaryomyces hansenii* were observed during winter and summer, which might contribute to the final qualitiy. Further research to assess whether *Debaryomyces hansenii* and *Yarrowia lipolytica* affect the flavour, aromatic compounds and organoleptic characteristics of the cheeses, under different simulated environmental conditions (pH, A_w, temperature and salt concentration), seems very promising.

The objectives of Chapter 4 were: the quantitative evolution of the microbial groups during the ripening of Camembert and Brie cheeses; to assess the possible areas of contamination during the processing of these cheeses and to perform chemical analysis (organic acids, sugars and pH) of the cheeses during manufacturing and throughout the ripening period. The yeasts, moulds, lactic acid and coliform bacteria present in Camembert and Brie cheeses during processing and maturation were monitored in a single cheese factory during a 56 day ripening period. The lactic acid bacteria counts were high in the cheese throughout processing and ripening, both on the surface as well as in the centre of the curd. Despite the predominance of lactic acid bacteria during the making of Camembert and Brie, yeasts and moulds play a significant role in the ripening process reaching counts as high as 10^6 cfu.g^{-1} at the later stages of ripening. The sources of microbial contamination that may lead to contamination of the curd were also determined. The whey, brine and equipment surfaces were responsible for the highest yield of contaminating yeasts and coliforms. The results of the quality control of the cheese factory showed that a better sanitation program should be implemented to ensure the proper hygienic conditions for cheese making. The milk to be used for the production of soft cheeses should be adequately pasteurized to eliminate the high populations of coliform bacteria that cause defects in the cheeses. Future research to improve product uniformity and to prevent wild moulds from contaminating the cheeses should also be performed.

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Chapter 6

Summary

Surface mould-ripened cheeses represent a small proportion of world cheese production. However, these cheeses are becoming increasingly popular with the consumer, as there is an increasing demand for it (Gripon, 1987). In Chapter 1 the integrated roles of moulds, yeasts and bacteria involved in the production and maturation of white surface mould-ripened cheeses, and the assistance of the microflora in the development of the characteristic flavour and texture of mould-ripened cheeses, were thoroughly discussed.

Due to the fact that yeast colonies are often overgrown by mould spreaders, we intended to develop a medium that inhibits moulds and bacteria, but supports the growth of all yeasts present in white-mould cheeses. Ten selective mycological media were evaluated and compared statistically for their suitability to enumerate yeasts and simultaneously suppress moulds in white-moulds cheeses. Malt extract agar (MEA) supplemented with 0.5% sodium propionate (SP), proved to be the only medium that totally suppressed mould growth. However, the medium also restricted yeast development, as the mean yeast counts obtained on this medium differed significantly ($p<0.5$) compared to the other media. Dichloran Rose Bengal agar (DRBC) and Oxytetracyline Gentamycin Glucose Yeast Extract agar (OGGYA) retarded mould growth and supported the growth of the six most dominant yeast species recovered from white-mould cheeses, making these media suitable for the isolation and enumeration of yeasts in the presence of moulds.

The yeasts present in Camembert and Brie cheeses during processing were monitored in a single cheese factory during summer and winter, to determine the seasonal diversity of yeasts over a ripening period of 56 days. Despite the predominance of lactic acid bacteria during the making of Camembert and Brie, yeasts play a significant role in the ripening process reaching counts as high as 10^6 cfu.g^{-1} at the later stages of ripening. A diverse variety of 20 yeast species representing 10 genera were present during the winter period associated with the factory environment, during processing and ripening, whereas only seven yeast species representing six genera were isolated during summer. Although a broad spectrum of yeasts were isolated from Camembert and Brie cheeses, *Debaryomyces hansenii* was the most

abundant yeast isolated. Other species encountered were *Yarrowia lipolytica*, *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa*, *Rhodotorula minuta*, and various species of *Candida*.

The yeasts, moulds, lactic acid and coliform bacteria present in Camembert and Brie cheeses during processing and maturation were monitored in a single cheese factory during a 56 day ripening period. The sources of microbial contamination that may lead to contamination of the curd were also determined. The whey, brine and equipment surfaces were responsible for the highest yield of contaminating yeasts and coliforms. Samples were taken at critical control points during the manufacturing process and the microbial populations enumerated after incubation at 25°C for 96h. Samples taken during manufacturing and ripening were also analyzed for organic acid (lactic, acetic, succinic and iso-butyric acids) and sugar (glucose, fructose, galactose and lactose) content using HPLC.