The Microflora of Blue Stilton Cheese

By
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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, May 2002

Acknowledgements

This study would not have been possible without the support of Long Clawson Dairy, in particular Mark Hough and Kim Kettle. They provided not only cheese for the experimental work, but also tremendous expertise and technical support. I hope that the results of the study are of benefit to the company.

Thanks go to The Department of Land-Based Studies at the Nottingham Trent University, for financial support, encouragement and a period of sabbatical time in order to complete this thesis.

I would like to express especial thanks to Professor Will Waites of the University of Nottingham, who supported me through the entire study and to his colleagues who helped with specific areas. Particular thanks go to Dr Christian Salles, who carried out the GC-MS olfactometry.

Finally, thanks go to my husband, for his constant support and understanding during the years of this study.

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Abstract

Blue Stilton is a blue-veined cheese manufactured in a restricted area of the UK, using lactic starter cultures plus a secondary culture of *Penicillium roqueforti.* The aim of this study was to determine the change in microflora during ripening of the cheese and to investigate potential microbial interactions. Additionally, the volatile compounds present in mature samples of cheeses exhibiting few blue veins were compared with those in good quality cheeses, showing ample blue veining.

Experiments on cheeses from a single dairy, monitored during the ripening process, showed that the total *Lactobacillus* count increased from levels of around 10^3 cfu g^{-1} on day one to around 10^7 cfu g^{-1} after 8 weeks of ripening. This is comparable to values found in other cheeses including both mould–ripened and non mould-ripened varieties. Yeast counts were generally higher than those found in other cheeses and also increased to levels in the region of 10^7 g^{-1}. The total viable count (TVC) decreased from around 10^9 g^{-1} initially, reflecting the presence of the starter bacteria, to 10^7 g^{-1}, suggesting a decline in the starter bacteria similar to that found in other cheeses.

Mature cheeses always exhibited similar numbers of microorganisms although the species varied between cheeses. High quality, mature, cheeses were compared with sub-standard cheeses from the same production site. The predominant species of lactobacilli in good quality cheeses were *Lb. plantarum* and *Lb. curvatus,* whereas in poor quality cheeses *Lb. brevis* predominated. This corresponded to the results of gas chromatography-olfactometry, which indicated the presence of fruity off flavours in poor quality cheeses. Several strains of these species were isolated, as indicated by differing capabilities in utilisation of a range of carbon sources.

Yeast species also varied between good and poor quality cheeses with *Candida sphaerica* and *C. catenulata* predominating in good cheeses and *C. famata, C. lipolytica* and *C. catenulata* also occurring in both good and poor quality samples. Strain differences were

observed by the biochemical profiles and two strains of *C. famata* demonstrated inhibitory effects against *P. roqueforti* when incubated under anaerobiosis. It was concluded that these strains may affect the development of blue veins in Stilton cheese when maturation conditions encourage their proliferation.

Comparisons were made between samples of cheeses from several Stilton producers and the results suggested that although the levels of the groups of microorganisms tested were similar, the species of lactobacilli and yeasts present were different. This suggests that the indigenous microflora may have a significant impact on the flavour of cheeses from individual production sites.

It was concluded that the microflora of Blue Stilton cheese may have a significant impact on the quality of the product both in terms of flavour and the development of the blue veins.

CHAPTER 1

1 General Introduction

Blue Stilton cheese is known as "the King of English cheeses". It is manufactured only in the three counties of Leicestershire, Nottinghamshire and Derbyshire by a total of six companies across eight creameries. The organoleptic properties of cheeses produced at these creameries vary considerably, as indeed they may vary from day to day within individual creameries. The relationship between the microflora of cheeses and their organoleptic qualities has been well researched, particularly in Cheddar cheese (McSweeney *et al.*, 1995) but a review of recent literature showed that the microflora of Blue Stilton cheese has not been examined.

The influence of non-starter lactic acid bacteria (NSLAB) on cheese flavour is also widely recognised (McSweeney *et al.*, 1995; Lane *et al.*, 1996) but there is no evidence to suggest that these bacteria vary in type or in proportions from creamery to creamery. The role of *P. roqueforti* in the flavour of Roquefort and Gorgonzola cheese has been examined (Gripon, 1993; Hansen and Jakobsen, 1997) but its role in affecting the microflora of the cheeses is unknown. Similarly there is little evidence to indicate the effect of the microflora on the growth of the mould.

The purpose of this study was to investigate the microflora of blue Stilton Cheese in order to identify the effects of blue mould growth as well as the effect of other microorganisms on the growth of *P. roqueforti*. This microflora can then be related to flavour development within a creamery's cheese. Secondly the study aimed to determine the reasons for differences between cheeses of different creameries. Since the starter cultures are basically the same, it is likely that NSLAB have a significant role in this area and the study examined whether or not different creameries possess proportions of NSLAB species unique to themselves.

It is intended that this will enable manufacturers to select starter cultures that will produce a consistent flavour that is acceptable to the consumer.

1.1 Aim of the study

The aim of this work was to investigate the possibility of relationships between those microorganisms growing in mature Blue Stilton cheese which may affect the microflora and thus the flavour profile of such cheese. Initially this focused on the relationship between the bacterial flora and the growth of the blue mould *P. roqueforti* and subsequently interactions of fungal species were investigated.

1.2 Objectives

1.2.1 To determine whether or not there is a relationship between the growth of *P. roqueforti* and the bacterial flora of Blue Stilton cheese.

1.2.2 To determine whether or not growth of individual genera or species of both bacteria and fungi may affect the growth of *P. roqueforti* and hence the typical flavour and appearance of the cheese.

1.2.3 To compare the microflora of Blue Stilton cheese with that of other cheeses as investigated by others.

1.3 Background information and review of literature

1.3.1 Introduction to Blue Stilton Cheese

Blue Stilton cheese is a semi-soft mould ripened cheese variety made only in the counties of Leicestershire, Nottinghamshire and Derbyshire. Its origin is suggested by many tales but it is generally associated with the cheesemaker Mrs Frances Pawlett and her husband William who sold the cheese in Stilton on the Great North Road in the mid-eighteenth century (Hickman, 1995). A keen purchaser was Cooper Thornhill, the innkeeper of the Bell Inn at the village of Stilton. His customers much enjoyed the cheese and they began to ask for "the cheese from Stilton". It was also sold by the other three inns in the town, as well as by others in the locality and so Stilton cheese was born. As communications improved the cheese began to be sold direct to retailers in London with the market in Stilton declining as a consequence (Hickman, 1995).

The Stilton Cheese Makers Association (SCMA) was formed in June 1936 to represent the interests of members and the cheese was one of the first cheeses in the world to become registered (SCMA, 1998). This took place in 1968 and as a consequence the cheese gained the equivalent of *Appellation d'origine controlée* which restricted its production to the three counties.

Stilton is still known as the "King of English cheeses" and much myth and lore surrounds the cheese. For example, an old saying, "Stilton is like a baby", suggests that it is a difficult if not temperamental cheese to manufacture, largely due to difficulties in the past to ensure blueing of the cheese. A number of methods existed for getting fungal spores into the vat of milk, including keeping a mouldy lemon in the vat room, with the lemon being added to the milk. Another technique was to keep an old saddle in the vat room, which would be banged to

release the spores of the mould which grew upon it. Modern manufacturers add a suspension of commercially produced spores of *P. roqueforti* to the pasteurised milk but each dairy still produces cheese possessing characteristics typical of that dairy, which may be due to local influences or to the particular manufacturing method of the cheese maker. The flavour and texture of the cheese can vary widely and a good cheese will have an open, creamy texture with an even growth of the blue mould running along the network of veins. The flavour should be clean, typical of blue cheese and with no evidence of bitterness. Plate 1.1 shows the typical appearance of a good quality Stilton with the characteristic open texture and even growth of the blue mould throughout the cheese.

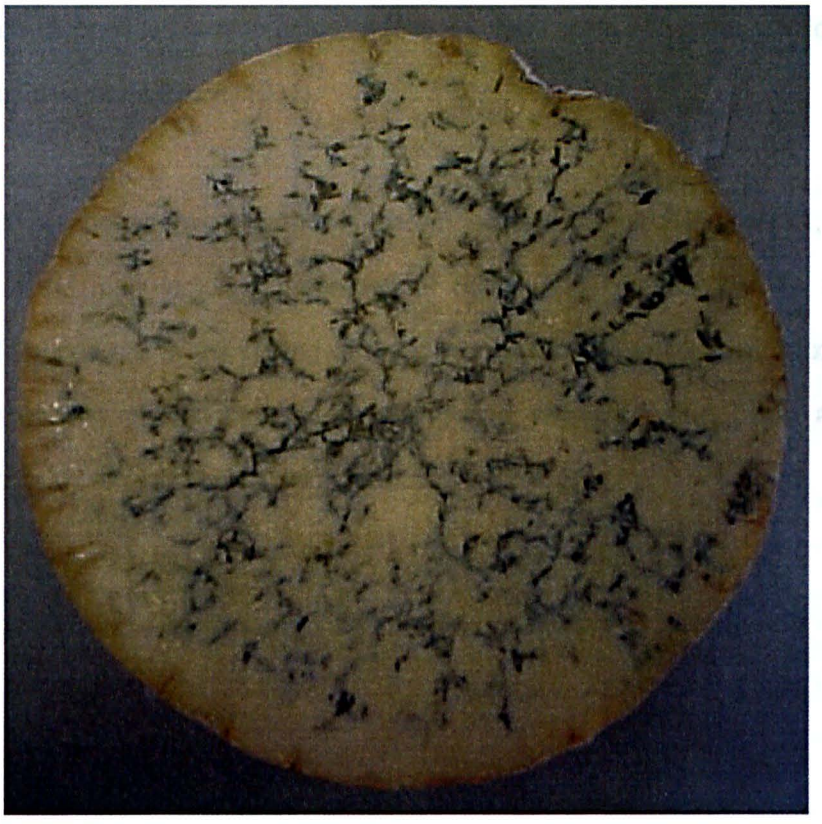


Plate 1.1 Half Stilton cheese at 8 weeks of age (Long Clawson Dairy)

This study aimed to determine the microbiological factors that affect the flavour of the cheese and the ability of the mould, *P. roqueforti*, to grow.

1.3.2 Cheese Manufacture

1.3.2.1 General Aspects of cheese manufacture

The manufacture of cheese involves the bacterial fermentation of lactose to produce lactic acid, resulting in a reduction of the pH of the milk.

$$C_{12}H_{22}O_{11} \Longrightarrow 2C_6H_{12}O_6 \Longrightarrow 4CH_3CHOHCOOH$$

In addition, the starter and other micro-organisms in the milk produce further metabolic end products such as peptides, amino acids, aldehydes, fatty acids and organic acids, including citric and propionic acids (Scott, 1981).

The process of cheese manufacture is shown schematically in Figure 1.1, where the differences in the manufacturing processes of Stilton and Cheddar can be seen. The principal differences are in the cutting of the curd, the lack of scalding of Stilton curd, the overnight incubation of Stilton curds, pressing and skewering and these aspects are discussed more thoroughly in sections 1.3.2.3 and 1.3.2.4.

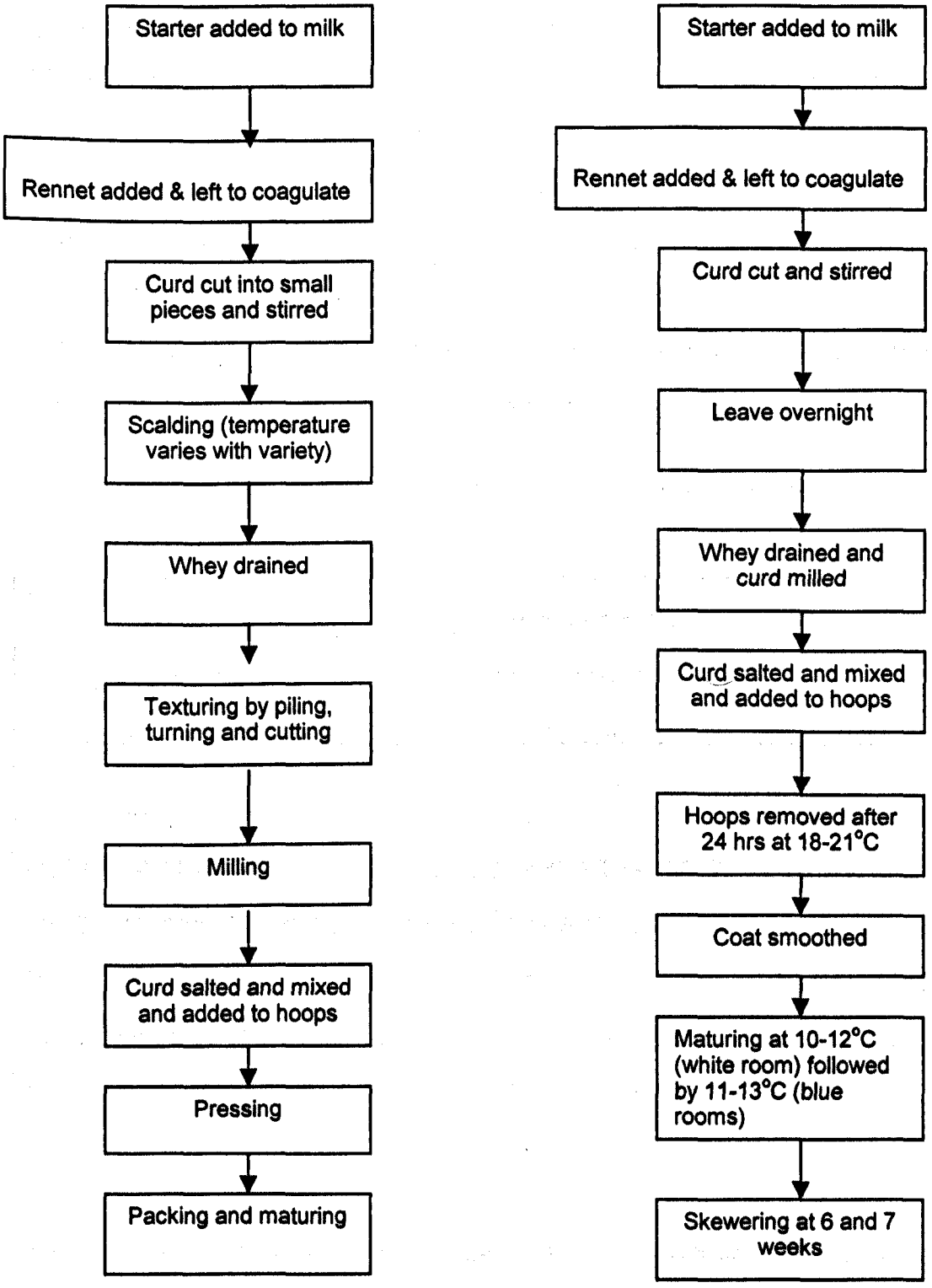


Figure 1.1 Comparison of the production of hard cheeses with that of Stilton cheese

During the production of most cheeses, milk, either raw or pasteurised, is warmed to 21 - 30 °C in a vat and the starter culture is added. Until recently not all milk for the production of Stilton cheese was pasteurised, but as a consequence of an outbreak of food poisoning thought, though never proven, to be attributed to Stilton, Senior Environmental Health officers recommended the pasteurisation of all milk for the production of this cheese (Hough, 2001, pers. comm.). The starter culture usually contains two or more species of microorganisms selected for their metabolic end products such as lactic acid or flavour components. Examples of bacteria used as starter cultures are shown in Table 1.1.

Table 1.1 Species used as starter bacteria in cheesemaking (Scott, 1986).

Species	**Primary function**
Lactococcus lactis subsp. *lactis*	Lactic acid production
Lactococcus lactis subsp. *cremoris*	Lactic acid production
Lactococcus lactis subsp. *lactis* var. *diacetylactis*	Diacetyl production
Leuconostoc citrovorum	Citric acid production
Leuconostoc mesenteroides subsp. *cremoris*	Gas production
Lactobacillus helveticus	Lactic acid production in high scald cheese
Lactobacillus bulgaricus	Lactic acid production in high scald cheese
Propionibacterium shermanii	Propionic acid production

The amount of starter used varies with the type of cheese to be produced and may vary from 0 to 1 or more percent. In the production of Stilton cheese as little as 0.005 - 0.001% v/v is used, although the curd is left overnight for the acidity to develop. This mirrors the traditional methods of manufacture for many cheeses, where starter cultures were not used and so a long ripening period was necessary. The starters used in the production of Stilton include

Lactococcus lactis subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and may also include *Leuconostoc mesenteroides* and *L. lactis* subsp. *lactis* var. *diacetylactis*. Members of the genus *Lactobacillus* are not used as starters in this type of cheese.

Until relatively recently, starters were cultured on site and liquid starter was added to the vat of milk. More recently, however, direct vat inoculation (DVI) starters have been used to some extent, although not all dairies use them.

1.3.2.2 Comparison of DVI starter with bulk starter, composition and preparation

The DVI starter is a mixture of lactic starters prepared from pelleted deep frozen, concentrated starters, intended for direct inoculation into the vat. Because of the small inoculation levels needed for Stilton cheese production these are further prepared to make a liquid live starter suitable for inoculation. This is carried out by adding 11.25 grams *Lactococcus lactis* subsp. *lactis* (M531, supplied by Rhodia Texel Ltd., Stockport, Cheshire UK) and 3.75g MH1/2 (*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* var. *diacetylactis*, *L. lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris*) to freshly pasteurised milk. This mix is designed to give 8.33% citrate fermenters in the final starter (Hough, 2000, pers. comm.). The mixture is then stored under refrigeration, without further incubation and 15ml of this is added to each 4800 litre vat. Whilst in theory the ratio of organisms stays the same, in practice this is unlikely as, despite refrigeration, the growth characteristics of each individual species may vary and the resultant starter may not conform to the original ratio.

Bulk starter is composed of the same mixture of species and is prepared in a similar manner using Dri-Vac CHN01 (Christian Hansen, Denmark). The method used for preparation is less controlled since approximate measurements are made: approximately 0.5 g CHN01 is added to 500ml of sterilised milk and incubated at 26°C for 18-21 hours. Ten ml of this is subcultured into a further 500 ml of sterilised milk and incubated at 26°C for 18-21 hours. This step is repeated twice more and the culture is ready for use at 3-4 days after initial inoculation (Hough, 2001, pers. comm.). Clearly this method is more likely to give variations

in strains and species within the starter, since the original mix is subcultured and then incubated three times.

Since only six companies now manufacture blue Stilton cheese and each dairy's individual manufacturing technique is a closely guarded secret, it is unlikely that all dairies will follow the same starter preparation technique exactly, but it is likely that similar methods will be used.

The starter for Stilton cheese manufacture is different from that used for Cheddar and other English territorial varieties in that it may contain either *Leuconostoc mesenteroides* subsp. *mesenteroides* or *Leuconostoc mesenteroides* subsp. *cremoris* for the production of carbon dioxide to lend a more open curd texture (Stanley, 1999, pers. comm.). This allows ingress of oxygen after piercing the cheese, which in turn stimulates sporulation of the *P. roqueforti* and thus blueing is able to progress more rapidly (Stanley, 1999, pers. comm.). Small amounts of carbon dioxide have been produced by naturally occurring *Ln. mesenteroides* subsp. *cremoris* but it is found that *Ln. mesenteroides* subsp *mesenteroides* produces larger quantities and thus a more open texture (Stanley, 1999, pers. comm.). However, the subspecies *cremoris* is more popular than the subspecies *mesenteroides*, as the latter has been found to produce less consistent quality in the end product (Hough, 2001, pers. comm.).

Starter cultures of moulds are used in mould ripened soft cheeses and are added at the same time as the bacterial starter culture. *P. roqueforti* is used in the manufacture of Stilton and other blue cheeses (such as Roquefort, Blue Wensleydale, Shropshire Blue and Buxton Blue), whilst *P. camemberti* is used in the manufacture of Brie and Camembert. The inoculation rate for the mould starter in Stilton is 1 gram in 4800 litres of milk (Hough, 2000, pers. comm.).

The milk and starter are usually left for a ripening period, which if an enzymic (rennet) coagulation is to follow is often less than one hour and may be omitted completely. Following the ripening period the coagulating agent, rennet, is added at a rate of 1200 ml to 4800 litres of milk, in order to bring about the coagulation of the casein in the milk. Rennet is a mixture of pepsin and chymosin and acts by splitting the K - casein at the bond between the amino acids phenylalanine and methionine at positions 105-106. This results in the production of para K - casein and the destabilisation of the casein micelle. Subsequently the casein combines with calcium to form insoluble calcium caseinate and the remaining components are bound within the coagulum thus formed. Following formation of the coagulum the tertiary phase of rennet activity commences, resulting in proteolysis of the alpha and beta caseins (Scott, 1986).

Once the coagulum reaches the desired firmness the curds are cut to a size specific for the variety of the cheese to be produced, using specialised curd knives, which are similar in appearance to a rectangular harp. The process of cutting can be seen in Plate 1.2 and the "American knives" are visible. The wires or blades are suspended from the horizontal bar indicated.

American knives, with wires or blades suspended between an upper and lower bar.

Plate 1.2 Cutting Stilton curd (courtesy Long Clawson Dairy)

The aim at this stage is to release whey from the curd, thus controlling the moisture and development of acidity. The smaller the curd is cut the more whey is released and typically Cheddar is cut to form pieces the size of wheat grains, Double Gloucester curd is pea sized, Stilton is chip sized (see 1.3.2.3) and soft cheese is uncut. Once cut, the curd particles are able to heal by joining the cut fibrils to form a network around the particles and so the curd is kept gently moving to prevent the curd forming a solid mass once more. In the production of Stilton cheese the curd is cut once down the length and across the width of the vat with the vertical knives and once down the length of the vat with the horizontal knives. This gives very large pieces, approximately chip sized, leaving much of the whey (and thus lactose) enmeshed within the coagulum.

1.3.2.3 Aspects of manufacture specific to Stilton cheese

The production of Stilton differs from that of other British hard cheeses in that it is a two-day curd production with no scalding period. The dairy from whence the samples were drawn additionally standardises the milk to give a fat:protein ratio of 0.87:0.84, since it has been found that this produces more consistently good quality product (Hough, 2000 pers. comm.). The pasteurised standardised milk is cooled to 30°C before being added to the vats where the starter culture is added at a rate of 0.005% and the mixture is then allowed to ripen for 40 minutes. Rennet is added at a rate of 1.2 litres to 4800 litres of milk and after approximately 40 minutes coagulation will have taken place. After cutting and stirring the coagulated curd as described above, the coagulum is left to settle to the bottom of the vat for a period and the whey is then slowly drained from the curd, leaving sufficient whey to cover the curds. The curd is left overnight (20 hours) in order to allow the acidity to increase to between 1.0 and 1.2 percent lactic acid.

The following morning, once the acidity has reached 1.2% the solid curd is cut into blocks of approximately 15cm width, weighing approximately 1 kg, to allow the remaining excess whey to drain off and the curd is then milled into small pieces of about 9cm^3 although there is some

variation in the size of the pieces (typically ranging from $1cm^3$ to $12cm^3$). Salt is added at a rate of 2-2.5% after the milling process and is mixed thoroughly into the curd using an automated mixing belt (plate 1.3).

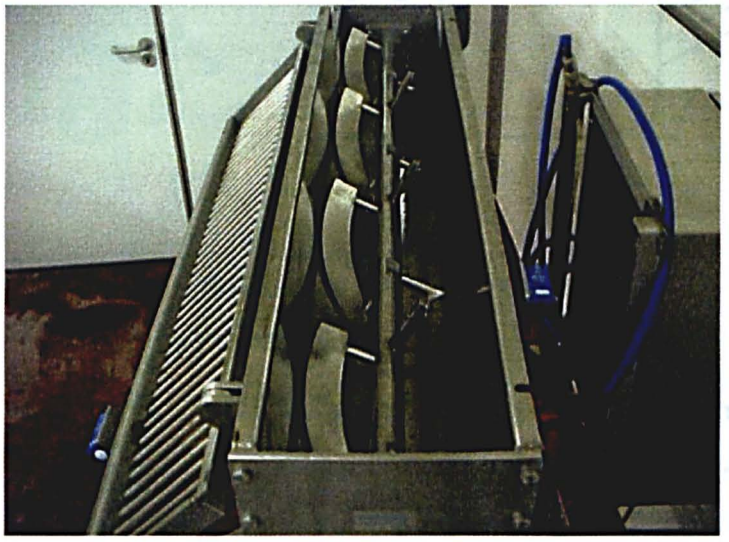


Plate 1.3 Salt mixing belt (Long Clawson Dairy)

The finished curd is then put into stainless steel hoops and allowed to drain, with the hoops being turned daily for the first few days. At this stage the immature cheeses are kept at a temperature of 18 - 21° C in the "hastener" room and after 5 days the hoops are removed and the coats of the cheeses are rubbed with a knife to smooth the coat and seal the holes in the curd. The cheeses are stored at a temperature of 10 - 12° C for three days before being removed to maturing rooms at 11-13° C and 85-90% relative humidity. After 6 weeks from the date of production the cheeses are pierced to allow oxygen to enter the curd network. This permits the growth of the aerobic mould and also allows the ammonia and carbon dioxide produced to escape.

The skewering needles are spaced approximately one inch apart vertically and enter the cheese at an angle of approximately 40°, also at intervals of approximately one inch around the circumference of the cheese, thus allowing ample aeration of the curd (Figure 1.2).

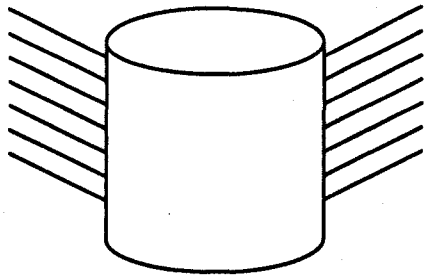


Figure 1.2 Arrangement of skewering needles

After a further week the cheeses are skewered once more to increase this gaseous exchange and are ready for the market about one week after this.

The final typical compositional analyses of Stilton, Cheddar and Brie at this stage are compared in Table 1.2. The differences can largely be attributed to the cutting and scalding stages with Cheddar being cut into wheat-sized pieces, Stilton to larger pencils of curd and Brie larger still (Scott, 1981). Cheddar is scalded to 39°C whilst Stilton and Brie have no scald hence the moisture contents of these two cheeses are higher than that of cheddar.

Table 1.2 Comparison of the typical composition of Stilton, Cheddar and Brie

Variety	% Maximum Moisture	% minimum Fat in dry matter
Stilton	42	48
Cheddar	39	48
Brie	60	20

Typical analysis of mature blue Stilton (Table 1.3) may vary from the maxima shown in Table 1.2 and, in fact, there are likely to be significant variations from these typical values, resulting in individuality among individual cheeses and between production sites.

Table 1.3 Typical analysis of mature blue Stilton (Hough, pers. comm. 2001)

Parameter	Typical value %
Moisture	36
Fat	34
Salt	2
Fat in Dry Matter (FIDM)	52
Salt in Moisture (SIM)	5.5

The typical values shown in Table 1.3 suggest that normally the moisture is much lower than the maximum quoted in Table 1.2 and a SIM of 5.5% would suggest a low a_w in the product. Turner and Thomas (1980) showed that high SIM levels (about 6%) may inhibit growth of starter bacteria and result in high levels of residual lactose and that consequently NSLAB may take over lactose metabolism. Their study, using Cheddar cheese, showed that in cheeses with a lower SIM (in the region of 4%) lactose was completely utilised by day eight of ripening.

1.3.2.4 *Aspects of manufacture of other English Hard cheeses*

During the production of the traditional territorial hard cheeses such as Cheddar, Leicester and Wensleydale, as well as blue versions of hard cheeses such as Buxton Blue and Blue Wensleydale, the curd undergoes a heating or scalding period after the cutting and healing. The aim of this stage is to expel moisture from the curd and so produce a drier curd, with less lactose remaining, and to provide more control over the acidity development. However, such cheeses are made using a greater initial inoculum of starter (typically 1-2%) and the curd is left to develop its acidity on the day of coagulation. Such cheeses normally achieve a pH of approximately 5.4 after 6 hours, whereas in the production of Stilton the pH develops more slowly but reaches a much lower value (typically 4.5) at salting.

After scalding the curds are drained, stacked and turned continuously until the final acidity is reached after which milling, salting and hooping (putting the curd into the hoops or moulds) take place. This is usually carried out at between three and five hours after starter addition and at this stage acidity development is curtailed by the addition of the salt. The final curd is then pressed for two to three days at a pressure that depends on the variety being produced. For example, Cheddar is pressed at 200 kNm^{-2}, whilst Leicester is pressed at 75-100 kNm^{-2} (Scott, 1981), whereas Stilton receives no pressure, other than that brought about by the weight of the curd in the hoop.

1.3.3 The Starter organisms in Stilton Cheese

Starter organisms have a number of different functions in fermented products: in cheese these are acid, flavour and texture production. The starters in Stilton include both the lactic acid bacteria and a blue mould. The principal lactic starter organisms are members of the genus *Lactococcus* which are characterised as Gram positive, catalase negative, non-motile cocci occurring in pairs or chains (Mundt, 1986). Members of the genus are known to grow at 10°C but not at 45°C and are not able to grow in broth containing 6.5% NaCl. They are microaerophilic and ferment lactose to produce lactic acid but no gas and some strains are able to produce the bacteriocin, nisin. Surface colonies on agar are very small and discrete (Mundt, 1986).

1.3.3.1 The role of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* in cheese starters

These organisms are the principal lactose fermenters in the mix of starters used in the production of Stilton cheese. Figure 1.3 shows the fermentation of lactose by the species and can be compared with Figure 1.4 which represents the production of other flavour compounds by *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*. The homofermentative subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* produce L-lactate whereas *L. lactis* subsp. *lactis* var. *diacetylactis* produces D-lactate (Marshall, 1987).

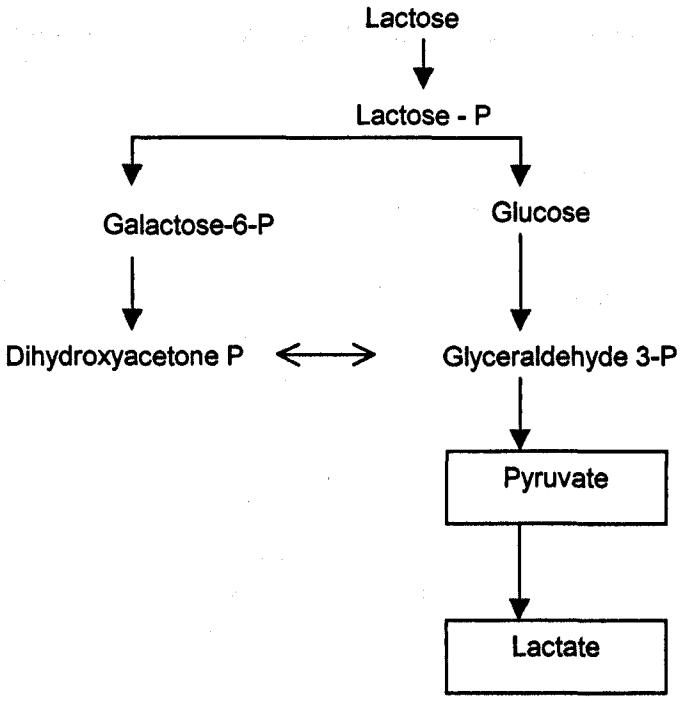


Figure 1.3 **Fermentation by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris***

(after Marshall,1987)

1.3.3.2 The role of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*

Lactococcus lactis subsp. *lactis* var. *diacetylactis* produces the flavour compounds diacetyl and acetoin. This results in buttery flavours. In lactic acid bacteria, diacetyl is not produced from carbohydrate unless an additional source of pyruvate is present (Marshall, 1987). The reason for this is that the enzymes responsible for the production of diacetyl, along with acetoin, are repressed. Citrate metabolism provides this pyruvate although it does not provide an energy source for the species (Marshall, 1987).

During the initial stages of metabolism, citrate is converted to oxaloacetate and acetate by the enzyme citrate lyase (Hugenholtz, 1993) and this oxaloacetate is then decarboxylated to pyruvate resulting in the production of carbon dioxide. This process is important in Stilton cheese as the formation of carbon dioxide aids the open texture and subsequent formation of the blue veins. Hugenholtz (1993) has indicated that citrate may only be fermented by a

limited number of lactic acid bacteria and that the products of this fermentation include not only diacetyl but also acetoin, butanediol and acetaldehyde (Figure 1.4).

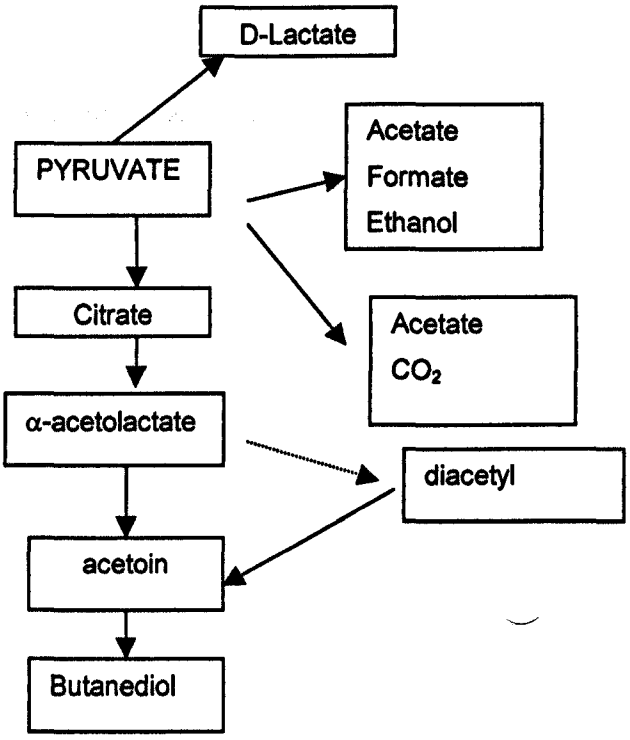


Figure 1.4 Citrate and carbohydrate metabolism in *L. lactis* subsp. *lactis* var. *diacetylactis*

(Hugenholtz, 1993)

The amount of the volatiles produced depends upon the growth conditions but Hugenholtz and Starrenburg (1992) found that strains of this species produced approximately 1-7 mM of α-acetolactate and 3-12 mM of acetoin in three hours of incubation at 30°C, although <1mM of diacetyl was produced under these conditions. In the same study, continued incubation resulted in the conversion of some of the acetoin to butane-diol and the complete disappearance of the α-acetolactate. In a study by Mauriello *et al.* (2001) 78 strains of lactic acid bacteria belonging to the genera *Lactococcus, Lactobacillus, Leuconostoc, Enterococcus* and *Streptococcus* were investigated. Using whey as the medium, their main flavour compounds were analysed using high resolution gas chromatography. They found

that citrate fermenting subspecies of *L. lactis* produced levels of acetoin varying from 1-12.75 ppm whilst *Ln. mesenteroides* produced less than 1ppm. The strains of the species that were unable to ferment citrate, were able to produce small amounts (0.012-0.186 ppm) of the compound. In this study the lactococci were found to produce 12 flavour compounds whereas the leuconostocs produced only six (Table 1.4). All 12 compounds were detected in citrate fermenting strains of the lactococci whereas 6 of these compounds were not detected from the citrate negative strains.

Table 1.4 Volatile compounds isolated in whey culture extracts of strains of lactococci and leuconostocs

(Mauriello *et al.*, 2001)

Compound	Odour description	Production by lactococci		Production by Leuconostocs
		Cit. +	Cit -	
3-hydroxy-2-butanone (acetoin)	Buttery	+	+	+
2-methyl-1-butanol	Whisky	+	-	-
3-methyl butanal	Malt	+	-	-
Furfural	Sweet, woody, almond, fragrant, baked bread	-	-	+
Ethyl benzene		+	+	-
Compound not identified		+	-	-
3-hydroxy-methyl butanoate	Fruity	+	-	-
Phenol	Disinfectant	+	+	+
Compound not identified		-	-	+
2-hydroxy benzaldehyde	Pungent, phenolic, spicy, almond, burning taste	+	+	-
Maltol	Malt, toasted	+	+	-
Compound not identified		+	+	+
Naphthalene	Camphor	+	-	-
Compound not identified		+	-	-
Compound not identified		-	-	+

It is clear from Table 1.4 that citrate fermenting strains of *L. lactis* are important in the generation of flavour compounds in the production of cheese. However, as the study

measured these compounds in whey, it is not certain that they would be retained in the cheese.

Kempler and McKay (1979) demonstrated the presence of a citrate plasmid in several citrate utilising strains of *L. lactis*. It is now widely recognised that the ability of *L. lactis* subsp. *lactis* var. *diacetylactis* to ferment citrate is plasmid encoded and as such is considered to be an unstable characteristic. Citrate metabolising activity in the species is at its highest in the pH range 5.0-6.0 (Hugenholtz, 1993) and so would decrease during the progress of the lactic fermentation, which reduces the pH to around 4.5. However, in Stilton this could potentially increase again as the growth of the mould, *P. roqueforti*, brings about an increase in pH of the curd.

1.3.3.3 The role of *Leuconostoc mesenteroides* subsp. *cremoris*

There are three subspecies of *Ln. mesenteroides*: *cremoris*, *lactis* and *mesenteroides* (Bergey *et al.*, 1986). The first two are considered to be the significant subspecies in aroma production (Marshall, 1987) and are known to produce diacetyl and acetoin. Marshall (1987) stated that the subspecies *cremoris* only grows in milk when supplemented with yeast extract or amino acids but the presence of proteases from other starters or microorganisms may provide the essential amino acids for growth. Cogan and Jordan (1994) have suggested that this type of symbiotic relationship may exist in cheese production. Furthermore, it is known that *P. roqueforti* produces several proteases (Gripon, 1993) and it may be that these too, encourage the growth of the *Ln. mesenteroides* subsp. *cremoris* by producing the amino acids necessary for its growth. In addition, potentially, proteases resulting from the growth of other adventitious or spoilage microorganisms may stimulate its growth.

Figure 1.5 shows the fermentation pathways used by this organism to produce a range of end products, most significant of which, to this cheese, is carbon dioxide. It is included in order to give a slightly more open texture (Hough, 2000, pers. comm.), which subsequently permits the gaseous exchange necessary to enable the growth of the blue mould *P.*

roqueforti. However, leuconostocs are able to produce diacetyl and thus are important flavour producing bacteria (Vedamuthu, 1994).

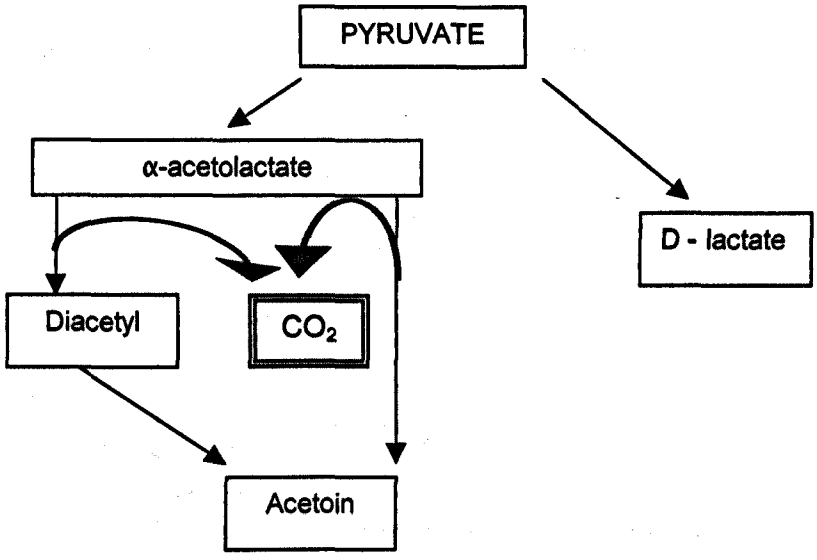


Figure 1.5 The role of *Leuconostoc mesenteroides* in the production of carbon dioxide and flavour compounds (after Stanley, 1999, pers. comm.)

Members of the genus *Leuconostoc* metabolise citrate in a similar way to *L. lactis* subsp. *lactis* var. *diacetylactis* (Hugenholtz, 1993), although Hugenholtz and Starrenburg (1992) found that little or no acetoin or butanediol were produced from citrate and there was no accumulation of α-acetolactate due to its conversion to diacetyl and carbon dioxide. This supports the study of Mauriello *et al.* (2001) who found that leuconostocs produced less than 1ppm of acetoin during fermentation of citrate in whey. The lack of production of acetoin could be attributed to the low acidity achieved in the whey cultures since the citrate transport mechanism is known to be induced by the production of acid (Garcia-Quintans *et al.*, 1998). During the production of cheese, however, these organisms grow in conjunction with other lactic acid bacteria that are able to produce significant quantities of lactic acid and so the leuconostocs produce high amounts of acetoin during the fermentation (Levata-Jovanovic and Sandine, 1996).

1.3.3.4 Flavour production by the starter lactic acid bacteria

Lactic acid bacteria play a significant role in the production of flavour compounds. Figure 1.6 demonstrates the range of flavour components produced by the breakdown of pyruvate, some of which are end products of metabolism. Other flavour components are intermediates and as a consequence may be less significant as maturation takes place.

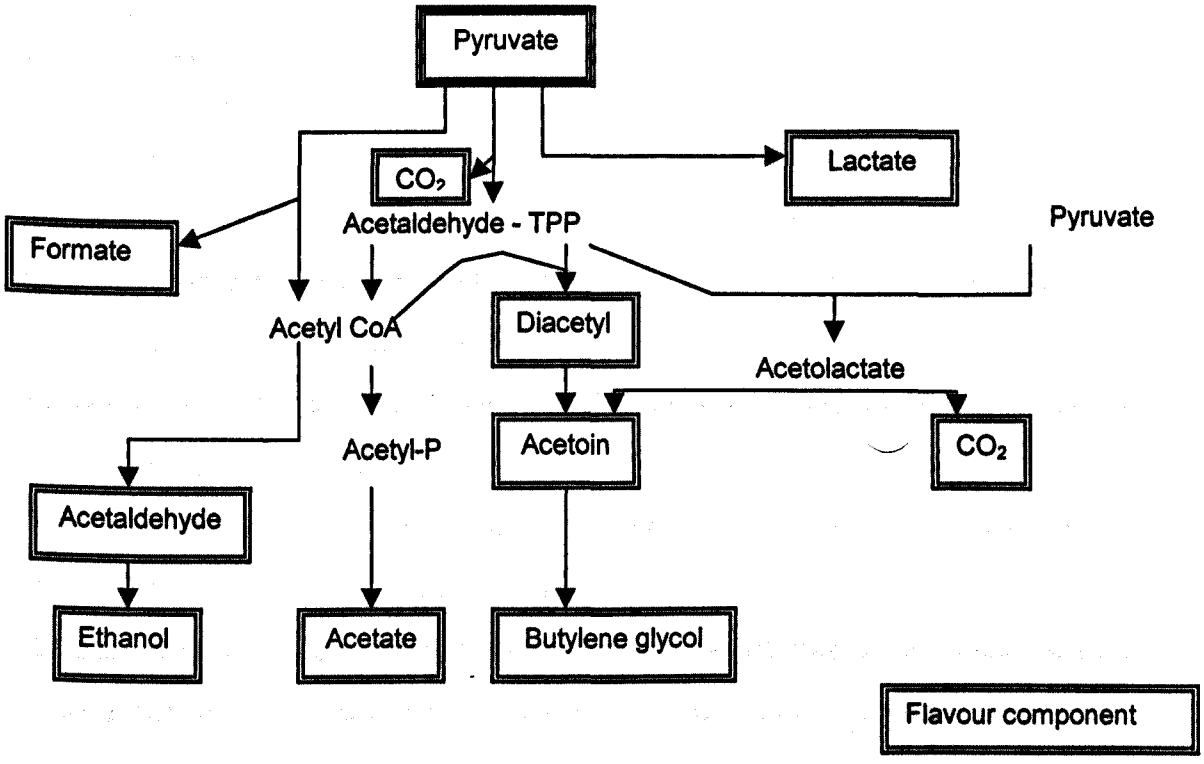


Figure 1.6 The fate of pyruvate in lactic acid bacteria

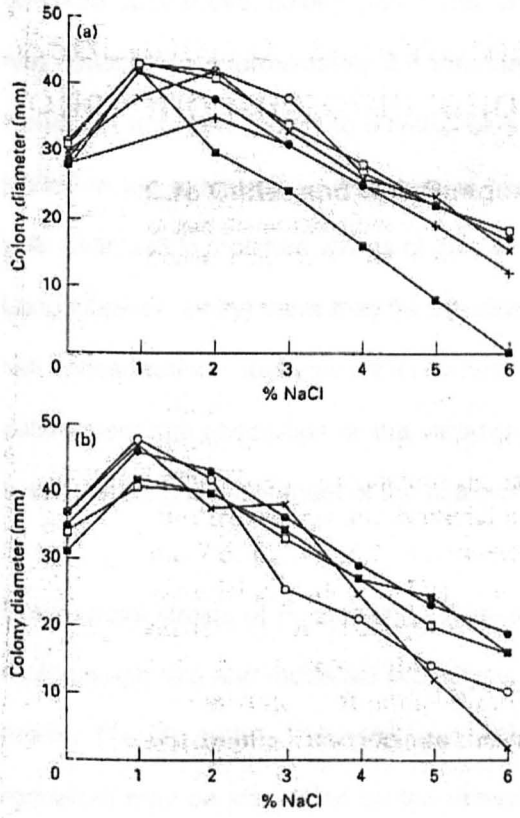
(after Marshall, 1987)

In blue Stilton, the lactic starters are selected to incorporate most of the flavour components indicated in Figure 1.6. The incorporation of *L. lactis* subsp. *lactis* var. *diacetylactis* is specifically for the production of diacetyl, although the amount present should decrease during maturation as conversion to acetoin takes place. Vedamuthu (1993) reported that early studies on the production of diacetyl in butter using *Leuconostoc* starters achieved diacetyl concentrations ranging from 4.48 to 11.84 ppm, but there are no documented reports of the level of diacetyl achieved in Stilton or other cheeses. Mauriello *et al.* (2001)

found that species of *Leuconostoc* produced less than 1 ppm of acetoin during citrate fermentation in whey which may suggest that equally low amounts of diacetyl were present, although these were not measured. However, it is known that high quantities are produced during co-fermentation with other lactic acid bacteria (Levata-Jovanovic and Sandine, 1996). Lindsay *et al.* (1965) found that generally 1.6 to 4.0 ppm of diacetyl was necessary to produce a typical flavour in dairy products.

1.3.3.5 Characteristics of *Penicillium roqueforti*

The fungus *P. roqueforti* is characterised by the production of conidiophores with wrinkled walls and exhibiting a green/blue colour varying from light to dark (Texel, 1998). Members of the species are proteolytic and lipolytic with different strains exhibiting varying degrees of activity (Lopez-Diaz *et al.*, 1996). The strain PV (Visbyvac®, Danisco) is used to produce the Stilton cheese used in the current study and is strongly proteolytic and lipolytic as well as having a high tolerance to salts (Danisco, 1999). *P. roqueforti* possesses the ability to grow over a wide pH range (4-7), as well as being cold tolerant, with an optimum for growth of 20-25°C and showing growth at 8°C but not at 0°C. The species is micro-aerophilic with the ability to grow at 5% oxygen (Texel, 1998). *P. roqueforti* is also able to grow in the presence of low concentrations of salt and Lopez-Diaz *et al.* (1996) showed that concentrations of 1-3% NaCl were stimulatory to the organism. The study also showed that NaCl levels greater than 3% were restrictive, although limited growth was still able to occur at NaCl concentrations of 6%. Stimulation was measured by the colony diameter after 5 days incubation on malt extract agar at 25°C and showed that up to 3% NaCl gave an increase on colony diameter over no addition of NaCl (Figure 1.7). It was also shown that different strains exhibited different tolerances to NaCl.



a) Effect of NaCl on the 5-day colony size at 25°C of *Penicillium roqueforti* strains (PR1-PR6).
■, PR1; □, PR2; ●, PR3; ○, PR4; X, PR5; +, PR6

b) Effect of NaCl on the 5-day colony size at 25°C of *Penicillium roqueforti* strains (PR7-PR9, S1 = MUCL 29148, S2 = MUCL 29151).
■, PR7; □, PR8; ●, PR9; ○, S1; X, S2

Figure 1.7 The effect of NaCl on the 5-day colony size of selected *P. roqueforti* strains (Lopez-Diaz *et al.*, 1996)

A study by Godinho and Fox (1981) also found that low salt concentrations, particularly 1%, were stimulatory to the species, although they showed that a strain isolated from Stilton cheese was found to be an exception. This strain, when tested on malt extract agar, exhibited maximal stimulation at 3% NaCl and the three day colony diameter was inhibited less by concentrations of 10% NaCl than strains from Roquefort, Danish Blue or Irish Blue cheeses and a commercial spore preparation. Valik *et al.* (1999) used mathematical modelling to predict the lag time of *P. roqueforti* at a range of a_w values using salt as the humectant. The study measured the diameters of the colonies, grown on Sabouraud agar, and an average of four diameters were used for modelling. Growth curves were produced using a range of a_w (0.995, 0.97, 0.96, 0.92, 0.91, 0.87) and modelling enabled graphs of predicted colony growth rate and lag time to be produced. These showed that at $a_w > 0.92$ the lag time did not change much, but at $a_w < 0.92$ there was a large increase. At the

optimum a_w of 0.998, colony growth rate was calculated as 13.4 mm/day, but at a_w 0.92 this was reduced to approximately 2.8 mm/day. It is likely, therefore, that over-salted cheese would not only take longer to develop blue veins due to the increased lag time, but also be limited in the extent of development of blueing as a result of the restriction created by the salt. With salt in moisture values of greater than 5% being found in Stilton (unpublished data, Long Clawson Dairy) there may be significant inhibition of growth. It is also possible that the salt concentration could vary within a cheese due to the differences in curd size at milling and subsequent salt absorption or the variation in local moisture content. This could potentially result in patchy development of the blue veins in the cheese.

Commercial strains of *P. roqueforti* which are used for cheese manufacture are selected for rapid growth rate and moderate proteolysis and lipolysis leading to moderate development of aroma (Texel, 1998). Hanson and Jakobson (1997) demonstrated that growth of *P. roqueforti* may be stimulated by the release of amino acids such as arginine and lysine by lactic acid bacteria and also showed that among lactic acid bacteria, arginine is only produced by *Leuconostoc mesenteroides* subsp. *cremoris*. This study focussed on Danablu cheese and tested 20 strains of *P. roqueforti* and 15 strains of starter lactic acid bacteria from the following species: *Ln. lactis; Ln. mesenteroides* subsp. *cremoris; Streptococcus thermophilus; Lb. delbrueckii* subsp. *lactis; Lb casei* subsp. *casei; L. lactis* subsp. *lactis; L. lactis* subsp. *cremoris; L. lactis* subsp. *lactis* var. *diacetylactis; Lb. plantarum*. The study showed that there was variation in the stimulatory effect depending on the strain of *P. roqueforti*. The strain Roq 15 was not stimulated by any of the lactic acid bacteria (LAB) under test, whilst six strains were stimulated by 10-12 of the LAB. *Leuconostoc mesenteroides* subsp. *cremoris* stimulated 16/20 strains of *P. roqueforti* as characterised by faster growth, stimulation of sporulation, more intense blue-green colour and thicker mycelial growth. Subsequent amino acid analysis showed that *Ln. mesenteroides* subsp. *cremoris* increased the level of arginine present, along with other amino acids, while the other LAB did not affect the level of this amino acid, although they did produce an increase in other amino acids such as alanine, glycine and leucine. This may indicate a necessity for inclusion of the

latter in starters for blue cheeses. Indeed, there may be a symbiotic relationship between the two organisms, with *P. roqueforti* producing growth factors essential for *Ln. mesenteroides.*

The proteolytic activity of *P. roqueforti* has been discussed by Gripon (1993) and proteolysis has been shown to be more extensive in blue than in other types of cheeses. A study by Trieu-Cuot *et al.* (1982) showed that β-casein was degraded to a greater extent in blue cheese, as a consequence of the effect of the microbial proteases, with those of *P. roqueforti* being dominant. *P. roqueforti* has been shown to have both endo- and exo-peptidase action. Gripon (1993) found that the proteases produced by *P. roqueforti* and *P. camemberti* were similar. These proteases are; metalloproteinase, aspartate proteinase, acid carboxypeptidase and alkaline aminopeptidase. *P. roqueforti* is also capable of producing alkaline carboxypeptidases and an alkaline proteinase.

Zarmpoutis *et al.* (1996) showed that proteolysis during ripening of Chetwynd blue, an Irish farmhouse cheese, was initially due to chymosin, but during the later stages was due to the fungal endo- and exo-peptidases. The result was extensive protein hydrolysis. This was also the case in a study on Gorgonzola cheese (Gobbetti *et al.*, 1997) when the water soluble nitrogen (N) in the core of the cheese was found to be about 53% of the total N at 63 days. The authors considered this to be indicative of extensive proteolysis. Several other studies have produced similar conclusions (Ottogalli *et al.*, 1971; Fernandez-Salguerol *et al.*, 1989).

Gripon (1993) also suggested that lipolysis is greater in mould ripened cheeses than in others and stated that *P. roqueforti* produces two lipases, one with an alkaline optimum for activity (pH 7.5-8.0) and the other with an acid optimum (6.0-6.5). The acid lipase is more active in cheese as would be expected from the pH values found in the product, including blue cheeses. Studies by Schwartz and Boyd (1963) and Schwartz and Parks (1963) showed that methyl ketones were the significant flavour compounds in blue cheese ripening with heptanone predominating. In the study of Roquefort cheese by Schwartz and Boyd (1963), heptan-2-one was most commonly produced in levels ranging from 4.2 to 15.6μM

$10g^{-1}$, as compared to 2.6 – 12.9μM $10g^{-1}$ of nonanone. In the study by Schwartz and Parks (1963), which examined three commercial varieties of blue cheese (varieties not stated), heptanone also predominated and larger methyl ketones with 11 or more carbons were found only in very small amounts. They postulated that the smaller methyl ketones may contribute more potent flavours to the cheese.

Penicillium roqueforti is known to produce the mycotoxin *Penicillium roqueforti* (PR) toxin as a secondary metabolite, which may be present in cheeses ripened by the organism. PR toxin was discovered by Wei *et al.* (1973) and was found to be lethal to rats, mice and cats with LD_{50} values of approximately 1-15 mg kg^{-1} via intra-peritoneal injection (Wei *et al.*, 1976; Chen *et al.*, 1982). It has also been shown to inhibit some metabolic activities such as RNA and protein synthesis (Moule *et al.*, 1976; Moule *et al.*, 1978) and to alter the genetic activities of *Saccharomyces cerevisae* and *Neurospora crassa* (Wei *et al.*, 1979). However, Chang *et al.* (1998) suggested that the toxin is soon (within a few days) degraded sequentially to PR-imine, PR acid and PR-amide and so was unlikely to be of significance in blue cheese and related products. Siemens and Zawistowski (1993) found PR imine in 50/60 samples of mature Danish Blue cheese in levels ranging from 19 to 42μg kg^{-1}, although it was not found in blue cheese dressing (six samples). PR toxin was not detected in any sample using a minimum detection rate of 1.5ng and Siemens and Zawistowski (1993) proposed that this was due to the conversion of the toxin to PR imine.

The toxins Roquefortine C, patulin, penicillic acid mycophenolic acid and isofumigoclavin A and B are also produced by *P. roqueforti* (Engel and Teuber, 1989). A study by Finoli *et al.* (2001) concluded that blue cheese was safe for the consumer as low levels of Roquefortine C, a toxin with neurotoxic properties, were produced in the cheeses tested. In this study, 20 Gorgonzola cheeses and 10 other blue cheeses (Danablu, Roquefort, Edelpilkäse and Stilton) were tested for the presence of Roquefortine C and PR toxin. Levels of Roquefortine C ranged from 0.05-1.47 mg kg^{-1}, whereas PR toxin was never detected, due to its conversion to PR imine, PR amide and PR acid as mentioned previously. The levels of

Roquefortine C detected in the Stilton samples were 0.21 and 0.65 mg kg^{-1}. Although not previously suggested, it is possible that this toxin, along with others such as PR toxin, could have a negative influence on the growth of bacterial species and thus its presence in blue cheese may affect the diversity of bacterial species present.

1.3.4 Cheese ripening

Cheese ripening is a combination of biochemical changes brought about by starter bacteria, natural milk enzymes, the secondary flora e.g. moulds and NSLAB, the cheese making processes such as acidity control and rennet coagulation and the environmental conditions (pH, Eh, a_w salt, temperature and humidity). Stilton may have micro-environments within the cheese due to its open texture and the growth of *P. roqueforti* along the veins is likely to accentuate these. There is potential for salt concentration to vary due to the variability in size of curd particles at milling, as mentioned previously and additionally there could be localised moisture variations. In addition, the veining might give rise to variations in Eh with higher values possibly occurring in the veins than in the curd. Depending on the extent of growth of *P. roqueforti* the pH may be higher in the blue veined areas than in the white areas since proteolysis may be greater in the areas of luxuriant fungal growth.

During the storage and maturation of cheese, the microflora changes continually both in number and variety of species represented. Figure 1.8 demonstrates a typical change in microflora in a hard cheese and shows the decline in numbers of starter LAB during ripening whilst the lactobacilli increase. In a previous study the author showed that this was also true in Stilton cheese, despite the absence of lactobacilli in the starter (Whitley *et al.*, 2000). In this study, mature Stilton cheeses were stored for up to 10 weeks under a range of modified atmospheres (N_2:CO_2:O_2 = 80:10:10, 100:0:0, 80:20:0 and no modification). During storage it was noted that the total viable count decreased, whilst the lactobacilli increased for the first five weeks, after which time a decrease in the number of these organisms was also noted. This decrease was attributed to competition for the limited oxygen, but the change in ratio of

total bacteria to lactobacilli in the early part of the study reflects changes reported in studies on other cheeses (Haque *et al.*, 1997). NSLAB have been the focus of a number of studies which have investigated their role in cheese ripening (Lane *et al.*, 1996; Walsh *et al.*, 1996).

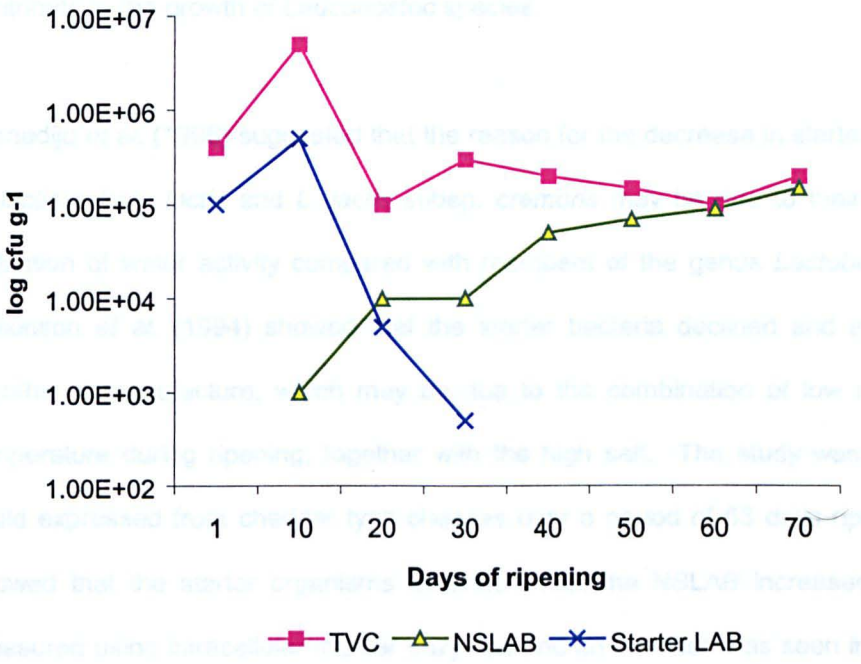


Figure 1.8 Changes in microbial flora of a typical hard cheese during ripening

(after Scott, 1981)

Figure 1.8 shows that although the total viable count may vary by small amounts during ripening, the lactococci decline whilst the lactobacilli come to predominate, despite the fact that members of this genus may not be added as the starter bacteria. These NSLAB are derived mainly from the milk or the factory environment (Martley and Crow, 1993; Jordan and Cogan, 1999) and it has been shown that they become the dominant flora in mature hard and semi-hard cheese (Naylor and Sharpe, 1958a). They are thought to play a role in flavour development (McSweeny *et al.*, 1995) although the energy source is unclear as only small amounts of lactose remain after cheese manufacture. Peterson and Marshall (1990) suggested that lactobacilli sustain growth in cheese depleted of fermentable carbohydrate by

metabolising peptides and amino acids released from the enzymic hydrolysis of casein and the products of degrading starter bacteria. These lactobacilli are also able to produce proteases and peptidases (Peterson and Marshall, 1990) and thus will potentially contribute to the development of bitter flavours. However, amino acids resulting from their action may contribute to the growth of *Leuconostoc* species.

Tornadijo *et al.* (1995) suggested that the reason for the decrease in starter bacteria such as *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* may be due to their sensitivity to the reduction of water activity compared with members of the genus *Lactobacillus*. However, Wilkinson *et al.* (1994) showed that the starter bacteria declined and autolysed within 3 months of manufacture, which may be due to the combination of low moisture, pH and temperature during ripening, together with the high salt. The study was conducted using liquid expressed from cheddar type cheeses over a period of 63 days ripening and results showed that the starter organisms declined whilst the NSLAB increased. Autolysis was measured using intracellular marker enzymes and an increase was seen in these over time. Several LAB are able to produce anti-bacterial substances, including H_2O_2, nisin and other lantibiotics (Lindgren and Dobrogosz, 1990; Karunaratne *et al.*, 1990; Van de Guchte *et al.*, 2001) so it may also be possible that the production of anti-bacterial substances by NSLAB has an effect on the starter population. This aspect is discussed more fully in section 1.3.5.

NSLAB typically reach levels of $10^7 - 10^8$ and are thought to contribute towards ripening since unpasteurised milk cheeses ripen faster and develop more flavour than those made from pasteurised milk (Wilkinson, 1993). In a study comparing raw, pasteurised and micro-filtered milks, McSweeney *et al.* (1991) found that indigenous microbes had the most significant effect on ripening. The cheese made from pasteurised and micro-filtered milks were indistinguishable from each other whereas the raw milk cheese underwent greater proteolysis and the flavour was more intense. However, commercial graders found the raw milk cheese unacceptable and Stanley (1998) points out that if NSLAB are present in

numbers in excess of 10^8, defects may arise such as gas, green spot development and development of calcium lactate crystals, along with unclean and acid flavours. This supports the statement made by Peterson and Marshall (1990) that lactobacilli produce proteases and peptidases as the activity of these enzymes could give rise to bitter flavours which would be unacceptable to commercial graders.

A study by Olarte *et al.* (2000) found that after 60 days of maturation *Lactobacillus* was the predominant organism in goat's cheese made with either raw milk or raw milk plus starter bacteria and that *Lb. plantarum* was the predominant species. However, in the same study, cheese made with pasteurised milk plus starter yielded only lactococci with no lactobacilli after 60 days. In terms of sensory quality the cheeses made with raw milk plus starter were considered to be superior to the other cheeses, having a good aroma and taste and an acceptable texture. To some extent this conflicts with the study by McSweeney *et al.* (1991) in that the raw milk cheese in that study was considered to be unacceptable, whereas the study by Olarte *et al.* (2000) found them acceptable, but it is possible that the addition of the starter bacteria prevented over-activity of the indigenous microorganisms in this study and hence limited the development of undesirable flavours. Equally, there might be some difference arising from the milk (goat as opposed to cow) or subjective differences might be significant in that the personal taste of those carrying out organoleptic assessment may vary.

Studies on NSLAB in cheddar cheese have revealed levels ranging from $10 - 10^4$ per gram in the first 10 days rising to 10^8 within a few weeks (Peterson and Marshall, 1990). Whitley *et al.* (2000) found levels of lactobacilli in mature (8 weeks old) blue Stilton cheese reached 10^7, suggesting that this cheese undergoes changes in the lactic flora that are similar to non-blue cheeses.

Adjunct cultures may be used to affect flavour development in cheese. These cultures were defined by El Soda *et al.* (1999) as "selected strains of cheese related microorganisms that are added to the cheese milk to improve development of sensory quality". Lynch *et al.* (1996) investigated the influence of adjunct cultures on cheddar cheese ripening and found

that there was a favourable effect on flavour with an increase in small peptides and amino acids in the cheese. A review of the effects of adjunct cultures by El Soda *et al.* (1999) also suggested that the addition of homofermentative cultures such as *Lb. casei* and *Lb. paracasei* produces desirable flavours in the cheese. However, Puchades *et al.* (1989) found that the addition of *Lb. brevis* led to the presence of off flavours and gas. Madkor *et al.* (2000) manufactured Cheddar cheese using added attenuated adjunct cultures of *Lb. helveticus* and *Lb. casei* and found that cheeses made with the *Lb. helveticus* culture received higher flavour and aroma scores, but that all the adjunct treated cheeses exhibited better body and texture than the non-adjunct treated samples. Freeze shocking of cultures was carried out by freezing the cell suspension at –20°C for 24 hours, followed by thawing in a water bath at 40°C for approximately 10 minutes before use. Sensory evaluation was carried out with five people experienced in Cheddar cheese evaluation, using a 10 point scale, where > 7 indicated high quality, 4-6 fair quality and < 4 poor quality. The control samples, containing no adjunct, received mean scores of 4.7 ± 0.5 at three months of ripening as compared to 7.1± 0.4 for cheese with added freeze-shocked *Lb. helveticus* and 5.4 ± 0.3 for that with added freeze-shocked *Lb. casei*.

There appears, therefore, to be conflicting opinion as to the desirability of the presence of NSLAB and Martley and Crow (1993) have suggested that the addition of adjunct starters may inhibit their growth. Their experiments included the addition of *Leuconostoc* 5201(a heat tolerant strain exhibiting characteristics similar to *Ln. mesenteroides* subsp. *dextranicum*) to cheese milk with the result that the growth of NSLAB was suppressed. However, it is likely that the range of flavours may be diminished by such action as it seems probable that NSLAB are important in this area.

1.3.5 Non-starter lactic acid bacteria

The anti-microbial effects of NSLAB and lactobacilli in particular are the subject of a number of studies (Wiseman and Marth, 1981; Karunaratne *et al.*, 1990; Niku-Paavola *et al.*, 1999; Van de Guchte *et al.*, 2001). Lactic acid bacteria have been shown to secrete chemicals antagonistic to other microbes resulting in changes to environmental conditions (Lindgren and Dobrogosz, 1990). These chemicals can be divided into low molecular weight chemicals and bacteriocins (with a molecular mass in excess of 1000). Bacteriocins have an inhibitory effect only on closely related species. The bacteriocin nisin has been used as a preservative in the food industry (Vandenburgh, 1993). Less is known about the low molecular weight compounds but they exhibit a wider spectrum of activity against Gram positive and Gram negative bacteria and fungi (Niku-Paavola *et al.*, 1999). Ouwehand (1998) reviewed the antimicrobial compounds produced by lactic acid bacteria and included in these not only the bacteriocins but also organic acids such as lactic and ethanoic acids as well as other chemicals such as hydrogen peroxide. One of the main preservative effects in cheese is the low pH brought about by lactic acid, but the undissociated acid may itself bring about growth inhibition (Ouwehand, 1998). This review also discussed the low molecular weight substances reuterin and 2-pyrorolidone-5-carboxylic acid (PCA) as well as bacteriocins. Reuterin is produced by the gut organism *Lb. reuteri* whilst PCA is produced by *Lb. casei* spp. *casei* and *Lb. casei* spp. *pseudoplantarum* and so PCA may be of significance in cheese containing these species as part of the NSLAB.

Bacteriocins may be divided into four main classes, :

Class I	Lantibiotics
Class II	Small moderate to high heat stable, non-lanthionine-containing membrane-active peptides
Class III	Large heat-labile proteins
Class IV	Complex bacteriocins: protein with lipid and/or carbohydrate.

Lactobacilli may produce substances found in all of these classes (Ouwehand, 1998).

Niku-Paavola *et al.* (1999) investigated anti-microbial compounds produced by *Lactobacillus plantarum* and found that the Gram negative test organism *Pantoea agglomerans* VTT E-90396 (formally *Enterobacter agglomerans*) was totally inhibited by the low molecular mass fraction of the culture filtrate. The active fraction included benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one and 3-(2-methylpropyl)-2,5,-piperazinedione and was found to be 100% effective when applied together with 1% (w/v) lactic acid. Individually the chemicals showed a 10-15% effectiveness and the lactic acid 40% effectiveness. This study also measured the inhibition of the fungus *Fusarium avenaceum* and found that inhibition was 10-15% individually and 20% in combination whilst the lactic acid had no inhibitory effect. It is possible that the NSLAB of Stilton cheese may have a similar antimicrobial effect on either the starter bacteria or the blue mould and so in certain conditions cause defects in the cheese.

A study by Okkers *et al.* (1999) demonstrated that *Lb. pentosus* TV35b produced a bacteriocin like peptide, pentocin TV35b. This was found to be inhibitory to a number of bacteria including *Clostridium tyrobutyricum, Propionibacterium* spp. *Lb. curvatus* and *Lb. fermentum* and the yeast *Candida albicans.* Production of the chemical was at a maximum towards the end of the logarithmic growth phase when grown in MRS broth.

Gourama and Bullerman (1995) discussed the possible inhibition of mould growth and mycotoxin production by lactic acid bacteria and suggested that these bacteria could improve the shelf life of fermented products and limit the health hazards associated with mycotoxins from spoilage fungi. Several moulds produce mycotoxins, such as aflatoxins and ochratoxins, both of which are known to be toxic. In particular, aflatoxins are produced by *Aspergillus flavus, A. flavus* subsp. *parasiticus* and *A. nomius* and have been found in a range of food products including cheese (Blanco *et al.*, 1988). Several have been shown to be carcinogenic but there is no evidence to suggest that they have been found in Stilton cheese. As discussed earlier, the mycotoxin PR toxin is produced by *P. roqueforti* and thus may be present in cheeses ripened by the organism.

Most studies on the antimycotic effects of lactic acid bacteria have focused on members of the genus *Aspergillus*. A study by El Gendy and Marth (1981) investigated the interaction between *A. parasiticus* and *Lb. casei* whilst Batish *et al.* (1989) screened 19 bacterial strains, including members of the genera *Lactococcus* and *Lactobacillus*, for antifungal activity against members of the genera *Aspergillus, Rhizopus* and *Candida*. It may be possible that the inhibitory effect demonstrated by lactic acid bacteria on some moulds could be present in Stilton resulting in a reduced level of growth of *P. roqueforti* and thus of blue veining in the cheese.

A number of studies have tried to identify the range of NSLAB in cheese, although these have focused on Cheddar cheese. Jordan and Cogan (1993) showed that the adventitious NSLAB flora of 8 weeks old commercial Irish cheddar was composed of 55% *Lb. paracasei*, 28% *Lb. plantarum* and 14% *Lb. curvatus*. This compared with 96.4% *Lb. paracasei*, 2.1% *Lb. plantarum*, 0.3% *Lb. curvatus* 0.3% *Lb. brevis* and 0.9% unidentified in mature (9-24 months) Cheddar cheese (Fitzsimons *et al.*, 1999). Fitzsimons *et al.* (2001) found that the mixture of NSLAB varied during ripening with *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* and unidentified species being found in Cheddar cheese up to 6 weeks of ripening, whereas after this point only *Lb. paracasei* was found. These studies suggest that *Lb. paracasei* and *Lb. plantarum* are the most commonly occurring species and may suggest that the latter predominates as ripening progresses.

In a study on the *Lactobacillus* strains in Ricotta Forte cheese, Baruzzi *et al.* (2000) found a wide range of species during the 12-month maturation, although the strain profile changed over time. Strain profile was measured using a combination of randomly amplified polymorphic DNA (RAPD) analysis, 16S rDNA sequencing and physiological tests, including citrate activity and proteolytic activity. At the beginning of ripening only *Lb. keferi* and *Lb. gasseri* were present, but by two months this had increased to five species, including six strains of *Lb. keferi*, eight of *Lb. paracasei* and three of *Lb. plantarum*. At the end of the

ripening period, *Lb. plantarum* had disappeared and only *Lb. paracasei* subsp. *paracasei, Lb. alimentarius, Lb. brevis* and *Lb. acetotolerans* were present. No starter cultures were used in the manufacture of this cheese and the authors suggested that the species survived pasteurisation.

1.3.6 The role of *P. roqueforti* in the ripening of blue cheeses

It is clear from the work of Schwartz and Boyd (1963), Schwartz and Parks (1963), Trieu-Cuot *et al.* (1982), Gripon (1993) and others that *P. roqueforti* has a very significant role in the ripening process of blue cheeses. These authors have shown that it produces a wide range of flavour compounds and that proteolysis, as a result of the proteases it produces, causes significant textural changes.

Madkor *et al.* (1987b) studied proteolysis in Stilton cheese, using commercially produced cheeses. Proteolysis was measured using a combination of pH measurement, fractionation of the nitrogen followed by analysis of the amino groups separated by this method and analysis of the free amino acids in the cheese. Changes in pH were noted ranging from approximately 4.8 in 4 day old cheeses to approximately 6.0 in mature (70 day old) cheeses, an increase which they attributed to proteolysis being much more extensive in blue cheeses than in non-blue varieties. In this study, the pH was measured by macerating the curd in a mortar with 10ml of distilled water and the pH of the slurry was measured. It is likely that this would preclude separate measurement of the blue and the white areas of the curd. The level of water soluble nitrogen increased to approximately 61% after 70 days of ripening and was used as an indicator of the extent of proteolysis. Gel electrophoresis showed that α_{s1}- and β-caseins had degraded extensively after four days and by 55-70 days the α_{s1}-casein was completely hydrolysed, whilst the degradation of the β-casein progressed more slowly.

Penicillium roqueforti also contributes to the flavour of blue cheeses through lipolysis, with the production of methyl ketones (Schwartz and Boyd, 1963; Schwartz and Parks, 1963;

Madkor *et al.*, 1987a) and free fatty acids (Madkor *et al.*, 1987a). Studies on the ripening of Stilton cheese showed that the levels of free fatty acids (FFA) increased slightly during the first 28 days of maturation and then increased rapidly up to the end of ripening at 70 days (Madkor *et al.*, 1987a). The predominant methyl ketones recovered in this study were C_7 and C_9 which echoed the findings of Schwartz and Boyd (1963) and Schwartz and Parks (1963) during studies on Roquefort and other similar blue cheeses. In the same study it was noted that the concentrations of individual FFA increased between 4 and 20 fold but that the longer chain FFA (C_{12}, C_{14}, C_{16}, C_{18}, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$) were more numerous than the shorter chain acids (C_4, C_6, C_8, C_{10}). The shorter chain acids, such as butyric, are more likely to contribute significant flavours so their presence in smaller amounts is still important to the flavour profile of the cheese. There is potential for over-intense flavour if these fatty acids are present in larger quantities.

Lack of blue veining in Blue Stilton is the most significant cause of downgrading (Hough, 2001, pers. comm.) since the customer expects a cheese with even growth of the blue mould. However, although the downgrading is due to the visual appearance, the defects are wider ranging with a close, firm, curdy texture and a reduction in the typical Stilton flavour. Since *P. roqueforti* starter is routinely added to the milk the failure of the curd to blue must be due to some inhibition of the mould. This may be due to physical characteristics such as low oxygen tension or inhibitory a_w or may be due to the presence of anti-microbial substances.

1.3.7 Yeast species occurring in cheese

Like the NSLAB, yeasts are not generally included in the starters of most cheeses, although they are frequently found in the end product. Gobbetti *et al.* (1997) reported yeast counts of 7.5 log cfu g^{-1} in samples taken from the core and under the rind of Gorgonzola cheese. The most frequently occurring genera were *Torulopsis, Candida* and *Pichia.* In a study of the microbial succession in Bethlehem St. Nectaire cheese, Marcellino and Benson (1992) found that the yeast species *Debaryomyces, Torulopsis* and *Candida* grew from the early stages of

ripening. Fleet and Mian (1986) isolated yeasts from 23 Cheddar cheese samples with counts ranging from 10^1 to 10^6 g^{-1}. The most frequently isolated species in this study were *Candida famata* (38% of samples), *Kluyveromyces marxianus* (19%), *Candida diffluens* (14%), *Cryptococcus flavus* (8%) and *Saccharomyces cerevisae* (8%). The authors suggested that the reason for the high yeast counts in the cheeses sampled was the low pH of the product, since Ingram (1958) proposed that the existence of a low pH creates a selective environment for yeast growth.

Van den Tempel and Jakobsen (1998) investigated the yeasts associated with Danablu produced at four dairies in Denmark. Samples were taken from the raw milk, whey, brine, cheese curd before and after salting and cheeses were sampled at the surface and in the interior at 1, 2, 3 and 4 weeks of maturation at 10°C. The numbers of yeasts in the raw milk ranged from 10^2 to 10^4 ml^{-1}, with 37 isolates being identified. The predominant species were *C. famata, C. catenulata, C. lipolytica, C. krusei* and *Trichosporon cutaneum. C. famata* was also the predominant species in the brine (21-23% NaCl) samples (where 29 isolates were identified) which the authors attributed to its salt tolerance. High numbers of yeasts (10^5 – 10^9 g^{-1}) were also found in the cheese samples with 100 isolates from the four dairies. The predominant isolates changed over the maturation period with *C. famata, Zygosaccharomyces* spp., *C. lipolytica, C. catenulata* and *T. cutaneum* being significant after day 1. Between days 1 and 14 *C. famata, Zygosaccharomyces* spp., *C. lipolytica* and *C. rugosa* were most notable and after 28 days only *C. famata* and *C. catenulata* were present in significant numbers. Isolates were distinguished using fermentation profiles and selected isolates were further distinguished using API 32C and as a result biochemical variations were noted, particularly for *C. famata*. Table 1.6 shows the results of biochemical differentiation of three species of *Candida* found in the study. The authors concluded that the wide-ranging biochemical activity of *C. famata* suggested that it made a positive contribution to the maturation of the cheese and had potential use as a starter culture, but noted that some isolates of the species produced a brownish pigment.

Table 1.5 Assimilation patterns (in API-32C) for strains of *Candida famata*, *Candida catenulata* and *Candida lipolytica* (Van den Temple and Jakobsen, 1998)

Substrate	*C. famata*	*C. catenulata*	*C. lipolytica*
Galactose	20/20	8/8	0/7
Actidione	0/20	5/8	7/7
Sucrose	20/20	0/8	0/7
N-acetyl-glucosamine	17/20	6/8	7/7
DL-lactate	19/20	8/8	7/7
L-arabinose	13/20	0/8	0/7
Cellobiose	16/20	0/8	0/7
Raffinose	19/20	0/8	0/7
Maltose	20/20	4/8	0/7
Trehalose	19/20	3/8	0/7
2 aceto-gluconate	20/20	0/8	0/7
α-methyl-D-glucoside	20/20	0/8	0/7
Sorbitol	20/20	8/8	6/7
D-xylose	7/20	0/8	0/7
Ribose	2/20	0/8	1/7
Glycerol	18/20	6/8	7/7
Rhamnose	10/20	0/8	0/7
Palatinose	20/20	0/8	0/7
Erythritol	3/20	0/8	7/7
Melibiose	1/20	0/8	0/7
Glucuronate	13/20	0/8	0/7
Melezitose	20/20	0/8	0/7
Gluconate	13/20	2/8	5/7
Levulinate	4/20	0/8	0/7
Mannitol	18/20	8/8	6/7
Lactose	18/20	0/8	0/7
Inositol	1/20	0/8	0/7
Glucose	20/20	8/8	7/7
Sorbose	19/20	0/8	0/7
Glucosamine	17/20	7/8	5/7
Aesculin	9/20	0/8	0/7

Yeasts have also been isolated from brines used in cheese production: eighteen samples of brine with NaCl content of 2.6-5.0% w/v taken from six factories producing feta cheese were found to contain levels of yeasts ranging from 5.5 x 10^2 to 3.4 x 10^6 (Kaminarides and Laskos, 1992). A total of 180 strains of yeast were isolated from the following species: *Saccharomyces cerevisae* (64), *S. cervisae (italicus)* (41), *C. famata* (31), *Pichia membranaefaciens* (21), *C. sphaerica* (9), *Torulaspora delbrueckii* (5), *C. colliculosa* (4), *C. robusta* (2), *C. tropicalis* (1), *S. exiquus* (1) and *S. cerevisae (chevalieri)* (1) and the study concluded that the yeasts may play an important role in the preservation of Feta cheese. The NaCl content of the brines examined in this study was not restrictive, but it is known that several species of yeast, including *C. famata, C. catenulata* and *C. lipolytica* are able to tolerate NaCl contents of 10% and the former is able to tolerate 16% (Barnett, 2000).

Although yeasts are not generally considered to be detrimental to cheese quality, some species have been found to cause defects. A study by Nichol and Harden (1993) found that enzymic browning in Gorgonzola style blue cheeses may be attributable to yeast species, including *C. catenulata, C. famata* and *C. lipolytica.* In this study, browning was observed to occur in the region close to the spike hole and generally radiated 10-20 mm from that hole, whilst growth within the spike hole was noted to be pink and crusty. These brown areas were sampled and homogenised with 10 volumes of sterile 2% trisodium citrate and then diluted with 90 volumes of peptone saline. Incubation of drops of this dilution on Tryptone Soy agar enabled culture isolation and the isolates were subsequently grown on Tryptone Soy agar + 10% milk to detect browning. *Candida catenulata* was found to exhibit the most browning whilst three strains of *C. lipolytica* also produced slight browning. Two strains of *C. famata* were detected, although only one strain (which was not identified further) was found to produce slight browning. In the study by Van den Temple and Jakobsen (1998) mentioned earlier, brown pigments were produced by some isolates of *C. famata*, although the study did not identify these strains.

Browning in cheeses was also found to be attributable to the action of yeasts in a study by Carreira *et al.* (1998) when surface discolouration on ewe's milk cheese was investigated. In

this study browning was chiefly attributed to *Yarrowia lipolytica*, whilst *C. catentulata* was not found to cause browning. *Candida lipolytica* is the asexual state of *Y. lipolytica*, whilst *C. famata* is that of *Debaryomyces hansenii* (Barnett *et al.*, 2000).

Jakobsen and Narvhus (1996) indicated that yeasts may interact with other microorganisms in dairy products in three ways: they may inhibit or eliminate undesirable competitors; they may inhibit the starter culture; they may contribute positively to the fermentation. This review of the effect of yeasts on the quality of dairy products highlighted several studies where yeasts have inhibited the growth of other microorganisms and included the potential for *D. hansenii* to inhibit the germination of *Clostridium butyricum* and *C. tyrobutyricum*. More recently Arras *et al.* (1998) showed that strains of *C. famata*, *C. sake* and *Pichia guilliermondii* were able to inhibit the growth of the blue mould *P. italicum*. In this study, 19 yeast isolates recovered from the fruits of citrus, tomato, grape, fig apple and olive were added to artificially wounded citrus fruits and one hour later 25µl of a spore suspension of *P. italicum* (10^5 spores ml^{-1}) was added to the wound. After incubation at 20°C for 4-6 days, percentage inhibition was calculated from visual examination. Three out of three isolates of *Pichia guilliermondii*, 3/6 of *C. famata* and 1/1 of *C. sake* were able to reduce the incidence of infection by 86-98%. D'hallewin *et al.* (1999) also showed that *C. famata* strain 22D was able to inhibit the decay of "Star Ruby" grapefruit by *P. digitatum*. Artificially wounded fruit were inoculated with 20µl of a conidial suspension (10^6 spores ml^{-1}) of *P. digitatum* and treated with the yeast by dipping in water containing 10^6 cells ml^{-1} *C. famata* 22D for 3 minutes. Control of infection was significant when the yeast was applied within 36h of inoculation with the mould but delayed application of the yeast (36-72h post-inoculation) did not significantly reduce decay.

Yeasts are also considered to contribute positively to cheese ripening by lipolysis and proteolysis (Besancon *et al.*, 1992), aroma formation (Adda *et al.*, 1982) and production of gas which may assist the development of *P. roqueforti* in blue cheeses (Fox and Law, 1991).

1.3.8 Studies on the microflora of Cheddar cheese

A number of studies have focussed on the microflora of Cheddar cheese (Cromie *et al.*, 1987; Lynch, *et al.*, 1997; Lues, *et al.*, 1999; Shakeel-Ur-Rehman *et al.*, 1999), whilst there have been several investigations on the role of lactobacilli as flavour producers, which have been conducted using Cheddar as the carrier food. A literature search revealed no studies on the microflora of Stilton, although lipolysis in Stilton cheese (Madkor *et al.*, 1987a) and proteolysis in Stilton (Madkor *et al.*, 1987b) have been investigated as discussed earlier in this review.

Cromie *et al.* (1987) investigated the effect of elevated storage temperatures on the microflora of Cheddar cheese and found that in control samples of cheese made in a pilot plant and stored at 8°C, the total bacterial counts did not change during 32 weeks of ripening, but the lactobacilli increased from log 1.7 at week 0 to log 6.7 at week 8 and thereafter stayed at or around this level. The starter lactococci decreased from log 6.4 at week 0 to log 6 at week 8 and were not able to be differentiated after this time due to the growth of non-starter microorganisms. The study found that increasing the storage temperature above 8°C resulted in an increase in total bacterial count, total lactic acid bacteria, lactobacilli and undesirable lactobacilli but depended on continued storage at elevated temperatures. Undesirable lactobacilli were classed as those that produced off-flavours and gas but were not specified. The study did not find any difference in the numbers of non-lactic acid bacteria in elevated temperature storage, compared with the levels found in the control.

The changes in the non-starter microflora of Cheddar cheese were studied by Lues *et al.* (1999) who found that the total mesophilic count decreased rapidly from around 10^8 to 10^6 g^{-1} within 5 days and reached 10^5 g^{-1} at the end of maturing (96 days). The study also found that the lactobacilli increased rapidly from around 10^6 to10^8 g^{-1} in the first few days of ripening and then fell back to levels of around 10^6 g^{-1} by 20 days maturation, at which level they stayed until the end of maturing. Homofermentative lactobacilli followed a similar pattern as might be expected, since heterofermentative lactobacilli were not detected during the

duration of the study. Other contaminants, such as coliforms and Enterobacteriaceae, were not detected in this study either, although yeast and mould counts showed levels of around 10^5 g^{-1} at the start of ripening followed by a gradual decline in numbers to around 10^4 g^{-1} by the end of maturing.

The decrease in lactococci and increase in lactobacilli has been noted during many studies (Wilkinson *et al.*, 1994; Tornadijo *et al.*, 1995; Haque *et al.*, 1997), although Marcellino and Benson (1992) proposed that the main lactic acid bacteria isolated from Bethlehem St. Nectaire cheese matured over 60 days were *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* var. *diacetylactis*, which may suggest that the lactobacilli behave differently in this cheese. Haque *et al.* (1997) studied the change in populations of microorganisms during maturation of low fat and full fat Cheddar cheese from one day after manufacture to five months. At day one the bacterial count in both sets of cheese was similar, but by five months the count in the full fat samples was significantly higher than that in the low fat samples. Electron microscopy revealed that the lactococci were more often associated with the fat phase than with the protein phase. However, in all samples the lactococcal counts decreased over time and the lactobacilli increased starting at around 10^2 g^{-1} after 2 months of aging and reaching approximately 10^5 g^{-1} after 5 months.

A study by Clark and Reinbold (1967) investigated the low temperature microflora of 967 commercial Cheddar cheese samples and found that *Lb. casei* and *Lb. plantarum* were the most commonly occurring lactobacilli. They suggested that lactobacilli began to predominate after the lactose supply was depleted and that this coincided with the reduction in numbers of streptococci. Fitzsimons *et al.* (2001) found a mixture of *Lb. paracasei, Lb. plantarum, Lb. rhamnosus* and unidentified strains in Cheddar cheese up to 6 weeks of maturation and thereafter only found *Lb. paracasei* strains. This study also found lactobacilli occurring at levels of approximately $10^7 g^{-1}$ after 8 weeks of ripening and $10^8 g^{-1}$ after 17 weeks. However, they found no difference in spatial distribution of the strains across the block of cheese, although there was a difference in microflora of different cheeses in that one of the three samples tested had no dominant strain whilst the others showed two dominant strains.

Whilst this suggested that individual cheeses had their own microflora, samples were from different production batches and so it was not known whether this extended to cheeses from the same batch.

The microflora of Cheddar cheese, made with sodium chloride, potassium chloride or mixtures of sodium and potassium chloride, was examined by Reddy and Marth (1995) and no significant difference was found between samples in terms of total aerobic microorganisms, LAB, NSLAB, aerobic spore formers, coliforms and yeasts and moulds. The aerobic plate count decreased over the 36 week ripening period from $10^9 g^{-1}$ to $10^7 g^{-1}$, a change that was also reflected in the LAB count. The NSLAB increased during the first 4 weeks from 10^4 –10^7 and then remained fairly constant until 24 weeks, thereafter declining to 10^5 by week 36. However, the NSLAB were not identified to species level. The numbers of coliforms observed during the study had generally reduced to <1 or <10 g^{-1} after 12 weeks (depending on the method of assay, <10 representing plate count and <1, tube method). Initial coliform counts were in the region of 10^1-$10^2 g^{-1}$.

It is generally thought that the NSLAB contribute to the flavour of mature cheese and early studies focussed on this aspect (Naylor and Sharpe, 1958b; Marth, 1963). More recently, investigations have concentrated on the possible addition of NSLAB as secondary starters for flavour enhancement or accelerated ripening in Cheddar cheese. Laleye *et al.* (1990) added strains of lactobacilli isolated from Cheddar cheese to milk with a commercial starter culture to produce Cheddar cheese and found variations in quality attributes between species. The study showed that addition of heterofermentative lactobacilli (*Lb. brevis, Lb. fermentum*) resulted in the production of fruity flavours and consequently caused the cheeses to be down-graded in terms of quality. The flavour defects were noticeable when grading at two months and were accentuated with ageing (at either 7 or 15°C) to ten months. The combined use of homofermentative species (*Lb. plantarum* and *Lb. casei-casei*) and heterofermentative lactobacilli produced higher quality cheeses after two months, with these cheeses being classed as first grade. However, the use of the homofermentative strain *Lb. casei-pseudoplantarum* (137-10/62) resulted in second grade cheese at the same age. After

10 months ripening there was no evidence of improved flavour in the cheeses made with added lactobacilli over the control cheeses that contained no added lactobacilli.

A study by Lee *et al.* (1990a) demonstrated that the addition of homofermentative strains of lactobacilli (*Lb. casei* subsp. *casei, Lb. casei* subsp. *pseudoplantarum* and *Lb. plantarum*) resulted in accelerated ripening accompanied by an increased rate of proteolysis. Cheddar cheese was manufactured with and without the addition of homofermentative strains and matured at either 7 or 15°C. Results showed that the numbers of lactobacilli were strongly influenced by storage time and temperature, supporting the study of Cromie *et al.* (1987) described earlier. The lactobacilli dominated the total microflora during ripening irrespective of whether or not they were added to the milk for manufacture and reached a maximum at about four months. However, the addition of lactobacilli with the starter bacteria enabled the normal dominant lactobacilli to become established earlier. The numbers of lactobacilli, streptococci and total bacteria were always higher in cheeses aged at 7°C than those at 15°C and it was noted that easily utilised nutrients such as sugar and organic acids were depleted faster at the higher temperature and therefore limited growth to a greater extent. The study also showed that the added lactobacilli contributed to proteolysis, as detected by soluble nitrogen, since in control cheeses proteolysis did not reach a maximum during the 10 months of the study, whereas in cheese with added lactobacilli, a maximum was reached in 8 months. It was suggested that since this maximum coincided with a noticeable decline in the number of lactobacilli, the lysis of cells was responsible for releasing proteolytic enzymes.

A further study by Lee *et al.* (1990b) demonstrated that the addition of specific strains of homofermentative lactobacilli to milk for cheese may overcome problems of open texture and off-flavour development. In this study 22 vats of cheddar cheese were compared and cheese with *Lb. casei* subsp. *casei* 119-10/62 and *Lb. casei* subsp. *pseudoplantarum* 137-10/62 added to the normal lactococcal starter were considered better than those without lactobacilli addition. It was further noted that other strains of the same species produced less desirable cheese.

Proteolysis in Cheddar cheese was investigated in a study by Lynch *et al.* (1997) when cheeses were manufactured with or without starter culture, with the addition of glucono-δ-lactone as an acidulant in those cheeses made without starter bacteria. Strains of lactobacilli (*Lb. casei* subsp. *casei, Lb. casei* subsp. *pseudoplantarum, Lb. plantarum* and *Lb. curvatus*) were added to one chemically acidified and one starter-acidified cheese before the addition of the rennet and the cheeses were measured for lactobacilli, total bacterial count, coliforms, composition and sensory properties as well as for proteolysis. The levels of water soluble nitrogen (WSN) and free amino acids (FAA) were greater in those cheeses manufactured with the addition of starter culture, although there was an increase in the FAA noted in both the chemically acidified and starter acidified cheeses when the *Lactobacillus* adjunct culture was used. It was concluded that the starter bacteria made a major contribution to proteolysis but that the NSLAB brought about an intensification of flavour.

The proteolytic capabilities of lactobacilli were further investigated by Muehlenkamp-Ulate and Warthesen (1999) by incubating Cheddar cheese slurries at 30°C for 12 days under anaerobic conditions. Fresh Cheddar curds were made using *L. lactis* subsp. *cremoris* and strains of four species of lactobacilli (*Lb. curvatus, Lb. paracasei, Lb. casei, Lb. helveticus*) were added to the slurries. Measurement of peptide profiles and amino acids by HPLC , in the slurries post-incubation, revealed that proteolysis was greater in those with added lactobacilli and that there was a difference in influence between strains. Higher levels of total free amino acids were detected in slurries with added lactobacilli, with *Lb. helveticus* and *Lb. casei* strains exhibiting greater influence than *Lb. curvatus* and *Lb. paracasei.*

It can be seen that the microflora of Cheddar cheese is complex and ripening is strongly influenced by the non-starter microflora. This non-starter microflora consists mostly of NSLAB, in particular lactobacilli, and within these lactobacilli, the effect of strains is varied. Research suggests (Clark and Reinbold, 1967; Fitzsimons *et al.*, 2001) that *Lb. paracasei, Lb. plantarum* or *Lb. casei* predominate the lactobacilli after several weeks of maturing, but it is clear that the microflora is dynamic and that many species and strains are present during

the ripening period, each of which may contribute to the flavour of the product. However, a study by McSweeney *et al.* (1993) showed that the indigenous microflora of milk affects the quality of Cheddar cheese made from raw milk. In this study, Cheddar cheeses were made using pasteurised, raw and microfiltered (MF) milk and were assessed for proteolysis, lipolysis, sensory quality and microbiological content. Microfiltration was found to be a very effective method of reducing the indigenous microflora of the milk, whilst pasteurisation was also efficient, although less so. Sensory assessment showed the cheeses produced from MF and pasteurised milk to be similar and more acceptable than those of the raw milk. Proteolysis and lipolysis measurements also showed similarities between the cheeses made from the treated milks, whilst there were differences between these and the raw milk cheeses which were attributed to the contribution of the NSLAB. This NSLAB flora was dominated by lactobacilli although again there was a difference in the cheeses, with the raw milk cheeses containing *Lb. casei* subsp. *casei, Lb. casei* subsp. *pseudoplantarum,* and *Lb. curvatus* whilst the cheeses made from MF and pasteurised milks contained mainly *Lb. casei* subsp. *casei.*

A study by Yiu (1985) used fluorescence microscopy to examine cheese microstructure in particular, but also to examine the microflora. Samples of several varieties of cheese, including Cheddar, Mozzarella, blue cheese (variety not stated), Brie and Camembert, were sectioned at –20°C to a thickness of 3-6 μm and stained with acridine orange and acriflavine (separately). Microscopic examination of sections of Camembert taken from the ripe area just below the surface of the cheese, revealed a few fungal hyphae penetrating the protein matrix. Mention was not made of the presence of hyphae in sections taken from the centre of the cheese, where ripening had not progressed, so it may be assumed that the hyphae had not extended to this area at the stage at which the cheeses were sampled. The lactic acid bacteria were studied in Cheddar and Mozzarella cheeses and it was noted that the lactococci were seen as orange–red bead like structures, whilst the lactobacilli, formed well defined colonies along the cracks of the casein matrix. The technique of cryosectioning followed by direct staining was also used to examine microbial growth *in-situ* in a number of foods in studies by Dodd and Waites (1991) and Dodd and Waites (1992). In these studies

toluidine blue was used as a stain mountant and the section was then covered with a coverslip and viewed mounted in the stain in order to minimise disruption of components. Microscopic examination at x100 and x400 magnification enabled microorganisms to be viewed as purple cells, whilst the food components stained different colours depending on their composition. For example in the former study, muscle tissue in fermented fresh sausage stained pale blue, green or yellow enabling the bacterial cells to be distinguished easily and bacteria were clearly seen at the junctions of curd granules of Cheddar type cheeses.

1.4 Conclusions

In summary, studies have revealed that the microflora of the varieties of cheese investigated are a complex mix of starter and non-starter bacteria, yeasts and moulds. The origin of non-starter microorganisms may be the raw material, with subsequent survival of pasteurisation, although equally some microorganisms have been shown to originate from the factory environment. Studies have demonstrated that these microorganisms play an important role in the quality of the product in terms of formation of typical or atypical flavour compounds resulting in either acceptable or unacceptable product depending on the species encountered. Furthermore, the activity of some species has been shown to result in textural defects, whilst others are considered to be important to the production of desirable textural qualities through proteolysis. Finally some microorganisms, in particular the lactic acid bacteria, have been shown to produce anti-microbial substances.

The current study is aimed at investigating the activity of the microflora in the production of blue Stilton cheese in order to determine whether there are similarities with other cheeses and to examine whether or not there is a relationship between the bacterial and fungal flora which may influence the quality of the cheese in terms of development of the characteristic blue veining. Studies on the flavour compounds present in poor and good quality Stilton cheese were also carried out.

CHAPTER 2

2 Materials and Methods

2.1 Sampling of cheeses

Samples of cheese were supplied by Long Clawson Dairy, Long Clawson, Leicestershire and were mature, usually whole or half cheeses, which enabled careful selection of the sample point to avoid external contamination. Samples from the blue veined areas of cheeses were all taken from within 1.5mm of the veins, whilst those from the white areas were taken to avoid contamination with blue veins. Both good quality cheeses, exhibiting ample blue veining, and poor quality cheeses, which were deficient in blue veins, were sampled at random times of the year.

2.2 Measurement of Total Viable (aerobic) Count

A sample (10g) was aseptically added to 90ml of Oxoid maximum recovery diluent (CM733, Oxoid, Basingstoke, U.K.) and blended in a Colworth stomacher (Seward Laboratory, Bury St. Edmunds, England) for one minute. Further dilutions were made to 10^{-6} and 10^{-7} and 1ml aliquots of these were plated out in Oxoid milk agar (CM 21) (Appendix 1). After incubating for three days at 30°C all colonies were counted and recorded as a count per gram.

2.3 Measurement of Total Viable *Lactobacillus* Count

One ml aliquots of 10^{-6} and 10^{-7} dilutions were also plated out using Oxoid De Man Rogosa Sharpe (MRS) agar (CM 361) (Appendix 1). The pour plate technique was used and the agar was overlaid with a second layer of agar. After incubating in anaerobic conditions, produced using the Merck Anaerocult (Merck KGaA, Germany) system in an anaerobic jar for three days at 30°C, all colonies were counted and recorded as a count per gram. Anaerobiosis was confirmed using Anaerotest (Merck KGaA, Germany) test strips which are blue in the presence of oxygen and turn white in anaerobic conditions.

The colonies were confirmed as *Lactobacillus* by the catalase test by adding 10% v/v hydrogen peroxide to samples of colonies on slides and examining for effervescence. No effervescence indicated a negative catalase reaction and the colonies were presumed to be *Lactobacillus* species.

2.4 Identification of *Lactobacillus* species

Colonies were further identified using the Gram stain and the appearance of the cells was recorded, taking note of the cell morphology and evidence of granulation. Subsequently the species was identified using the API 50 CHL system (Biomerieux, Lyon, France) and the results faxed to API for identification. The biochemical tests included in this system are listed in Appendix 4.

2.5 Total coliform count

One ml aliquots of dilutions prepared as in 2.2 were plated out using the pour plate technique, in Violet Red Bile Lactose Agar (VRBA) (Oxoid CM107). Once set the agar was overlaid with approximately 5 ml of agar to promote micro-aerophilic or anaerobic conditions. The inverted petri dishes were incubated for 24 hours at 30°C and then enumerated. Typical coliform colony formation on this agar is of purple colonies up to 0.5 mm in size and often elliptical in shape. This test was discontinued after results were negative on all samples in the early stages of the investigation.

2.6 Total yeast and mould count

One ml aliquots of the serial dilutions previously prepared as described in 2.2 were plated on Rose Bengal Chloramphenicol Agar (RBCA) (Oxoid CM549) or on Sabouraud Dextrose agar (SDA) (Oxoid CM41, Appendix 1) using the surface spread plate technique. Initially, RBCA was used, but SDA was found to be less restrictive, giving a higher count of organisms and was used in the later experiments. Plates were incubated at 22°C for 5 days and examined for the presence of yeasts and moulds. The moulds were then further examined

microscopically in order to perform preliminary identification by examination of the asexual spore formation. The genus *Penicillium* produces conidiospores, which are typically blue or green in colour. On batches where the growth of blue mould was poor typical yeast colonies were selected and identified using the API 20C AUX or API ID32C kit. The biochemical tests included are shown in Appendix 3.

2.7 Anaerobic yeast count

Where yeasts had been found to contaminate the MRS plates, an anaerobic yeast count was performed on SDA (Oxoid, CM41). This was done by preparing a pour plate as previously described and, once the agar was set, overlaying with a second layer of agar in order to encourage deep growth. The petri dishes were then incubated under anaerobic conditions as in 2.3.

2.8 Gram stain

The Gram stain (Harrigan & McCance, 1966) was carried out as follows:

A heat fixed smear was prepared by adding a drop of water to a clean slide and then aseptically transferring a small amount of typical culture to the slide. This was mixed thoroughly and allowed to air dry before passing the slide three times through a bunsen flame to heat fix.

The primary stain, crystal violet or methyl violet, was added to the fixed smear and allowed to soak for 1 minute. This was then rinsed off with water and the slide flooded with Gram's iodine and left for 30 seconds before rinsing with water. Alcohol was added and the slide rocked gently to and fro for 1 minute to remove the stain. Following further rinsing, safranin was added and left for one minute before rinsing with water. The slide was carefully blotted dry on white tissue and examined under the microscope.

Gram-positive organisms appear purple as they retain the primary stain whilst Gram-negative organisms appear pink as they lose the primary stain and take up only the pink counterstain. It is possible to see Gram variable reactions, with a mixture of Gram positive and Gram negative cells, although this is rare in lactobacilli (Kandler and Weiss, 1986). In addition, some members of the genus *Lactobacillus*, especially the homofermentative species, exhibit granulation whereby the cell appears mottled (Kandler and Weiss, 1986).

2.9 Identification of *Candida famata*

Suspect colonies of *Candida famata* were selected from petri dishes and streaked onto SDA or RBCA. These were incubated at 22°C for five days and then identified using the API ID32C or 20C AUX systems and the results faxed to API for identification. The API 20C AUX system was used for early detections and the ID32C for confirmation where results were ambiguous and for later identifications as the results were more consistent using this system.

2.10 Detection of inhibitory substances

Two methods were used to detect the presence of inhibitory substances produced by *C. famata*.

2.10.1 Zone of clearing around a stab inoculation

This was carried out on RBCA by preparing a petri dish of agar onto which 0.5 ml of *P. roqueforti*, selected from a cheese sample (after incubation at 22-25°C for 5 days), was spread using a sterile spreader. This was allowed to dry and then *C. famata* was inoculated by stabbing the agar with a loop of culture in four places. Samples were incubated either aerobically or anaerobically (using the system described in 2.3) for 5 days at 22-25°C before being examined for clear zones in the *P. roqueforti* around the stabs.

2.10.2 Well plate technique

This technique was carried out on SDA using several variations of technique. In the first instance petri dishes were prepared with 20 – 25 ml agar, which was allowed to dry. *P. roqueforti* suspension (0.5 ml) was then spread onto the agar and allowed to dry. Wells of agar were removed from the plates using a 3mm cork borer sterilised in methylated spirits with the excess being burned off before cooling.

Four wells were prepared on each plate and were inoculated with a culture of *C. famata* prepared in MRD at a concentration equivalent to 9.3×10^6 per ml. This was assessed by the plate count method prior to commencing the inhibition tests. Volumes of sample ranging from 0.1 ml to 50µl were inoculated initially in order to assess the most appropriate volume with 50 µl being finally selected. The wells were therefore inoculated with 9.3×10^5 to 4.65×10^3 cells, with the latter concentration being used in most experiments. Samples were then incubated aerobically and/or anaerobically as before and were examined for clear zones around the wells on each of the five days of incubation.

This technique was repeated using SDA which had been prepared by adding the *P. roqueforti* to the base of the petri dish before adding the agar and then mixed as a pour plate. This reduced the available oxygen in order to attempt to mirror conditions within cheese more closely. It also simplified the technique as the drying of the agar was quicker. Further repetitions were made using SDA with sodium chloride added to a level of 2% (w/v) to simulate the conditions in the cheese and again using SDA with sterile milk added, as a growth supplement, to a level of 5% (v/v).

A further series of inhibition tests were carried out by reversing the procedure ie by allowing the *C. famata* to grow before inoculating the wells with *P. roqueforti*. This was carried out in order to simulate the situation in cheese where the yeast may grow better in the conditions of low oxygen tension before the first skewering after which point the

mould should be encouraged to grow and sporulate. This may be especially significant in close textured cheese.

The technique was further refined by the use of contact plates instead of petri dishes. These had two advantages

- They enabled deeper wells to be prepared, since the plates can be poured with a raised meniscus and thus inoculation of the wells was made easier
- The plates were marked in cm^2 enabling easier measurement of zones of inhibition.

2.11 Preparation of cryogenic sections of cheese

Samples of cheese were frozen at –20°C and then 10μm sections were taken using cryosectioning as described by Dodd and Waites (1992). The sections were prepared by reducing the sample to –20°C in a Bright cryostat model OTF/AS with the chamber temperature at –25°C. The cutting temperature varied between –15 and –20°C.

Sections were stained using toluidine blue, using the method described by Flint and Firth (1988). The stain was applied directly, as a stain mountant, to the section on a microscope slide, left for approximately one minute and then covered with a cover slip. Excess stain was drawn off after a further minute and the samples were examined microscopically using a Nikon Optiphot light microscope with the overall magnification at x 100 and x 400 and photographed using a Nikon digital camera. Toluidine blue is a basic dye which stains bacterial cells blue with the acid curd staining yellow. Fat does not stain (Flint and Firth, 1988) and also appears yellow.

2.12 Microscopic examination using a simple methylene blue stain

As a comparison with the cryogenic sections described in 2.11, a culture of *P. roqueforti* taken from a cheese sample was examined microscopically, by suspending a loopful of culture in MRD and then applying a loopful of this suspension to a slide. After air-drying and heat fixing, this was stained using 1% methylene blue in a manner similar to above. A cover slip was placed over the stain and the excess methylene blue was removed using tissue. The same method was employed to examine a culture of *C. famata* taken from a cheese sample, in order to determine the method of asexual division. This process allowed comparison of the microscopic appearance of these species with the results obtained by cryosectioning and also enabled comparison of the size of the yeast cells with that of the conidiospores of the mould. Slides were examined at x 100, x 400 and x 1000 overall magnification using a Leica light microscope. This technique was used for simple identifications only, for example, when the size of cells was to be compared or for differentiation into rods and cocci on TVC plates.

2.13 Measurement of pH of the samples

The pH of the cheese samples was measured by direct contact. The pH meter (Jenway 3305, Jencons Scientific) was calibrated using pH 4 and pH 7 buffers (Prime Chemicals, Rotherham, UK) prior to use and the protective guard removed from the electrode, which was then inserted directly into the cheese at the point of sampling. This enabled more specific measurement of pH in either the blue or white areas of the cheese. The electrode was carefully washed between samples using distilled water and re-calibrated after every five samples.

2.14 The preparation and use of exposure plates

In order to determine whether or not *C. famata* was present in the atmosphere of the dairy and thus was able to enter the curd post-pasteurisation, exposure plates were placed in six

ripening rooms plus the vat room. The plates were prepared using RBCA and were exposed for a period of 20 minutes before being collected and incubated aerobically at 22-25°C. The rooms in which the plates were exposed were:

Vat room

Hastener

White room

Blue rooms 3, 4 and 6

The stage of cheese ripening in these rooms can be seen in Figure 1.1, whilst Figure 2.1 shows the ground floor layout of the dairy in order to demonstrate the positioning of the rooms relative to one another and the product flow through the dairy. Exposure plates were positioned in the centre of each room (blue rooms 3, 4 and 6 are on the first floor).

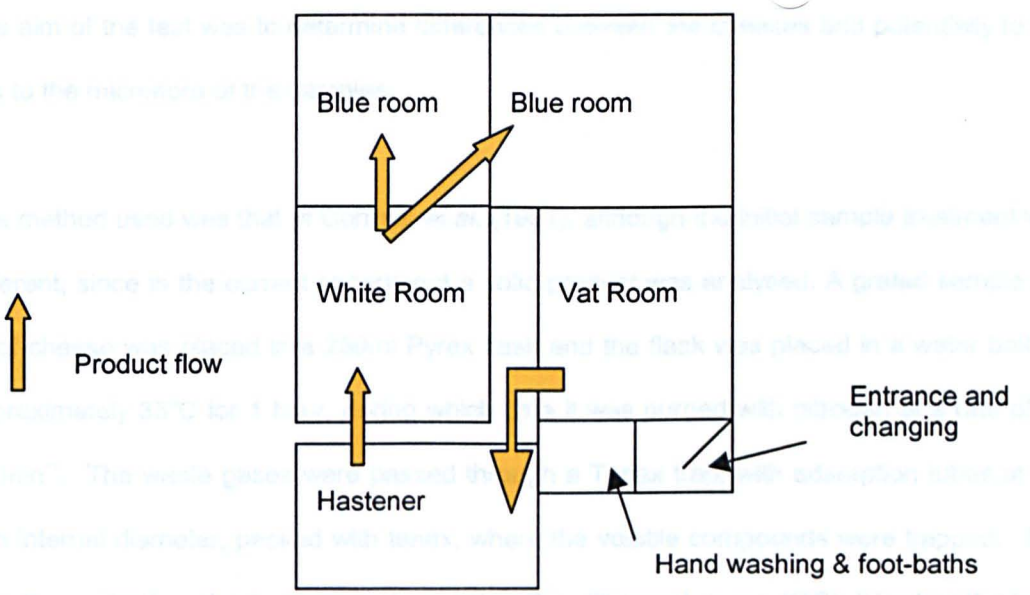


Figure 2.1 Schematic diagram of ground floor layout of dairy

(not to scale, other maturing rooms are on the first floor).

2.15 Statistical analysis

The Student's t-test was used to perform statistical analysis and was carried out using Minitab 13.30 on Microsoft Windows 2000. For analysis of individual experiments either the 2 tailed, paired t-test was used or the 2 tailed, 2 sample t-test depending on the relationship of samples to each other, but for analysis of all the data only the 2 tailed, 2 sample t-test was used, since the samples could not be directly related to each other.

2.16 Identification and olfactometry of volatile compounds in the headspace

This was carried out in conjunction with Dr Christian Salles, on samples of both good quality Stilton cheese and poor quality cheese, which exhibited little development of the blue veins. The aim of the test was to determine differences between the cheeses and potentially to link this to the microflora of the samples.

The method used was that of Cormier *et al.* (1991), although the initial sample treatment was different, since in the current experiment a solid product was analysed. A grated sample (15 g) of cheese was placed in a 250ml Pyrex flask and the flask was placed in a water bath at approximately 35°C for 1 hour, during which time it was purged with nitrogen at a rate of 50 ml min^{-1}. The waste gases were passed through a Tenax trap, with adsorption tubes of 3.0 mm internal diameter, packed with tenax, where the volatile compounds were trapped. This was then introduced into the injector of the Gas Chromatogram (GC) (Hewlett Packard, model 5890 II) where the volatiles were thermally desorbed and then separated on a DB1 GC column (30m).

The separation conditions used were 5 minutes at 30°C followed by a temperature gradient from 30°C to 240°C at 5°C per minute and finally a plateau at 240°C for 5 minutes. The flow at the end of the column was divided so that around 33% was directed to the mass

spectrometer, model MD800 (Fisons Instruments, UK), and around 66% to the sniffing port of the olfactometer. One panellist was used to assess odour intensity and to describe odours.

CHAPTER 3

3 Results of tests to determine changes in the microflora and pH of Stilton curd during ripening

In order to determine the change in the microflora of Stilton curd during the manufacturing and ripening process, a vat of milk and resultant cheese, produced at a Stilton cheese manufacturing creamery, was tracked and sampled on days 1, 5, 26, 35, 48 and 56 days. These samples corresponded to the following:

Day	Sample source
0	Milk from vat, before addition of starters and rennet
1	Curd at milling
5	Curd from hastener (20°C)
26	Early ripening
35	Pre-skewering
48	Between skewering 1 and 2
56	Mature cheese

Samples were tested for pH, total viable count on milk agar, total *Lactobacillus* count on MRS agar, and total yeasts and moulds on SDA. Ten percent of colonies growing on the SDA plates were isolated and identified using the API ID32C system. Similarly a sample of colonies growing on the MRS plates were confirmed as lactobacilli using the Gram reaction and catalase test and then identified using API 50 CHL. Results (Table 3.1) are means of two samples and the range is shown in order to indicate the relationship between the data sets.

Table 3.1 Results of tests to determine changes in the microflora during ripening

		cfu g-1									
Sample no.	Sample	TVC		*Lactobacillus*		Yeasts		Moulds		pH	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
1	Milk 20/6/01	5.3 E2	1 E2	1.0 E0	7 E0	0 E0	0	0 E0	0	6.6	0.15
2	Curd day 1	2.0 E9	7.9 E8	3.0 E2	1 E2	1.0 E3	0	6.8 E3	1.6 E3	4.5	0
3	Day 5	7.2 E9	8.0E8	3.0 E2	1.0 E2	1.0 E3	0	6.0 E3	1.0 E3	4.5	0
4	Day 26	2.0 E6	2.0 E6	3.0 E6	4.0 E6	4.0 E6	2.0 E6	<1.0 E6	0	5.1	0.1
5	Day 35	1.8 E7	6.0 E7	2.1 E7	6.0 E7	1.2 E7	4.0 E7	1.0 E6	0	5.29	0.18
6	Day 48	3.5 E7	8.0 E7	2.5 E7	1.0 E7	5.0 E7	2.0 E7	2.0 E6	2.0 E6	5.4	0.15
7	Day 56	4.3 E7	4.0 E7	2.5 E7	6.0 E7	2.0 E7	4.0 E7	9.0 E6	2.0 E6	5.8	0.17
8	MILK 11/7	1 E3	0	8 E0	0	0 E0	0	0 E0	0	6.6	0.16

The results of these tests (Table 3.1) showed similar trends to previous studies on other types of cheese (Naylor and Sharpe, 1958b; Tornadijo, 1995; Wilkinson *et al.*, 1994). The TVC of the curd samples declined over time from 2.69 x 10^9 at day 1 to 2 x 10^6 at day 26, during which time the *Lactobacillus* count (TLC) increased from 3 x 10^2 to 3 x 10^6. Figure 3.1 demonstrates these results graphically and can be compared with Figure 1.8 in Chapter 1, in that in both graphs the TVC shows an initial increase followed by a decline and thereafter this count remains at approximately the same level. The NSLAB (or lactobacilli in Figure 3.1) increase during ripening to approximately the same level as the TVC. Thus Figure 3.1 shows that Stilton, a typical blue cheese, exhibits microbial succession similar to Cheddar type cheeses.

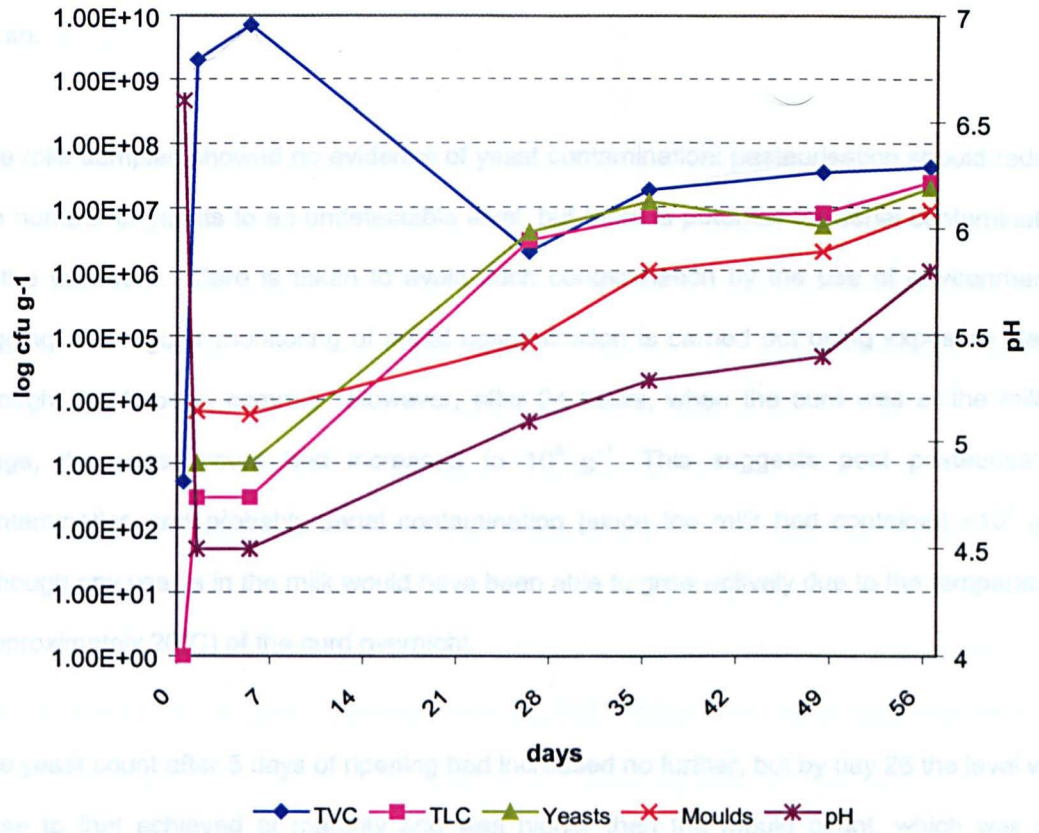


Figure 3.1 Change in microflora and pH of Stilton cheese during ripening

There was no detectable mould count in the milk sample, which was expected given that this was taken pre-inoculation. It was expected that the mould count in the curd would be in the region of 10^4 g^{-1}, since the inoculation rate was 1g/1200 litres milk, which is equivalent to 2.4

x 10^3 cfu ml^{-1} of milk. Experiments from a previous, unpublished, study by the author, using 350 litres of milk to make Stilton on pilot plant, had yielded mould counts of 10^4 g^{-1} of milk with lactic and *P. roqueforti* starters added, so it was expected that the level of moulds would be of this order. In fact, it was slightly less than this at 6.8 x 10^3 g^{-1} on day 1 and no significant increase was detected after 5 days of ripening. Furthermore, it was anticipated that significant growth would have occurred by 26 days of age (of the curd), to give levels close to the final expected count. Curd is pierced at 42 days after which sporulation is expected to take place and thus it had been expected to find significant levels of the mould. However, it is possible that there was some mycelial growth, but insufficient to be detected and that the levels of mould found in the cheese post piercing are due to the presence of large numbers of spores rather than representing a further increase in mycelial growth. Microscopic examination of a section of curd, as discussed in section 4.5.1, revealed this to be so.

The milk samples showed no evidence of yeast contamination: pasteurisation should reduce the number of yeasts to an undetectable level, but there is potential for aerial contamination in the vat room. Care is taken to avoid such contamination by the use of environmental fogging and regular monitoring of aerial contamination is carried out using exposure plates (Hough, 2001, pers. comm.). However, after 24 hours, when the curd was at the milling stage, the yeast count had increased to 10^3 g^{-1}. This suggests post pasteurisation contamination and probably aerial contamination (since the milk had contained $<10^1$ g^{-1}), although any yeasts in the milk would have been able to grow actively due to the temperature (approximately 20°C) of the curd overnight.

The yeast count after 5 days of ripening had increased no further, but by day 26 the level was close to that achieved at maturity and was higher than the mould count, which was still undetectable at the selected dilution. However, Van den Tempel and Jakobsen (1998) found levels of yeasts in the region of 10^5 to 10^9 g^{-1} in Danablu cheeses of 1, 2, 3 and 4 weeks of age produced at four dairies, so counts of the order of 10^4 would not have been unlikely in the current study.

Lues *et al.* (1999) found that the yeast and mould counts in Cheddar cheese decreased gradually from 10^6 cfu g^{-1} at day one to 10^4 cfu g^{-1} at the end of ripening, although it is not clear whether the cheese was made from pasteurised milk. Gobbetti *et al.* (1997) also found yeasts at levels of approximately 10^5 cfu g^{-1} on day one of ripening in Gorgonzola cheese, although this study noted an increase during ripening to yield levels of around 10^8 g^{-1} by day 86 but again it is not clear whether or not the cheese was made using pasteurised milk. Fleet and Mian (1986) found yeast levels ranging from 10^1 to 10^6 in 23 samples of Cheddar cheese purchased at retail outlets, although this study did not investigate the levels at the beginning of ripening. Other studies have shown similarly high levels of yeasts at the end of ripening of blue and other mould ripened cheeses (Gobbetti *et al.*, 1997; Van den Tempel and Jakobsen, 1998). Fleet and Mian (1987) reviewed the occurrence of yeasts in cheese and suggested that the highest yeast populations probably occurred in softer cheeses, including blue cheeses.

The second milk sample (sample 8) was taken after the results of the first sample were received, in order to try to enrich the sample to determine the types of lactobacilli present. An initial count was taken prior to enrichment, to compare the sample with sample 1 and it was found that this sample had a much higher TVC than sample 1. Furthermore, eight colonies, all of the same colony characteristics, grew on the MRS plate and it was deemed unnecessary to enrich the sample further to determine the species of *Lactobacillus* present. Two of these, exhibiting colony characteristics identical to the remaining colonies on the plate, were tested using the API 50CHL test strip and both were found to be *Lb. acidophilus*. There is no documented evidence to suggest that this species has been found in samples of mature cheese. It has been suggested (Hough, 2001 pers. comm.) that there may be a link between the age of the milk used for manufacture and the incidence of poor quality cheese. It is possible that keeping the raw milk for greater than 24 hours allows the growth of some species of yeast and/or lactobacilli. These may then be responsible for the inhibition of the blue mould either through survival of pasteurisation and subsequent production of anti-microbial substances, or by the production of heat stable anti-microbial substances prior to pasteurisation.

Many studies have revealed the anti-microbial properties of lactobacilli (Niku-Paavola *et al.*, 1999; Boris *et al.*, 2001; van den Guchte *et al.*, 2001) and these include anti-mycotic effects. El Gendy and Marth (1981) investigated the potential anti-mycotic action of *Lb. casei* against *Aspergillus parasiticus* and found that when the fungus was added to a 3 day old culture of *Lb. casei* growth of the fungus was reduced. Frank and Lee (1988) suggested that the cheese microflora was able to produce anti-mycotic substances, when eight of twelve retail cheese samples tested against *P. crysogenum* on potato dextrose agar were found to exhibit some antifungal activity. These samples all slowed growth of the test mould during the first 3 days of incubation, but only one sample slowed growth over the seven days of the trial. When the trial was repeated using sterile skim milk inoculated with cheese sample and incubated before adding to potato dextrose agar, six samples produced some mould inhibition and three of these exhibited marked inhibition, although this was not quantified.

It is highly possible for the lactobacilli to have survived pasteurisation, since Jordan and Cogan (1999) found that *Lb. paracasei* DPC2103 had the potential to survive pasteurisation temperatures. The study found that at 50 and 55°C there was no reduction in cell numbers even after 2 hours, but after 15 minutes at 60°C a linear reduction occurred and D-values (the time, in minutes, for a reduction in numbers of 1 log cycle to occur) of 22.5 ± 2.81 at 60.0°C, 3.71 ± 0.77 at 65.0°C and 0.32 ± 0.08 at 67.5°C were calculated. Two other strains, *Lb. plantarum* DPC2102 and *Lb. plantarum* DPC1919, did not exhibit such heat resistance. All strains were isolated from Irish Cheddar cheese samples. The heat resistance of *Lb. acidophilus* is not known, but fermentation by the species results in the production of ethanol, diacetyl and acetone as well as lactic acid and it is also known to produce other anti-microbial agents (Ouwehand, 1998). The heat resistance of anti-microbial substances produced by *Lb. plantarum* VTT E-78076 was demonstrated in a study by Niku-Paavola *et al.* (1999) when the antimicrobial activity of the culture filtrate was unaffected by heating at 120°C for 15 minutes. It seems highly possible that the growth of lactobacilli in the raw milk could result in the presence of antimicrobial substances in the product. Whether or not these may have an effect on the growth of the blue mould, *P. roqueforti*, and hence on product quality, has yet to be established.

Lb. acidophilus was not detected in curd samples during this experiment and appears to have been quickly out-competed by other species of lactobacilli. The colonies detected on days one and five were all found to be *Lb. casei*, whilst the sample taken on day 26 yielded *Lb. casei* (1/3) and *Lb. plantarum* (2/3). The *Lactobacillus* counts of the samples taken on days 35, 48 and 56 were of a similar order and four presumptive *Lactobacillus* colonies were selected from each sample, in order to determine the species present. *Lb. plantarum* was found on each sample (3/4; 2/4; 3/4), whilst the remaining colonies tested were found to be *Lb. curvatus* (0/4; 1/4;1/4)and *Lb.casei* (1/4;1/4; 0/4). This suggests that *Lb. plantarum* becomes the dominant species during the ripening of the cheese, whilst *Lb. casei* gradually becomes less abundant.

Table 3.2 Species of *Lactobacillus* isolated during ripening of Stilton cheese

Day	Species
Milk	*Lb. acidophilus*
1	*Lb. casei* (3/3)
5	*Lb. casei* (3/3)
26	*Lb. casei* (1/3); *Lb. plantarum* (2/3)
35	*Lb. plantarum* (3/4); *Lb. casei* (1/4)
48	*Lb. plantarum* (2/4); *Lb. curvatus* (1/4); *Lb. casei* (1/4)
56	*Lb. plantarum* (3/4); *Lb. curvatus* (1/4)

The yeast species isolated most frequently from these samples was *C. famata*, with this being the sole species isolated from the samples taken on days 1,5, and 26. By day 35 *C. sphaerica* was also detectable (1/4) along with *C. famata* (3/4) and samples taken on day 42 and 56 were found to contain these species plus *C. catenulata*. However, in both samples *C. famata* was most numerous (4/6; 3/6) whilst *C. sphaerica* and *C. catenulata* were detected in approximately equal proportions (1/6; 2/6 and 1/6; 1/6 respectively).

Other studies have found that *C. famata* occurs as the predominant species of yeast in cheese (Fleet and Mian, 1986; Van den Tempel and Jakobsen, 1998) and these studies also detected *C. sphaerica* frequently. This is likely to be partly because of their ability to utilise lactose as an energy source (Barnett, 2000).

During this experiment the pH of the samples increased from 4.5 on day one of the experiment, to 6.6 at maturity. This increase in pH seems to be related to the growth and sporulation of *P. roqueforti* and is probably a result of proteolysis by the species. Madkor *et al.*, (1987b) demonstrated the proteolytic effects of the species and showed that this corresponds to an increase in pH.

3.1 Comparison of the development of pH in Stilton and Cheddar cheese

The pH of Stilton curd at salting is much lower than that of Cheddar type cheeses and it was decided to compare the development of acidity in the early stages of production. A commercially produced vat of Stilton cheese was monitored during the period from addition of the starter to salting of the curd and a pilot scale vat of cheddar cheese was monitored over the same timescale. Figure 3.2 demonstrates the development of pH in these two types of cheese and shows that a lower value is reached by Cheddar cheese, after 5 hours, but that Stilton curd reaches a lower pH at salting. This is attributable to the increased proportion of starter used in Cheddar (causing a more rapid development of acidity) and the delay in addition of salt in Stilton (which permits continued development of acidity).

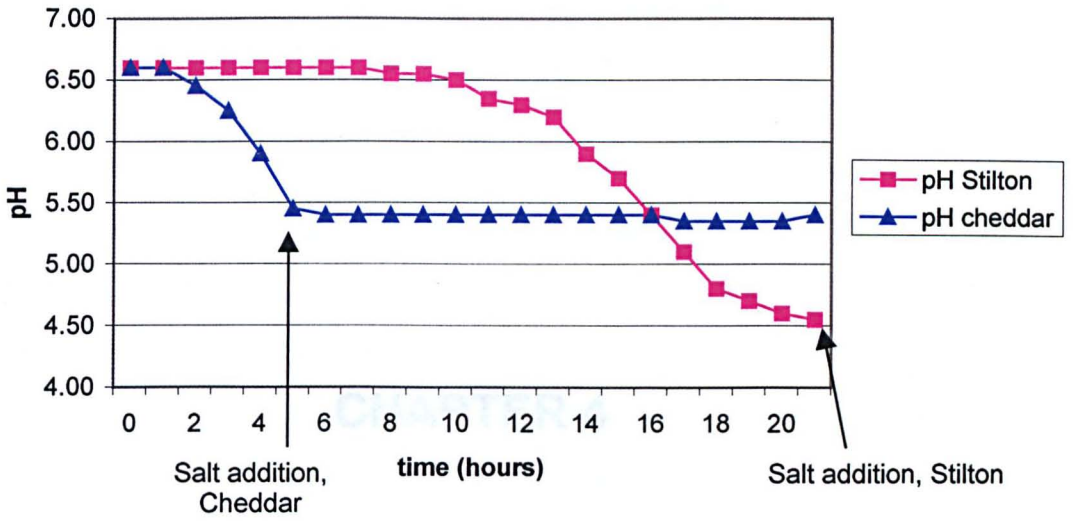


Figure 3.2 Comparison of pH development in Stilton and Cheddar cheeses

In summary, these experiments have shown that in numerical terms, the microbial flora of Stilton cheese progresses in a similar way to other types of cheese. Additionally, the development of pH in Stilton is considerably different from that in hard, Cheddar type cheeses, being lower at the start of ripening and increasing with growth of the blue mould. In terms of species growing in the samples it would appear that there are dominant species, but where these appear as a result of environmental or post-pasteurisation contamination these might be specific to individual dairies. Hence, cheeses produced from individual dairies could potentially exhibit characteristics specific to that dairy.

CHAPTER 4

4 Results of investigation into the microflora of mature Stilton cheese

The aim of this section of the study was to determine whether or not there is a relationship between the growth of *Penicillium roqueforti* and the microflora of mature blue Stilton cheese; in particular, to determine whether or not individual genera may affect the growth of the mould. In order to do this, mature blue Stilton cheeses of eight to twelve weeks of age were sampled and tested for total viable count, total lactobacilli, yeasts and moulds and coliforms. Subsequently, the lactobacilli were subjected to biochemical testing to determine the genera present. Table 4.1 lists the dates of production of samples and shows the quality of the cheese (in terms of proliferation of blue veins). The type of starter used for manufacture of the cheese is also indicated.

Table 4.1 Samples of Stilton cheese used during experiments

Sample number	Date of production	Quality (Good/Poor)	Starter
1	28.12.98	Good	DVI
2	28.12.98	Poor	DVI
3	30.12.98	Good	Bulk
4-9	29.12.98	Poor	DVI
10-13	28.4.99	Poor	DVI
14-16	28.4.99	Good	DVI
17-22	04.00	Good	Bulk
23-26	25.8.99	Poor	DVI
27, 28	15.9.99	Poor	DVI
29, 30	16.9.99	Poor	DVI
31-37	10.12.99	Good	DVI
38, 39	3.1.00	Good	DVI
40, 41	04.00	Poor	DVI
42-45	22-30.4.01	Poor	DVI
46-52	22-30.4.01	Good	DVI
53-55	23-29.7.01	Poor	DVI
56, 57	2,5.8.01	Poor	DVI
58-62	5-3.6.01	Good	DVI

4.1 Initial tests

Initially three mature (nine week old) cheeses were sampled in order to

1. assess the sample dilution rates necessary,
2. carry out an initial comparison of good and poor quality cheeses,
3. compare cheeses made with Direct Vat Inoculation (DVI) starter with those made with bulk, liquid starter and

4. deduce a preliminary ratio of total viable organisms, total lactobacilli, yeasts, moulds and coliforms. The last group were tested in order to determine any potential relationship between non-blueing of cheeses and high counts of the coliform group.

The cheeses were manufactured in the same creamery and were produced using the method described in Chapter 1. Two cheeses were manufactured using DVI starter whilst the third was made using bulk starter, as described in Section 1.3.2.2, and manufacture was on consecutive days.

Table 4.2 Results of initial tests comparing mature (9 weeks) cheeses, of both good and poor quality, made with direct vat inoculation (DVI) type starter with that made with bulk starter

Date of test	2.3.99	cfu g^{-1}				
Sample No	Sample	TVC	*Lactobacillus*	Yeasts	Moulds	Coliforms
1	DVI starter – good quality cheese made 28.12.98	1.9 E7	6.1 E7	>3 E6	>3 E6	<10
2	DVI – Poor quality cheese made 29.12.98	1 E6	1.8 E7	>3 E6	7.2 E5	<10
3	Bulk starter – good quality cheese Made 30.12.98	<1 E6	4.4 E8	>3 E6	>3 E6	<10

Initial results (Table 4.2) showed that the yeast and mould counts were higher than expected from previous experience of cheese analysis. The minimum detectable number of coliforms was 10 cfu g^{-1} and the counts were always lower than this.

Comparing the good cheese, made with the DVI starter, with the poor quality cheese, it is noticeable that the TVC and the TLC were considerably higher in the former. The cheeses made with DVI starter showed a higher total aerobic count (TVC) than that made with bulk starter, whilst in all cases the total *Lactobacillus* count (TLC) was higher than the TVC. The poor quality DVI cheese had a lower mould count than either of the good cheeses, although

visual observation showed that the mould on the bulk starter cheese developed more slowly and at five days of incubation had no evidence of sporulation (detected as blue colour development), whereas both samples from the DVI cheese had developed blue colouration in the areas of mould growth. It took a further two days for this to develop on the samples from the bulk starter cheese, despite the mould starters being identical. Also, although the yeast count was in excess of 3×10^6 g^{-1} and was uncountable at the selected dilutions, a visual estimation suggested that there were slightly fewer yeasts in the bulk starter sample than in the others.

The TLC of the bulk starter cheese was much higher than the TVC (in excess of 10-fold greater). The difference was less significant in the cheeses made with the DVI starter and it may be that the strains of lactococci and leuconostocs used in the DVI starters are more able to tolerate the conditions at maturity of the cheese than those in the bulk starter. These results suggest that in Stilton, as in other cheeses such as Cheddar (Naylor and Sharpe, 1958a; Peterson and Marshall, 1990; Olarte, *et al.,* 2000) lactobacilli predominate in the microflora after a few weeks maturation, confirming the findings of the experiments described in Chapter 3.

A preliminary investigation of the colonies on the MRS and milk agar plates of each cheese sample was carried out and three colonies from each of two plates were examined microscopically after Gram staining. On the MRS plates all colonies (which were either elliptical or circular in shape) were Gram positive rods and were also found to be catalase negative. On the milk agar both Gram positive cocci and rods were found, presumably the former resulting from the starter bacteria. These results suggest that the colonies growing on the MRS agar were indeed lactobacilli, whilst on the milk agar (TVC) there was a mixture of starter bacteria and non-starter bacteria which may have been lactobacilli. This may further suggest that since the total count could be made up of a mixture of starter and non-starter bacteria and was similar to the TLC, the starter bacteria had declined whilst the non-starter bacteria had increased. This has been found in a number of studies on Cheddar and other cheeses (Naylor and Sharpe 1958a;Tornadijo *et al.*, 1995; Fitzsimons *et al.*, 2001), as well as in the experiments detailed in Chapter 3.

4.2 Comparison of blue veins with white areas of cheese

Poor quality cheeses of the same batch, made with DVI starter, were then sub-sampled with samples being taken along the limited blue veining present within 1.5mm of the veins, as well as from the white only areas. This was in order to attempt to determine a difference in numbers of microorganisms in these areas, although at this stage no attempt was made to identify the species present.

Table 4.3 Results of tests comparing the areas of blue veining with the areas devoid of blue veins in poor quality, mature (9 weeks), cheeses, made 29.12.98, using DVI starters

Date of test	12.3.99	cfu g^{-1}				
Sample No	Sample	TVC	*Lactobacillus*	Yeasts	Moulds	Coliforms
4	Blue area	>3 E8	>3 E8	3.7 E7	2 E8	<10
5	Blue area	4.3 E7	3.3 E7	3 E6	1.9 E7	<10
6	Blue area	1.07 E8	5.2 E7	1.6 E7	1.3 E7	<10
Geo mean		1.11 E8	8.02 E7	1.21 E7	3.67 E7	
7	White area	4.1 E7	2.7 E7	1 E7	3 E6	<10
8	White area	1.81 E8	8.3 E7	1.9 E7	4 E6	<10
9	White area	1.22 E8	9.6 E7	1.8 E7	1.3 E7	<10
Geo mean		9.67 E7	5.99 E7	1.51 E7	5.38 E6	

Plates with crowded growth, which were too numerous to count, were recorded as greater than 300 and for the purpose of statistical analysis, counts of greater than 3×10^8 were always taken as 3.01×10^8 and those of less than 10^6 were taken as 9×10^5.

Consideration of the results in Table 4.3 shows that there was no significant difference in the TVC results of the white and blue areas of the cheeses ($P = 0.79$). Comparison of the TLC

of the blue and white areas also shows no significant difference (P = 0.63) and neither was the yeast count significantly higher in the white areas than in the blue areas (P = 0.83). In addition, comparison of the TVC with the TLC in both blue and white areas showed that there was no significant difference (P > 0.05). It is possible that the TVC is mostly composed of lactobacilli and other non-starter organisms, with the remainder being a small number of starter bacteria.

As may be expected, the mould count was lower in those areas devoid of blue veins (P = 0.034). However, a geometric mean count of 5.38 x 10^6 in the white area suggests that there had been some growth of *P. roqueforti*, but sporulation, which gives the characteristic blue colouration, had been prevented. It is possible that this was due to low fungal cell density or to an absence of oxygen, perhaps because of a dense curd structure, but it could also potentially be caused by the presence of inhibitory substances. In theory, the curd density should not vary considerably from day to day as the curd is measured into the hoops and the height of cheeses varies little (Hough, 2001, pers. comm.). Hoops are filled to 335mm and at grading, moisture loss has reduced this to an average of 223mm, ie an average loss of 33% in height. In practice, mature cheeses may vary from this average by approximately 20mm (223 ± 10mm) and this height difference may be sufficient to cause density variations, which may be significant to the oxygen levels in the cheese. The density of the curd may be influenced by other factors including the moisture content.

There was a gap between the initial tests and these tests of 10 days, during which time the counts appear to have increased for all except the mould count. The counts were in the range log 6 to log 8. In the experiment described in the previous chapter the initial mould count (at day one) was 6.8 x 10^3 cfu g^{-1}, so in the current experiment growth had clearly taken place, but had remained at between 10^6 and 10^8 g^{-1} at maturity, with little development of blue colour. The reason for this could be the presence of an antimycotic substance, although clearly this was not totally preventing growth, but might have been inhibiting sporulation.

4.3 Examination of a batch of poor quality cheese made on 28.4.99 with DVI starter

It was decided to investigate a number of poor quality batches of cheese as they arose in order to determine potential causes for the non-blueing and also to determine an overall picture of the microflora of the cheeses. A batch of mature (9 weeks) cheese exhibiting few blue veins, made on April 28th 1999, was sampled from the blue veined areas and from the white areas. The samples from the blue veined areas were taken within 1.5mm of the veins. The results are shown in Table 4.4.

Table 4.4 Results of tests comparing the areas of blue veining with the areas devoid of blue veins in poor quality, mature (9 weeks) cheeses made with DVI starters on 28.4.99

Date of test	28.6.99	cfu g^{-1}				
Sample No	Sample	TVC	TLC (*Lactobacillus*)	Yeasts	Moulds	Coliforms
10	Blue area	4.2 E8	7.1 E7	6 E6	2.2 E7	<10
11	Blue area	5.6 E7	2.6 E8	8 E6	1.8 E7	<10
Geo mean		1.15 E8	1.36 E8	6.93 E6	1.99 E7	
12	White area	<1 E6	2.1 E7	7 E6	1 E6	<10
13	White area	7 E6	2.7 E7	6 E6	<1 E6	<10
Geo Mean		2.51 E6	2.38 E7	6.48 E6	9.49 E5	
	Blue vs White P =	0.43	0.36	0.8	0.07	

The TVC, TLC and the mould count were slightly, but not significantly, higher in the blue areas than in the white areas, whilst there was little difference in the total yeast count (P=0.8). However, when the log number of colonies was used to determine the significance, a significant difference is shown in the mould count of the blue and white areas (P=<0.01). This might be expected given the presence of spores in the blue areas. In the blue areas the TVC and TLC were similar, whilst in the white area the TLC was almost one log cycle greater

than the TVC. Unusually, the TLC plates (MRS agar) had colonies of yeasts, which appeared as shiny, glistening colonies, growing both on the surface and deep within the agar of those samples from the blue area. These were confirmed by staining with methylene blue stain. This type of colony was not evident on samples from the white areas.

The mean counts of moulds in both the blue and the white areas of the cheeses were of the same order as those in the previous samples (4-6), while the counts in the white areas of the cheese were lower than those in the blue areas. This suggests that the mould was again present in significant numbers (of the order of log 6) but was unable to sporulate and thus create the characteristic blue veins. Limited blue veining was apparent in the cheeses, but it was not able to develop throughout, despite the presence of viable mould cells.

4.3.1 Anaerobic yeast detection

Since yeasts had grown on the MRS agar incubated anaerobically, samples of the cheeses were tested for the yeasts by preparing plates as in method 2.7 and subsequently incubating in anaerobic conditions using the "Anaerocult" system. The samples were taken randomly from the same cheeses rather than differentiating between blue and white areas. Furthermore, cheeses made with bulk starter were also checked by this method as a comparison. Table 4.5 shows the results, which were of the same order as was obtained using the standard method ($P = 0.16$).

Table 4.5 Results of yeast count conducted by incubating under anaerobic conditions

Sample	Anaerobic yeasts cfu g^{-1}
DVI Poor quality	1.51 E7
DVI Poor quality	1.4 E7
Bulk Poor quality	3.12 E7
Bulk Poor quality	2.6 E6
Geometric mean	1.14 E7

Although only two samples of cheeses made with the bulk starter were examined the counts appeared more variable, with a ten-fold difference between the results. However, this variation may be due to the difference in sample locations within the cheese. It is difficult to compare the counts in the DVI cheese with those in the bulk on the basis of such low numbers of samples, but it is interesting that the use of this method yielded high yeast counts on all samples.

4.4 Examination of poor quality, organic, cheeses made 25th August 1999 using DVI starter

Further samples of poor quality cheese, exhibiting few blue veins, were checked on 6.10.99, in order to determine both the number of microorganisms and the species of lactobacilli present. These cheeses were also different from previous cheeses since they were produced organically. Hence, while unknown chemical traces could have been responsible for the previous poor quality cheeses, this possibility did not apply here. In addition, it was necessary to investigate the potential link between the yeast count and blue vein appearance. Sampling could not differentiate between the blue and the white areas of the cheeses since 50% of the samples exhibited no blue veining whilst the remainder had very minor amounts of blueing. Plate 4.1 indicates the appearance of such cheeses in cross section.

Plate 4.1 Poorly blued cheese in cross section

(photographed using Kodak MCX digital camera)

As indicated previously, these cheeses were "organic" cheeses with the milk used for manufacture sourced from registered organic producers. Additionally, Soil Association approved ingredients, Hannilase rennet (Hansen, Denmark) and Ezal MA400 starter culture (Rhodia, France), were used. The remainder of the manufacturing process was exactly the same as that of other, non-organic, samples, as detailed in Chapter 1. Table 4.6 shows the results of tests on these cheeses and includes the pH of the area from whence the sample had been taken. The sample identity relates to the vat from which the samples originated, FF3 being vat 3 and FF2, vat 2, whilst 1945 indicates the date of manufacture (25th August 1999). The pH was tested in order to start to determine the possibility of pH influencing either the growth of the blue mould or other species within the sample and hence affecting the quality. There is potential for this factor to be affected either way, ie by pH affecting the microflora or the microflora affecting the pH.

Table 4.6 Results of tests on poor quality, mature (12 weeks) cheeses, made using organic ingredients

Sample No	Sample	cfu g^{-1}				pH
		TVC	TLC (*Lactobacillus*)	Yeasts	Moulds	
23	FF3 1945	4.4 E7	6.2 E7	4.8 E7	2.4 E6	5.40
24	FF3 1945	1.37 E8	7.9 E7	7.2 E7	4.2 E6	5.12
25	FF2 1945	2.8 E7	2.3 E7	2.4 E7	3 E5	4.82
26	FF2 1945	2.5 E7	3.8 E7	6.4 E7	1.3 E6	5.06
Geo. mean		4.53E7	4.55E7	4.80E7	1.41E6	5.10

The TVC and TLC were again very similar in these samples, suggesting that the TVC may consist mostly of lactobacilli. However, Gram staining of approximately 10% of colonies from the TVC plates indicated the presence of Gram positive cocci (presumably, the starter organisms) as well as Gram positive rods and yeasts. In addition, it is unlikely that lactobacilli would flourish as well on milk agar as on MRS agar, so the TVC clearly represented a mixture of microorganisms which included both starter and non starter bacteria. The *Lactobacillus* counts were comparable between samples, although the MRS agar plates again showed evidence of yeasts growing, despite being incubated under anaerobic conditions. The presence of these anaerobic yeasts reflects the results obtained in the previous experiment. The yeast count was at least 10-fold higher than in the previous samples and the presence of high numbers may explain their recovery on the other agars.

The numbers of moulds in the curd was similar to previous results obtained from white areas of curd. This was not unexpected since sporulation had not been able to take place. The amount of *P. roqueforti* in the curd of this cheese on day one of ripening was not known but experiments detailed in Chapter 3 suggested levels in the region of 10^3 g^{-1}, so it seems likely that a small amount of growth of the mould starter had taken place. Conidiospores, such as are produced by *P. roqueforti*, are usually formed by vegetatively growing colonies, but in an area of the colony that will not contribute further to vegetative growth (Deacon, 1984). Thus, on agar plates, spores are formed to within about 2 mm of the edge of the colony and in

Stilton they only appear to develop in the curd void, whilst, presumably, mycelial growth extends further into the curd. Spore production is stimulated by a deficiency in the level of available nitrogen, which restricts vegetative growth (Deacon, 1984). In these samples mycelial growth appeared to have taken place, although spore production had not and this may have been due to an over-abundance of available nitrogen. This in turn may have been as a result of metabolism by other microorganisms which might have released nitrogen into a form which the mould could utilise.

A sample of three yeast colonies from the MRS plates was examined microscopically and were confirmed as yeasts, so it was decided to carry out a yeast detection under anaerobic conditions of incubation, by the method described in 4.3.1. Table 4.7 shows the results of these tests, which can be compared with those in Table 4.5. The geometric means of these tests under anaerobic conditions were comparable at 1.14×10^7 for those tested in 4.3.1(Table 4.5) and 1.73×10^7 for those shown in Table 4.7. Furthermore, in both these sets of samples the anaerobic yeast count appears to be of a similar order to the aerobic yeast count.

Table 4.7 Results of yeast count conducted by incubating under anaerobic conditions

Sample	**Anaerobic yeasts cfu g^{-1}**
23	1.74 E7
24	5.3 E7
25	2.7 E6
26	3.6 E7
Geometric mean	1.73 E7

Since two sets of cheeses, produced several months apart, had been found to combine a deficiency in blue veins with a high level of yeasts able to grow anaerobically, it was decided to carry out biochemical identification tests on the yeasts, using the API 20C test kit. Duplicate colonies (8 in total) exhibiting similar colony characteristics and typical of the

majority of colonies on the plates, were checked using this system and were found to be *Candida famata*, with no strain differences being apparent (Appendix 3). Subsequently, it was decided to perform inhibition tests to determine the potential anti-microbial activity of this yeast against the *P. roqueforti* starter mould.

In these initial tests for anti-microbial activity the stab method (2.10, 1) was used. After incubating duplicate sets of plates aerobically and anaerobically it was noted that those incubated under aerobic conditions showed no evidence of anti-microbial activity, whilst those incubated under anaerobiosis showed a 1mm zone of clearing in the growth of the *P. roqueforti* around the *C. famata* inoculum. This may suggest that anaerobiosis stimulates the production of a substance inhibitory to *P. roqueforti* or that the mould was more susceptible to an inhibitory substance when grown anaerobically. The mould was able to sporulate under anaerobiosis, although less so than in those samples incubated aerobically, as might be anticipated given its growth requirements. This was demonstrated in later experiments and is shown in Plate 4.8. The ability of the mould to sporulate in the absence of oxygen indicates that the density of the curd should not significantly affect blueing, although the formation of the blue veins may be limited in extent. Potentially the veins might be absent as a result of close curd structure, but once pierced the blue colouration should be able to develop along the lines of the piercing even under conditions of reduced oxygen tension. This is seen in the cheese shown in Plate 4.1, which shows clear evidence of growth of *P. roqueforti* along the holes made during piercing of the cheese, but very little growth is noticeable elsewhere in this cheese.

The pH of samples 23-26 was similar to that of white areas of Stilton obtained during comparison of the pH of white and blue areas of mature blue Stilton cheeses (Appendix 2), using the method described in Chapter 2. All of the samples showed an increase in pH from that normally achieved at salting (4.50). However, sample 23 showed a higher pH (5.40) than the other samples, with this pH being closer to that of cheeses exhibiting blue veins (Table 4.13). This suggests that some proteolysis had occurred within the cheeses, despite the lack of abundant development of the blue veins and it is possible that this was due, in

part, to the action of the non starter lactic acid bacteria (NSLAB) as well as to the *P. roqueforti.*

Further samples of poor quality cheeses, in terms of poor development of blue veins, were sampled as available in order to compare batches. All were from the same dairy and were made using the same method and starter type, which, by this time, had been standardised as DVI.

4.5 Investigation of cheeses manufactured in September 1999, exhibiting poor blue vein development

In January 2000 a number of cheeses were downgraded due to poor blue veining. The batch numbers were LV191923 and LV 201924, corresponding to production on 15/9/99 and 16/9/99. In this experiment there was sufficient blue veining for the blue and white areas to be sampled separately, with the blue areas being sampled within 1.5 mm of the veins.

Table 4.8 Results of tests on poor quality mature (12 weeks) cheeses made 15th and 16th September 1999

Sample No	Sample	cfu g^{-1}				pH
		TVC	TLC (*Lactobacillus*)	Yeasts	Moulds	
27	LV191923 white area	4 E6	4.9 E7	9 E6	2 E6	5.86
28	LV191923 blue area	1.3 E7	3.1 E7	1 E7	2.9 E7	5.76
29	LV201924 white area	2 E6	1.2 E7	<1 E6	4 E6	5.61
30	LV201924 blue area	1.4 E7	3.3 E7	2.3 E7	2.5 E7	5.7
Geo mean	white area	2.83 E6	2.43 E7	9.49 E5	2.83 E6	5.74
Geo mean	blue area	1.35 E7	3.2 E7	1.52 E7	2.69 E7	5.73
Geo mean	LV 191923	7.21 E6	3.90 E7	9.49 E6	7.62 E6	
Geo mean	LV 201924	5.29 E6	1.99 E7	4.55 E6	1.0 E7	
P (blue vs white)		0.01	0.94	0.27	0.007	0.97
P (TVC vs TLC)	White area	0.06				
P (TVC vs TLC)	Blue area	0.004				

Comparison of the results (Table 4.8) shows that the TVC of the blue area was 50-fold greater than that of the white area (P = 0.01). The *Lactobacillus* count was very similar in both the blue and the white areas (P = 0.94), suggesting that any difference in conditions in the micro-environment had little impact on members of the genus. However, it could be that the limited blue veining resulted in small differences in the micro-environment. The pH results support this as little difference is shown between the blue and the white areas, whereas in good cheese greater variations exist (Appendix 2). This might be expected since the growth of the blue mould brings about proteolysis and a study by Madkor *et al.* (1987) found that the pH of mature Stilton increased to approximately 6 at the end of ripening. However, the pH values obtained during this experiment were higher (P< 0.003) than those achieved on samples 23-26, in Table 4.6, which suggests that some proteolysis had taken place, both in the blue and the white areas. This could have been brought about by mycelial

growth and it was notable in these samples that blue veining was inhibited, although not prevented altogether. Cryosectioning across a blue vein of the same cheeses, discussed more fully in section 4.5.1, showed that sporulation and mycelial growth had taken place, although clearly there were insufficient blue veins to categorise the batch as first grade. Lactobacilli may also cause proteolysis and are known to contribute to flavour production through proteolysis in other cheeses (Muehlenkamp-Ulate and Warthesen, 1999). However, Muehlenkamp-Ulate and Warthesen (1999) found pH values of 4.8-5.1 in Cheddar cheese slurry as a result of proteolytic activity, so the higher pH values achieved in this experiment may be due to proteolysis by *P. roqueforti.* In the previous experiment (Table 4.6) the lower pH values in the curd, combined with the lower mould counts, suggested that proteolysis may have been due to the activity of the LAB as well as to *P. roqueforti.*

When the mould counts achieved in samples 23 – 30 are plotted against the pH of the samples (Figure 4.1), it can be seen that an increase in mould count leads to a higher pH. However, the relationship is fairly weak ($r^2 = 0.30$). It may be significant that two pairs of data have similar mould counts but the pH values of these samples differ. The samples from the current experiment (27 and 29) have higher pH values than those from the previous experiment (samples 23 and 24), despite similar mould counts. Plotting the results of the total *Lactobacillus* count against pH on the same graph shows that the number of lactobacilli did not affect the pH difference in the samples ($r^2 = 0.167$), since there is little difference in the numbers of lactobacilli in these samples. Nevertheless, that is not to say that the lactobacilli did not contribute to the pH changes. It is more likely, however, that the contribution of *P. roqueforti* was the more significant factor.

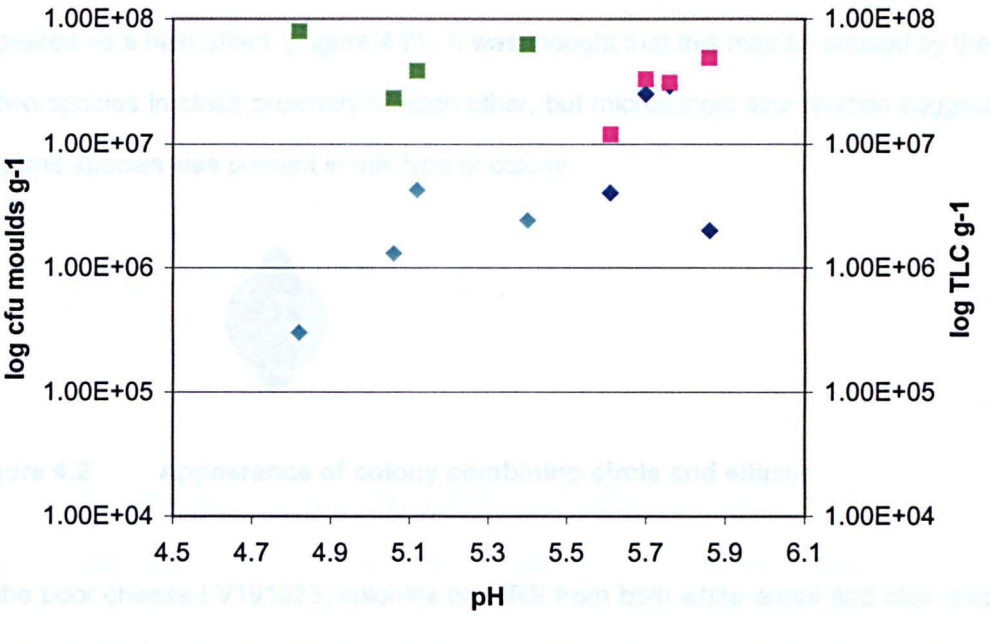


Figure 4.1 Comparison of counts of *P. roqueforti* and pH in blue Stilton cheese

Table 4.8 also shows that the TVC was less than the TLC by 1 log cycle in the white areas of the cheese, whilst in the blue areas the difference was much less, being only a factor of two, although the TVC was still less than the TLC. Significance testing (Table 4.8) suggests that the difference between the TVC and TLC in the blue areas of these samples is highly significant ($P < 0.01$), whilst the difference between the TVC and TLC in the white areas is less so ($P = 0.06$). This may be attributable to the variance in the sample results within the white areas as the results were much closer to each other in the blue areas. It is important to note that statistical analysis based on such small numbers of data may give misleading results and as such it is the cumulative statistics which are likely to be more accurate.

The yeast counts varied within the two white samples by approximately 90-fold, as did the mould counts, although the mean yeast count was lower in the white areas than in the blue areas of the cheese. All moulds on these plates were blue moulds, a common occurrence throughout the study.

Colonies from the MRS plates were checked for catalase activity, Gram stained and examined microscopically and were all found to be Gram positive, catalase negative rods.

Colony appearance was either elliptical, circular or a combination of both, where the circle appeared as a halo effect (Figure 4.2). It was thought that this may be caused by the growth of two species in close proximity to each other, but microscopic examination suggested that only one species was present in this type of colony.

Figure 4.2 Appearance of colony combining circle and ellipse

In the poor cheese LV191923, colonies on MRS from both white areas and blue areas were made up of 50% elliptical and 50% circular colonies. The elliptical colonies, when checked by the API 50 CHL system, were found to be *Lb. plantarum* and *Lb. brevis*, whilst the circular colonies were found to be only *Lb. plantarum*. The biochemical results of these tests are shown in Appendix 4.

In the poor cheese LV 201924, 90% of colonies in the white area were elliptical and were shown to be *Lb. brevis*. In the blue area 20% of colonies were elliptical whilst 80% were the combination of circle and ellipse and both types were found to be *Lb. plantarum* or *Lb. brevis*. It would appear that the colony characteristics of these two species are almost identical on this agar and that the macro-appearance is no guide to the species. The results of the biochemical tests are shown in Appendix 4 and it can be seen that there was very little difference in the strains occurring in the two cheeses. Two strains of *Lb. plantarum* are indicated but these only differ in the utilisation of gluconate.

There was no evidence of the growth of yeasts on the MRS plates, suggesting that the anaerobic yeasts found previously were not present in this sample. This batch exhibited counts of the same order as or lower than good samples (31-39 and 58-62) but still was lacking in blue veins. The yeast count was not significantly higher than previous samples, although the possible presence of *C. famata* was not investigated. It seems that the lack of

blue veining in this batch was not attributable simply to the numbers of those microorganisms described here, but may have been due to the presence of other inhibitory factors. These may include the species of microorganisms present but may also be due to physical parameters such as low a_w or oxygen tension or restrictive salt content.

4.5.1 Cryogenic sectioning of the cheeses

In order to try to determine the potential presence of micro-environments within the cheeses, a sample of cheese LV191923 (poor cheese) was cryosectioned across a blue vein and stained with Toluidine blue stain (Method 2.11). Plates 4.2 to 4.4 show the results obtained with the yellow areas being curd or fat and bacteria and fungi staining purple to blue.

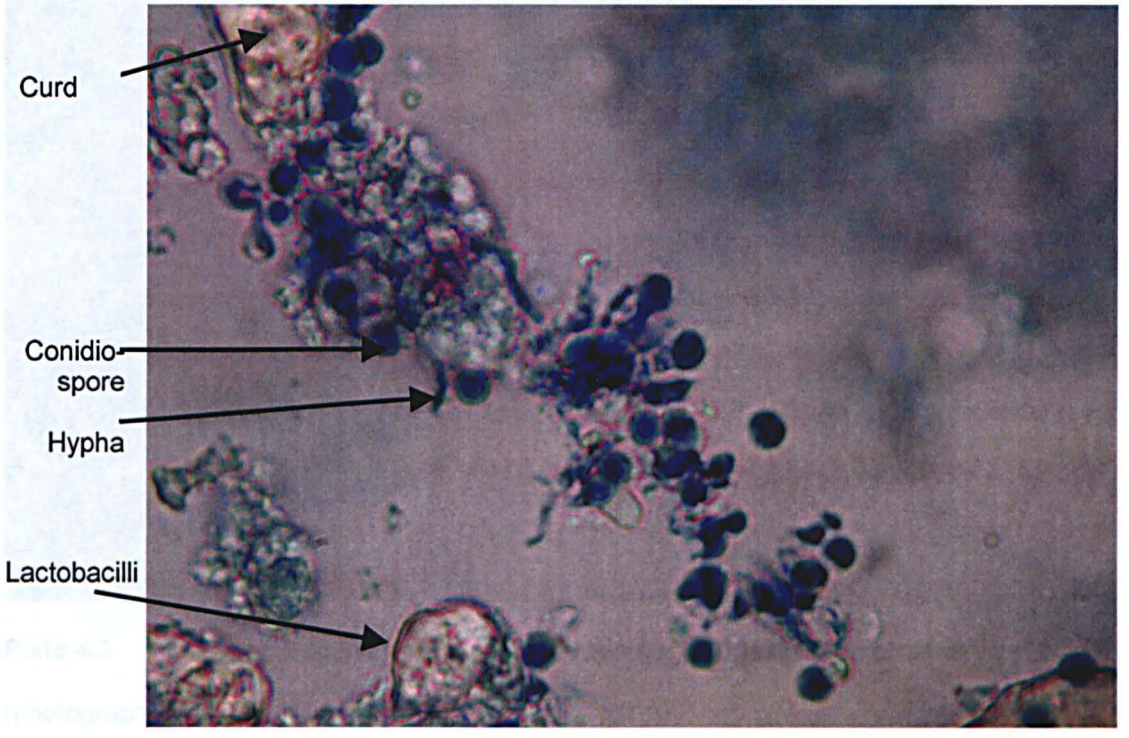


Plate 4.2 Cryogenic section of Stilton examined at X 400 magnification and photographed using Nikon digital camera

It can be seen in Plate 4.2 that there is evidence of conidiospores of *P. roqueforti* as well as rod shaped bacteria, thought to be lactobacilli, whilst there was no evidence of the presence of yeast cells, despite counts in the region of 10^6 g^{-1} on the samples, ie similar to the number of moulds. This may be due to the small section (10μm), which was taken across a blue

vein, where one would expect to find mould spores. In addition, it is not clear how many hyphae make up a colony forming unit of the *P. roqueforti*. Due to the localised growth of organisms, the presence of particular genera in the section may not relate to the viable cell count. Dodd and Waites (1991) and Dodd and Waites (1992) described similar findings in other foods such as fermented sausage. There is, however, potential for confusion in identification since the conidiospores are similar in size to the yeast cell, but *C. famata* replicates by budding and there was no evidence of budding cells, whilst there was evidence of mycelium.

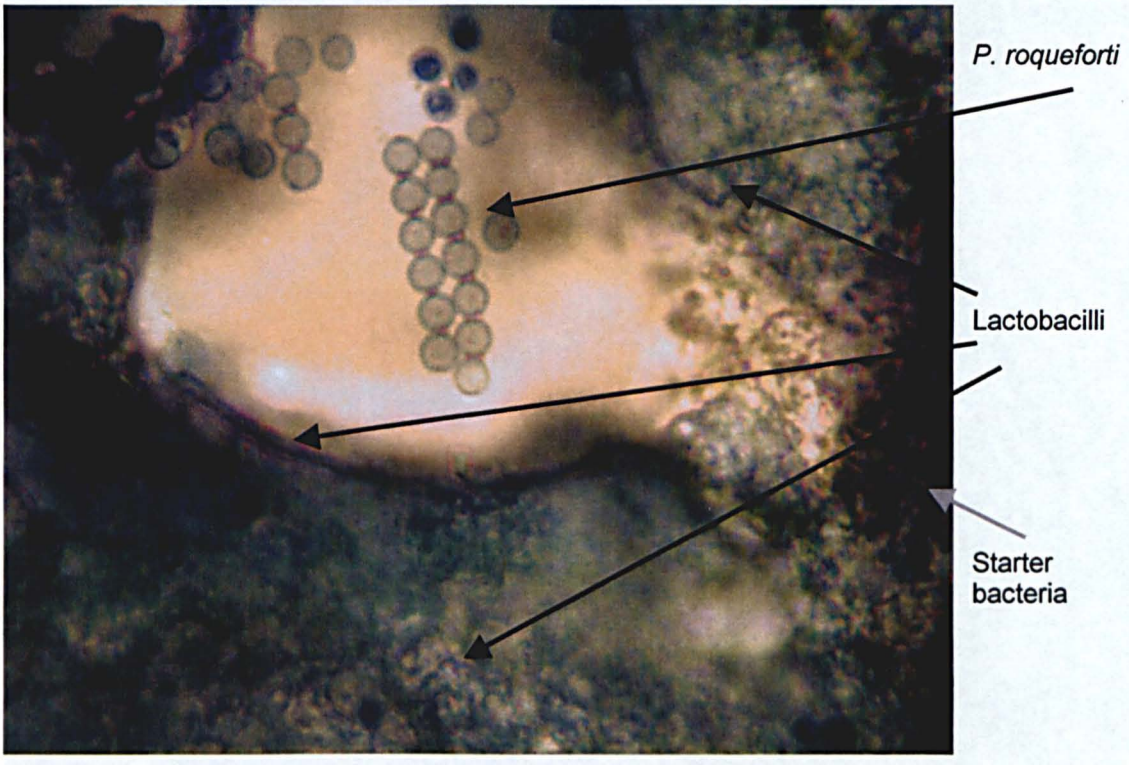
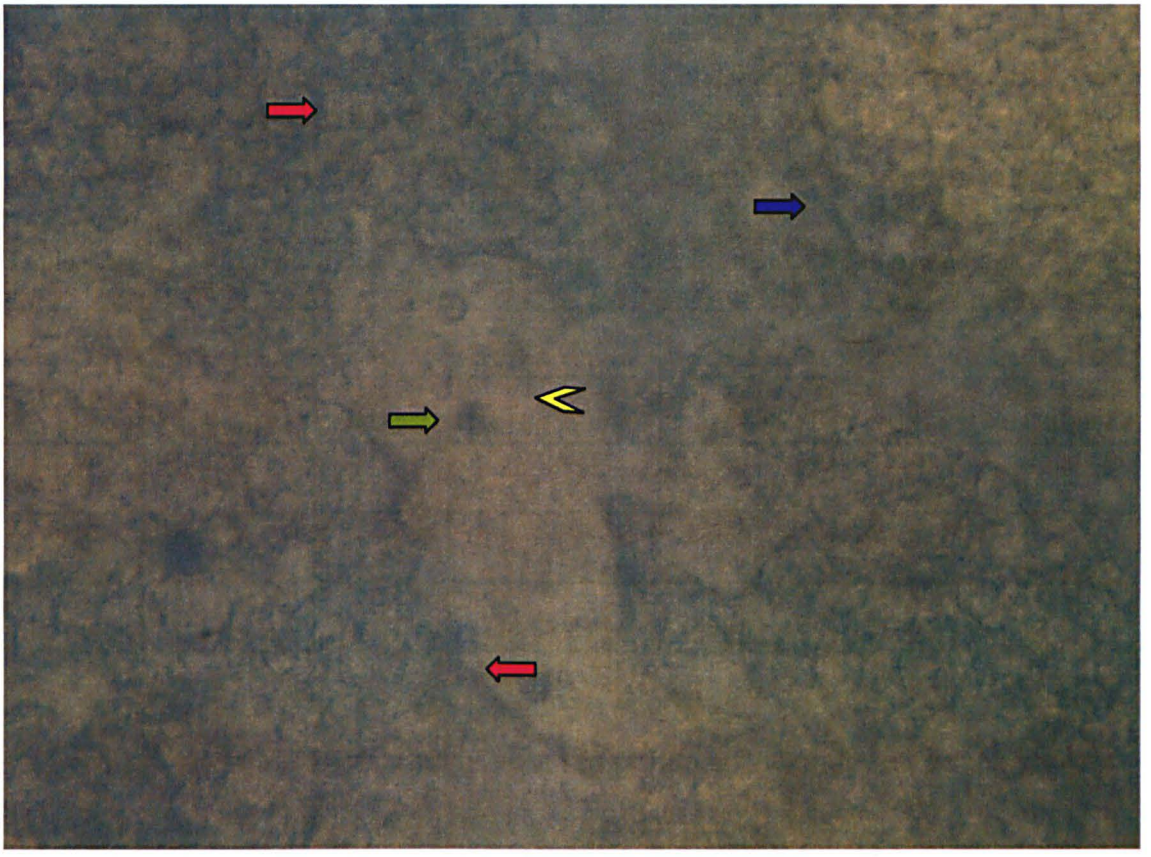


Plate 4.3 Cryogenic section across a blue vein of Blue Stilton cheese
(photographed using a Nikon digital camera)

Plates 4.2 and 4.3 show the presence of mould spores in the open (veined) area whilst in the curd area, rod shaped bacteria are aligned around the curd particles. There were some cocci present, which were assumed to be starter bacteria, although they appear to be present in a complex mass, which stained deep purple to blue, rather than appearing in chains, which are typical of lactococci.

This distribution of species may be due to the variation in pH level, with a lower pH being more likely within the curd than in areas with growth of the blue mould. Lactobacilli are more tolerant of lower pH levels than lactococci and could be expected to be found in the curd areas where the pH may be lower. Cryosectioning cannot differentiate between species of rods but does indicate variation in the distribution of non starter and starter lactic acid bacteria. Neither will this method differentiate between dead and live cells, so that the presence of clusters of starter bacteria does not necessarily indicate that they are viable, although the plate count result would suggest that significant numbers are.



Mould spore; lactobacilli; cocci; curd particle

Plate 4.4 Cryogenic section of Blue Stilton cheese

(photographed using a Nikon digital camera)

Plate 4.4 shows a cryosection of a portion of curd, rather than the void where the vein occurred and also shows that the rods, presumably lactobacilli, are present predominantly in chains and are aligned around the curd particles whilst the cocci, presumably starter bacteria, are less clearly defined and are present in clumps. In this photograph the curd appears to be more broken than in Plates 4.2 and 4.3, although it could be that the lactobacilli are invading

the curd more than in the cryosections shown in these plates and are producing a net like appearance. There are individual fungal spores present, but there appears to be little evidence of fungal hyphae within the curd particles, although short (presumably young) hyphae are clearly visible emanating from spores in the curd spaces in plates 4.2 and 4.3.

4.6 Examination of poor cheese manufactured in April 2000

A batch of cheese, made in April 2000, which had exhibited very poor blueing quality at maturity (eight weeks), was examined in July 2000, when the cheeses were 12 weeks old. Sample 40 showed no blueing at all whilst sample 41 showed slight evidence of blue vein formation.

Table 4.9 Results of poor quality cheese examined at 12 weeks of age

Sample no		cfu g^{-1}				pH
	Sample	TVC	*Lactobacillus*	Yeasts	Moulds	
40	White	7 E6	6 E7	1.7 E7	<1 E6	4.76
41	White	2 E7	3.4 E7	2.2 E7	<1 E6	4.81
Geo mean		1.18 E7	4.52 E7	1.93 E7	<1.00 E6	4.79

The results (Table 4.9) show that both samples had TVC and TLC that were comparable with poor cheese examined previously (samples 23 to 30), but not dissimilar to good cheeses (samples 31 to 39). The pH values obtained were close to those at the beginning of ripening in blue cheeses and suggest that proteolysis had not progressed to any great extent. The pH achieved in these samples was lower that that found in previous samples (Table 4.6 and Table 4.8), although the lactobacilli were found in similar numbers. This suggests that the lactobacilli contributed little towards proteolysis in this experiment and may mean that the same was true in other experiments. However, Muehlenkamp-Ulate and Wartheson (1999) noted proteolysis due to the action of lactobacilli in Cheddar cheese slurries of 12 days ripening, so it could be expected that lactobacilli would contribute to proteolysis in Stilton

cheese of 8 – 12 weeks ripening as used in the current study. The mould counts were much lower in this experiment and it seems likely that the higher pH values achieved in 4.4 and 4.5 were mainly as a result of proteolysis produced by *P. roqueforti.*

Biochemical checks on 10% of colonies, using the API 50CHL system, showed the lactobacilli to be either *Lb. plantarum* or *Lb. brevis*, with the former being found twice as frequently as the latter. The strains of *Lb. plantarum* show very different biochemical profiles from those isolated from previous samples (Appendix 4) and four strains were differentiated during the current experiment. Two of the strains varied only in their ability to utilise three of the substrates used in the test, whereas the third and fourth strains showed much greater metabolic capabilities and differed from each other only in the ability to utilise rhamnose. It is likely that the wide range of strains present would contribute to the frequency with which this species was isolated, since more strains may be able to survive conditions found within the cheese. Only one strain of *Lb. brevis* was noted.

The yeast count was similar to that in good cheeses (samples 31 to 39), but since the physical appearance of these cheeses was similar to those sampled in 4.3 and 4.4, an anaerobic yeast count was carried out as a comparison.

Table 4.10 Results of yeast count conducted by incubating under anaerobic conditions

Sample	Anaerobic yeasts cfu g^{-1}
40	9 E6
41	4.9 E7
Geometric mean	2.1 E7

The number of yeasts able to grow anaerobically (Table 4.10) was very similar to that achieved using the aerobic yeast count, suggesting that the yeasts detected using aerobic incubation were equally able to proliferate in the absence of oxygen. Four colonies were checked using the API 20C AUX test kit with the following results:

1. Anaerobic cultured from sample 40, 10^6 plate – *Candida famata*
2. Anaerobic cultured from sample 41, 10^6 plate – *C. famata*
3. Aerobic cultured from sample 40, 10^6 plate – *C. dubliniensis*
4. Aerobic cultured from sample 41, 10^6 plate – *C. famata*

An inhibition check was then carried out using the stab method and again a 1mm zone of inhibition was produced around *C. famata* (isolate 1) when incubation was under anaerobic conditions, although aerobic incubation did not produce inhibition. Isolates of *Candida dubliniensis* were not checked for the ability to inhibit growth of *P. roqueforti* since this organism appeared to be fewer in number and since *C. famata* had demonstrated inhibition of *P. roqueforti* in previous experiments. When the biochemical profiles (Appendix 3) of 1, 2 and 4 above are compared with that of the yeast from the organic cheeses (Section 4.4) it can be seen that there are slight differences. The isolate from the organic cheese, which also produced a zone of inhibition, was able to ferment L-arabinose and D-xylose whereas this ability was absent in all of the isolates from the current experiment. Furthermore, two of the isolates in the current experiment (2 and 4) differed further in that they were unable to utilise trehalose, whereas both the isolate from the organic cheese and isolate number one in this experiment had this ability. None of these isolates were detected during environmental sampling as described in Section 4.14.

4.7 Comparison of blue with white areas in good quality cheeses made 28th April 1999 using DVI starters

As a direct comparison with samples from poor quality cheeses, three samples were taken from good quality, mature cheeses made with DVI starter on 28th April 1999 and the blue veined areas compared with the white areas. Such was the intensity of the growth of the blue mould that sampling the white areas proved exceptionally difficult and in fact only one sample of white area could be achieved without encroaching into the blue area.

Table 4.11 Results of tests comparing the areas of blue veining with the white areas between the blue veins in good quality, mature (8 weeks) cheeses made with DVI starters

Date of test	28.6.99	cfu g^{-1}				
Sample No	Sample	TVC	*Lactobacillus*	Yeasts	Moulds	Coliforms
14	Blue area	8.5 E7	3.3 E7	4 E6	2.1 E7	<10
15	Blue area	>3 E9	6.8 E7	2 E6	3.2 E7	<10
Geo mean		1.6 E9	4.74 E7	2.83 E6	2.59 E 7	
16	White area	2.7 E8	1.21 E8	6 E6	8 E6	<10

The results (Table 4.11) show that in these good quality cheeses, exhibiting ample blue veining, the TVC in the blue veined areas exceeded that in the white areas. It was higher than the TVC in the non-blued cheeses, made on the same day, by 1 log cycle and comparison of the white areas of samples 10-16 (Tables 4.3 and 4.11) shows a 100-fold difference between the good samples (14-16) and the poor cheeses (10-13) (Table 4.4), despite the use of identical manufacturing methods. In the samples used in this experiment, the TLC was lower than the TVC in both blue and white areas and was lower in the blue area than in the white area by approximately 50%. The TVC mean counts exceeded those from samples 4-13, although the TLC was less in the blue area of these samples than in previous samples (4-6,10,11). Comparison of the TVC of the blue veined areas of poor cheese produced on the same day (samples 10 & 11) with those of good cheese (14 & 15) shows a significant difference ($P = 0.01$). This suggests that although the manufacturing method was identical, the growth of *P. roqueforti* was inhibited in samples 10-13.

Ten percent of colonies growing on the MRS were examined and confirmed as *Lactobacillus*, being Gram positive, catalase negative rods. The colonies examined were selected as being representative in appearance of all colonies on the plates. In both the white and the blue areas, 3 colonies were checked from the 10^{-7} dilutions (ie 3/3, 3/7 and 3/12) and biochemical checks, using API 50 CHL, showed these to be *Lb. curvatus, Lb. casei* and *Lb. plantarum*, with each species being represented on each sample.

All moulds on the SDA were typical of the starter mould, *P. roqueforti*, with the exception of one darker green mould, which was also presumed to be *P. roqueforti* since its microscopic appearance was identical, although it was different from the inoculated species in colour and in appearance on the reverse of the agar. There was no significant difference in the yeast counts in the blue and white areas of the cheese, but the difference between the yeast and mould counts in the blue areas was greater, whilst in the white area these two counts were very similar. However, it would be expected that there would be greater number of mould colonies in the blue areas due to sporulation, which would result in the presence of large numbers of spores which would give more individual colonies than the undivided hyphae.

Since the coliform count was less than 10 g^{-1} in all of samples 1 to 16, this test was dropped from further experiments.

4.8 Comparison of the microflora of good quality cheese made from bulk starter

Good quality, mature (8 weeks) cheeses made in April 1999, using bulk starter, were sampled to compare the microflora within the blue areas with that in the white areas of curd and to establish any difference in the microflora of cheese made with bulk starter rather than DVI. Six samples were tested, three from the white area and three from the blue area and the results of these tests are shown in Table 4.12. The pH of the samples was not measured since these cheeses were tested in the early part of the study, before this had been added to the testing schedule.

Table 4.12 Results of tests comparing the areas of blue veining with the white areas between the blue veins in good quality, mature (8 weeks) cheeses made using bulk starter

Sample No	Sample	cfu g^{-1}			
		TVC	*Lactobacillus*	Yeasts	Moulds
20	blue 1	1.86E8	1.28E8	1.00E6	4.00E7
21	blue 2	1.32E8	2.07E8	4.00E6	2.10E7
22	blue3	1.98E8	2.34E8	<1.00 E6	5.10E7
Geo mean		1.69E8	1.84E8	1.53 E6	3.50E7
17	white 1	4.00E6	1.05E8	2.00E6	<1.00 E6
18	white 2	2.00E6	7.10E7	1.00E6	<1.00 E6
19	white 3	7.00E6	1.05E8	3.00E6	1.00E6
Geo mean		3.83E6	9.22E7	1.82E6	9.32 E5
P blue vs white		0.01	0.1	0.6	0.01

Comparison of results from the blue and the white areas shows that the TVC was higher in the areas of blue veining than that in the white areas (P = 0.01). The TLC was similar in the blue and white areas (P = 0.1) and there was also very little difference in the yeast count (P = 0.6). This contradicts the results shown in Table 4.11 (samples 14-16), on good cheese made with DVI starter, where the TLC was greater in the white area than in the blue and might be attributable to the difference in starter type.

The TLC results of samples 17-22 (Table 4.12) show greater similarity to the results of samples 10-13 (Table 4.4), which was poor cheese made with DVI starter. However, although the mean counts are similar in Tables 4.4 and 4.12, the difference between the TVC of the blue and white areas was not significant in samples 10-13 (Table 4.4), whereas it was in this experiment. Comparison of the ratio of TVC to TLC in Table 4.12 shows that in the white areas, the TLC was significantly greater than the TVC (P = 0.01), whereas in the blue

areas the TLC and the TVC were very similar (P = 0.70). Comparison of the results in Table 4.4 showed a difference in the white areas (P = 0.002), whereas the blue areas showed little difference (P = 0.45). These results suggest, therefore, that the difference in ratios of groups of microorganisms has little effect on the abundance of blue veins, although it may be that species variations within these groups is more significant.

In two samples, the mould count in the white areas was not detectable at the selected dilutions, being less than 1×10^6, whereas in the blue areas mean levels of 3.5×10^7 were detected, which is a level similar to that achieved in previous experiments. This difference (P = 0.01) may suggest that the blue mould did not grow actively in the white areas, where the available oxygen was lower and thus may have led to poor blue veining in dense cheeses. Attempts were made to measure the available oxygen in both the blue and white areas of the cheeses. However, this proved impossible to measure accurately, since distinguishing between locations within a cheese necessitated cutting to expose the surface, which allowed oxygen to enter the location and affected the results.

The lower mould counts in the white areas of the cheeses may also be a result of the production of inhibitory substances by lactic acid bacteria, especially NSLAB. However, the TVC and TLC of the blue areas would seem to contradict this suggestion, since the TVC was greater in the blue areas (P = 0.01) and the TLC was similar in both areas (P = 0.1). It is more likely that the difference in the mould counts in the two areas was either related to the presence of oxygen in the open areas of the curd or to the technique. The development of the blue veins is due to sporulation and the presence of spores would yield a higher plate count.

The higher TVC in the blue area might be due to the higher pH (since other samples have shown a higher pH in the area of blue veining), which might enable the starter bacteria to grow and return to levels similar to those found at the start of ripening (Chapter 3), whereas the lower pH in the white areas might limit growth of these bacteria. Furthermore, growth of *P. roqueforti* in the cheese may generate amino acids which the starter bacteria can utilise as

growth substrates. Marshall (1987) stated that *Ln. mesenteroides* subsp. *cremoris* needed amino acids or yeast extract in order to be able to grow in milk and the proteases produced by *P. roqueforti* may supply these amino acids (Gripon, 1993; Cogan and Jordan, 1994).

Low mould counts (of a similar order) were noted in samples 40 and 41 (Table 4.9), from poor quality cheeses which exhibited almost no blue veins and since the mould count at the start of ripening is typically of the order of 10^3 to 10^4 it appears that there was growth in samples 17-22, whereas there may not have been in samples 40 and 41. It seems likely that in the current experiment the spores were able to form in the veins, where oxygen was present, but that sporulation was limited in the white areas, possibly because of a lack of oxygen. In samples 40 and 41, taken from poor quality cheeses, no growth was detectable at the dilutions employed and this may have been due to the absence of oxygen but, equally, may have been due to the presence of some inhibitory substance, which may have been produced by LAB or by some other microorganism, given the isolation of *C. famata* which showed inhibitory activity under anaerobic conditions in Section 4.4.

Ten percent of colonies grown on MRS agar in the current experiment were sampled and all were catalase negative, Gram variable rods growing in chains, some of which appeared to show granulation. Biochemical checks demonstrated these also to be *Lb. curvatus, Lb. brevis* or *Lb. plantarum* (Appendix 4). Strain differences within each species were not apparent, although there were several differences in fermentation capabilities between the species. *Lb. plantarum* exhibited the broadest range of utilisable substrates, including the ability to utilise glycerol, melibiose and D-raffinose, which neither *Lb. curvatus* nor *Lb. brevis* were able to ferment. This might explain the frequent isolation of this species during this study. *Lb. curvatus* was able to utilise fewer substrates than the other two species. Both this species and *Lb. plantarum* are homofermentative, whilst *Lb. brevis* is heterofermentative.

Colonies taken from the TVC plates were also catalase negative, Gram positive rods, but in addition, yeasts were present on these plates, representing approximately 50% of the colonies detected in the white areas of the cheeses and about 10% of the colonies in the blue areas. No yeasts were detected on the MRS plates and it may be possible that the total

count is made up of yeasts and lactobacilli rather than starter bacteria, suggesting that the latter lost viability during ripening.

There were low numbers of yeasts in all samples and these were less than in previous samples of poor quality cheeses, but there was no significant difference between the number of yeasts in the blue and white areas ($P = 0.6$). It is possible that there is a link between the quality in terms of blue veining and the level of yeast contamination in the product, since the cheeses sampled in this experiment had slightly lower yeast counts than previous samples of poor quality cheeses. However, significance testing suggests that this may not be an important factor ($P = 0.11$). The presence of yeasts on the TVC plates of these good cheeses might contradict the theory that yeast species inhibit development of blue veins, although there appeared to be more yeasts in the TVC of the white area than that of the blue.

4.9 Examination of good quality cheeses manufactured using DVI starter in December 1999 and January 2000

Further samples of good quality mature (8 weeks) cheeses were tested in order to establish their microflora. Again the blue and white areas were sampled separately in order to establish differences in the microflora of the two regions, although one sample, LCD 49, had such confluent blue veining as to make differentiation impossible, so a combined blue and white sample was tested. Table 4.13 shows the results of tests on five cheeses (blue and white 1-3; 4 and 5, blue and white), produced in December 1999 (week 49) and January 2000 (week 1).

Table 4.13 Viable counts from good cheese tested at 8 weeks of ripening

Sample no	Sample	cfu g^{-1}				pH
		TVC	*Lactobacillus*	Yeasts	Moulds	
31	White 1 (49)	2 E7	1.2 E7	2.7 E7	3 E6	5.35
32	Blue 1 (49)	1.8 E7	3.2 E7	4.4 E7	2.1 E7	5.91
33	White 2 (49)	1.4 E7	4 E6	8 E6	6 E6	5.79
34	Blue 2 (49)	5.6 E8	6.8 E7	2.2 E7	3 E7	5.98
35	White 3 (49)	7.6 E7	4.8 E7	1.7 E7	4 E6	5.59
36	Blue 3 (49)	9 E6	3.5 E7	9 E6	2.5 E7	5.92
37	4 (49)	5.5 E7	7.6 E7	5 E6	4.1 E7	6.1
38	White 5 (1)	3.1 E7	6.7 E7	4 E6	2.5 E7	5.52
39	Blue 5 (1)	6.2 E7	5.7 E7	5 E6	4 E7	5.97
Geo mean white		2.85 E7	1.98 E7	1.10 E7	6.51 E6	5.55
Geo mean blue		4.87 E7	4.56 E7	1.44 E7	2.82 E7	5.95
Blue vs White	P =	0.44	0.46	0.38	0.002	0.02

There was little difference between the blue and white areas as regards TVC and TLC (P= 0.44 and 0.46, respectively). Comparison of the TVC with the TLC within each area also showed no significant difference (P >0.05) and this may suggest that the TVC was composed mainly of lactobacilli. The yeast counts were similar in both the blue and the white areas, although they were high (log 7) compared to other good cheeses. Previous samples of good cheeses (Tables 4.11 and 4.12) exhibited counts in the region of log 6 yeasts. Once more, the mould count was lower in the white area (P = 0.002), in these samples by approximately 75%.

The pH values measured in the white areas of the cheeses were lower than those in the blue areas (P = 0.02), but in both areas the pH had risen from the value normally obtained at the start of ripening (pH 4.5) and suggested that proteolysis had been extensive in both the white and blue areas, although it had been more extensive in the blue areas. Observations show

that Stilton cheeses exhibiting good blueing characteristics have a much creamier texture than those where the growth of the blue mould is limited. In poor cheeses the texture is much more akin to immature White Stilton (ie "curdy") and it is probable that this textural change is due to proteolysis caused by the blue mould. Comparison of the mould count with the pH of samples (Figure 4.3) suggests that increasing numbers of moulds yields a higher pH in the sample ($r^2 = 0.57$).

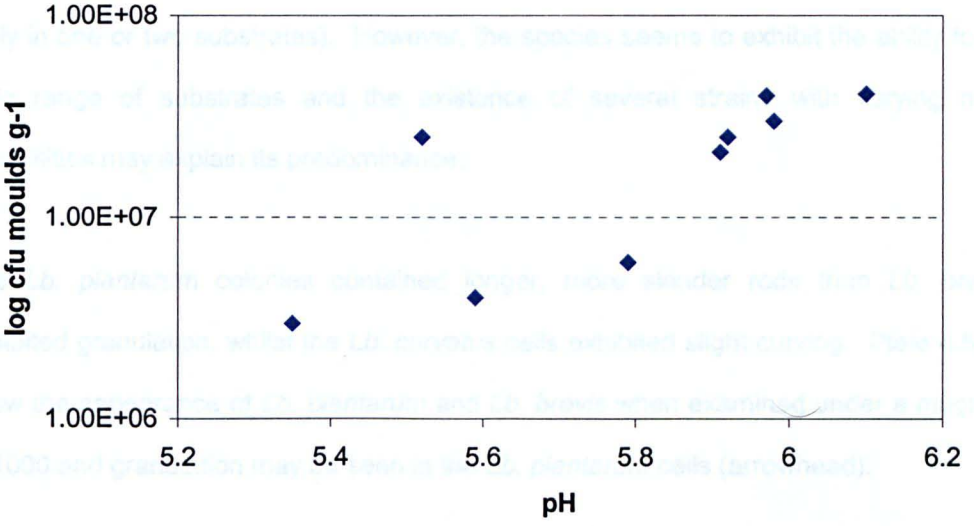


Figure 4.3 Comparison of viable count of moulds with pH of mature Stilton cheese

Colonies from the MRS plates from all samples were checked for catalase activity, Gram stained and examined microscopically and were all found to be Gram-positive, catalase negative rods, as in all previous experiments. There was no evidence of the growth of yeasts cells. Colony appearance was either elliptical, circular or a combination of both as before. Typical colonies were tested using the API 50 CHL test kit and the circular colonies were always *Lb. plantarum*, whilst the elliptical and the combination colonies were either *Lb.curvatus, Lb. plantarum* or *Lb. brevis* (Appendix 4). Two strains of *Lb. curvatus* were noted during this experiment, although they differed only in the ability to utilise cellobiose. The strain that was able to utilise this substrate was found in the white areas of the cheese, but not in the blue areas. This indicates a potential preference of different strains for different environments in the cheese and may be due to the presence of the substrate in the white areas, but not in the blue areas. Two strains of *Lb. brevis* were also noted, with these strains

differing in their abilities to utilise the substrates L-arabinose and mannitol. The strain occurring in the white area was able to utilise these substrates and again this may indicate the absence of the substrates in the blue area. However, *Lb. curvatus* was able to utilise mannitol in both blue and white areas of the cheese, so it seems more likely that the strain of *Lb. brevis* was unable to utilise or was out-competed for the substrate.

Three strains of *Lb. plantarum* were isolated from these samples, exhibiting slight differences (only in one or two substrates). However, the species seems to exhibit the ability to utilise a wide range of substrates and the existence of several strains with varying metabolic capabilities may explain its predominance.

The *Lb. plantarum* colonies contained longer, more slender rods than *Lb. brevis* and exhibited granulation, whilst the *Lb. curvatus* cells exhibited slight curving. Plate 4.5 and 4.6 show the appearance of *Lb. plantarum* and *Lb. brevis* when examined under a magnification of 1000 and granulation may be seen in the *Lb. plantarum* cells (arrowhead).

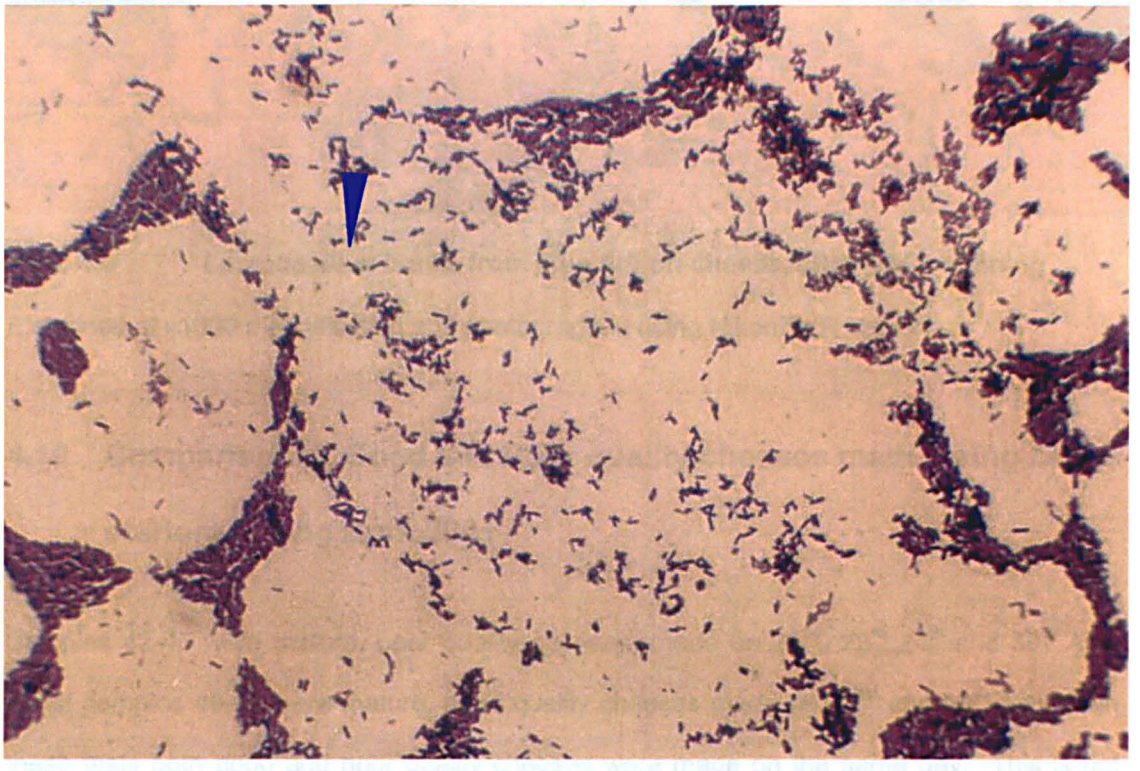


Plate 4.5 ***Lactobacillus plantarum*** **from blue Stilton cheese, after Gram staining**

Examined at x1000 magnification and photographed using Nikon SLR camera

Comparison of plates 4.5 and 4.6 shows that the *Lb. brevis* cells are much shorter, almost coccoid rods, and there is no evidence of granulation in these cells. Kandler and Weiss (1986) have written that "some species of the gas-producing lactobacilli (*L. fermentum, L. brevis*) always exhibit a mixture of long and short rods". They also stated that granulation is often seen in the homo-fermentative species such as *Lb. plantarum* (Kandler and Weiss, 1986).

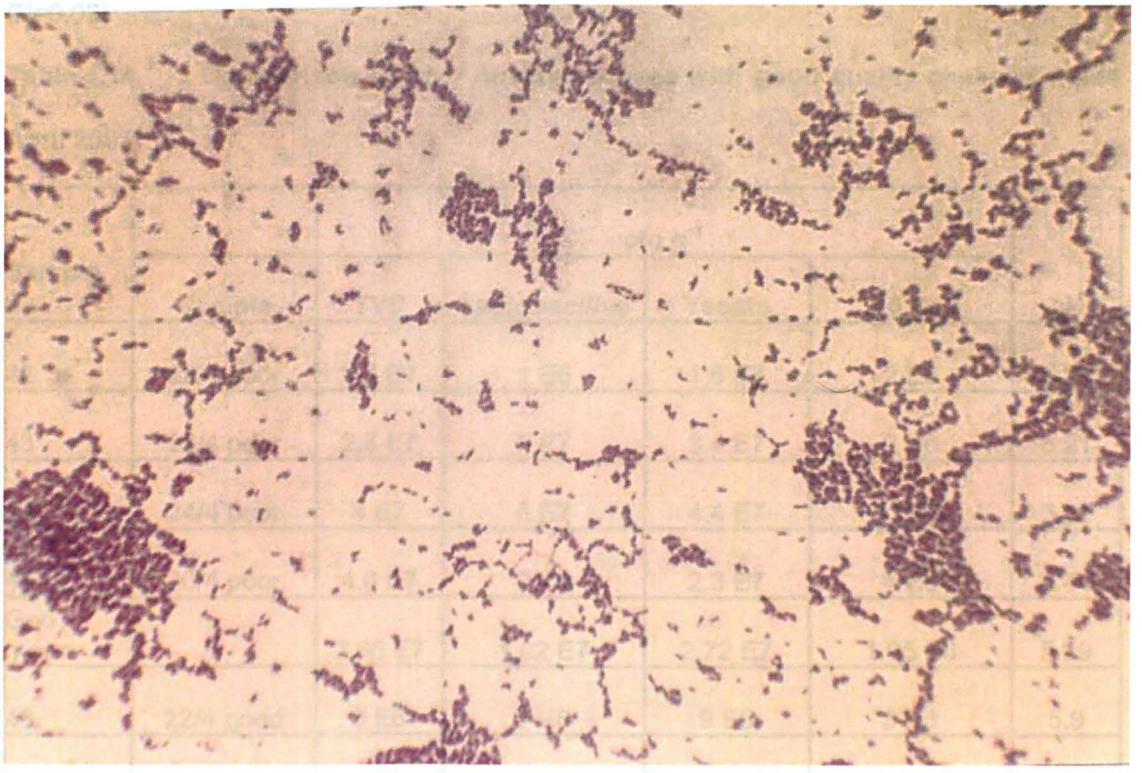


Plate 4.6 ***Lactobacillus brevis*** **from blue Stilton cheese, after Gram staining**

Examined at x1000 magnification and photographed using Nikon SLR camera

4.10 Comparison of Good and Poor quality cheeses made using DVI starters during April 2001

Samples 42-45 were mature, poor quality cheeses, made on 22nd, 23rd 24th and 30th April whilst samples 46-52 were mature, good quality cheeses made on 22nd and 30th April, ie in these tests both good and poor quality cheeses were made on the same day. This is not unusual in the manufacture of Blue Stilton cheese. Samples were tested at 12 weeks of age,

but were not divided into blue and white areas as on the non-blued cheeses there was insufficient blue veining to be sampled, whilst in the good samples blueing was so confluent as to preclude separation. Because of the difference in sample numbers (more good samples than poor quality ones) statistical analysis was performed using the 2 sample t test. Using arithmetic values a statistically significant difference between poor and good quality cheeses was noted in the TVC and mould counts, but not in the TLC and yeast count (Table 4.14). However, when the log value was used the difference in the TLC was also significant (P=0.02)

Table 4.14 Comparison of poor quality cheeses with good quality cheeses, made April 2001

Sample no	Sample	cfu g^{-1}				pH
		TVC	*Lactobacillus*	Yeasts	Moulds	
42	22/4 poor	1.6 E7	1 E6	1.6 E7	2 E6	5.21
43	23/4 poor	2.5 E7	2 E7	3.4 E7	2 E6	5.21
44	24/4 poor	4 E7	6 E7	4.4 E7	<1 E6	5.29
45	30/4 poor	4.8 E7	9 E6	2.3 E7	4 E6	5.44
Geo mean		2.96 E7	1.02 E7	2.72 E7	1.95 E6	5.29
46	22/4 good	8 E6	7 E6	9 E6	9 E6	5.9
47	30/4 good	1.68 E8	3 E6	1.4 E7	4.1 E7	5.85
48	30/4 good	1.17 E8	3 E6	1 E7	8 E6	5.63
49	30/4 good	1.06 E8	<1 E6	1 E7	1.5 E7	5.70
50	30/4 good	8.3 E7	1 E6	1.3 E7	1 E7	5.90
51	30/4 good	7.6 E7	1 E6	1.4 E7	1.3 E7	5.45
52	30/4 good	1 E8	1 E6	9 E6	1.1 E7	5.82
Geo mean		7.25 E7	1.78 E6	1.35 E7	1.14 E7	5.75
2 sample t-test (P)	Poor vs. good	0.04	0.06	0.13	0.05	0.001

Comparison of the pH values of the poor and good quality cheeses showed a significant difference (P = 0.001). However, the pH of the poor samples (mean 5.29) suggests that proteolysis was progressing albeit to a lesser extent than in the good cheese. The values detected in the poor samples reflected those noted in earlier experiments (Sections 4.4, 4.5 and 4.6) and similarly those in the good cheeses were of the same order (P>0.05) as those noted in the previous experiment on good cheese (Table 4.13). Figure 4.4 compares the mould count with the pH values obtained in samples 42-52 and show that generally pH increases with increasing numbers of viable mould counts (r^2=0.57).

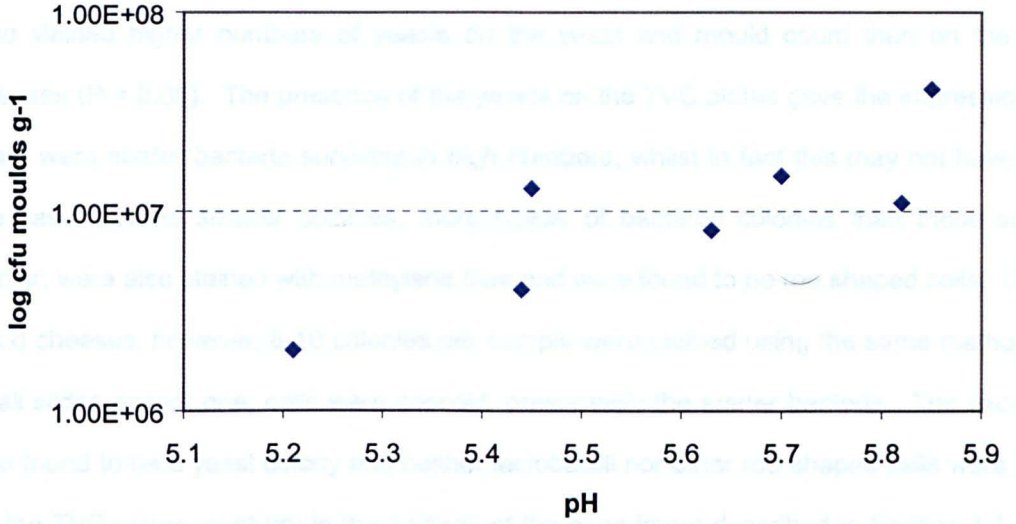


Figure 4.4 Comparison of mould count with pH of Stilton cheese samples (42-52)

Three colonies were selected from the MRS plates of each sample for further identification by Gram stain, catalase and API 50CHL (Appendix 4) and in each case was found to be *Lb. plantarum.* In all previous experiments more than one species of *Lactobacillus* has been isolated, although *Lb. plantarum* has been isolated in every case. The cheeses tested in the current experiment were ripened slightly longer (3-4 weeks longer) than samples from previous experiments. This might explain the detection of only one species of *Lactobacillus,* as other species may have lost viability over the additional ripening period, due to reduced quantities of growth substrate and the wider metabolic capabilities of *Lb. plantarum* as noted in the previous experiments (Sections 4.8 and 4.9). Four strains of *Lb. plantarum* were noted during the current experiment (Appendix 4), all of which had been isolated during these earlier experiments. Again two of the strains (1 and 2) utilised more substrates than the

remaining two (3 and 4) and these pairs of strains showed greater biochemical similarities to each other. The TLC was lower in the current experiment than in those discussed in Sections 4.8 and 4.9, particularly in the good cheeses and this, too, may be due to competition for substrates.

The poor quality cheeses had yeasts growing on the TVC plates, as identified by the glistening colony appearance and confirmed by a methylene blue stain (Section 2.12). Three of such colonies on the lowest dilution of each sample were checked and colonies with this appearance made up approximately 50% of the colonies on the TVC plates. These cheeses also yielded higher numbers of yeasts on the yeast and mould count than on the good cheeses ($P = 0.05$). The presence of the yeasts on the TVC plates gave the impression that there were starter bacteria surviving in high numbers, whilst in fact this may not have been the case. Three smaller colonies, more typical of bacterial colonies than those stained earlier, were also stained with methylene blue and were found to be rod shaped cells. On the good cheeses, however, 8-10 colonies per sample were stained using the same method and in all slides except one, cells were coccoid, presumably the starter bacteria. The exception was found to be a yeast colony and neither lactobacilli nor other rod shaped cells were found on the TVC plates, contrary to the findings of the experiment described in Section 4.4. This may support the suggestion that in areas of blue mould growth the increased pH and/or release of nutrients enables the starter bacteria to continue growing.

Three yeast colonies from the poor quality cheeses were selected for further identification. Two of these yeasts were growing deep within the agar, one in milk agar and one in SDA and since all tests used pour plates these species were clearly able to proliferate in the reduced oxygen conditions found below the agar surface. The following species were confirmed using the API 20C AUX test:

1. yeast growing deep in agar of TVC plate: *Rhodotorula glutinis*
2. Surface yeast from SDA plate: *Candida famata*
3. Deep yeast from SDA plate: *Candida sphaerica*

The biochemical profiles of these species are shown in Appendix 3 and it can be seen that they vary only in the ability to utilise raffinose as a substrate. However, the strain of *C. famata* isolated during this experiment had a very different biochemical profile from that isolated from the organic cheeses discussed in Section 4.4 and was able to utilise 19 of the 20 substrates of the API 20C.

Inhibition tests were carried out using *C. famata* isolated from these cheeses, using the well plate method (Section 2.9) but no inhibition was noted. Previously (Sections 4.4 and 4.6), the stab technique had been used but it was decided to use the well plate technique at this point as a known volume of yeast could be used which might give an indication of critical numbers necessary to induce inhibition. Several techniques were used to see if the conditions of growth influenced the production of anti-fungal substances. Initially *P. roqueforti* was added to the surface of the agar and allowed to dry before preparing the wells for the yeast, but no inhibition was detected by this method. The approach was then changed by adding the mould to the base of the dish, so that it was mixed into the agar, to enable more even distribution and to eliminate the need for drying the plates. This had the effect of making the technique easier to perform and avoided aerial contamination during drying, since the lids could be left on the dishes. Again no inhibition was noted so the process was then reversed, with the yeast being added to the agar and the *P. roqueforti* to the well but in no case was inhibition noted. All tests were carried out both using both aerobic and anaerobic incubation.

However, when a culture of *P. roqueforti* was inoculated onto a lawn of *C. famata* that had previously been inoculated into wells and incubated for 5 days, no growth of the mould was apparent (Plate 4.7). This may be due to the production of anti-fungal substances but may also be due to competition for growth substrates, since there had been confluent growth of the yeast. There is potential for this to prevent blue vein development in cheeses if the yeast species can become established prior to piercing of the cheese. If substrate competition is significant the mould may be unable to develop sufficiently post-piercing and sporulation may not take place. The fungal counts in all poor cheeses have been of the order of log 6 and this may simply be due to residual mould starter rather than actual growth of the species.

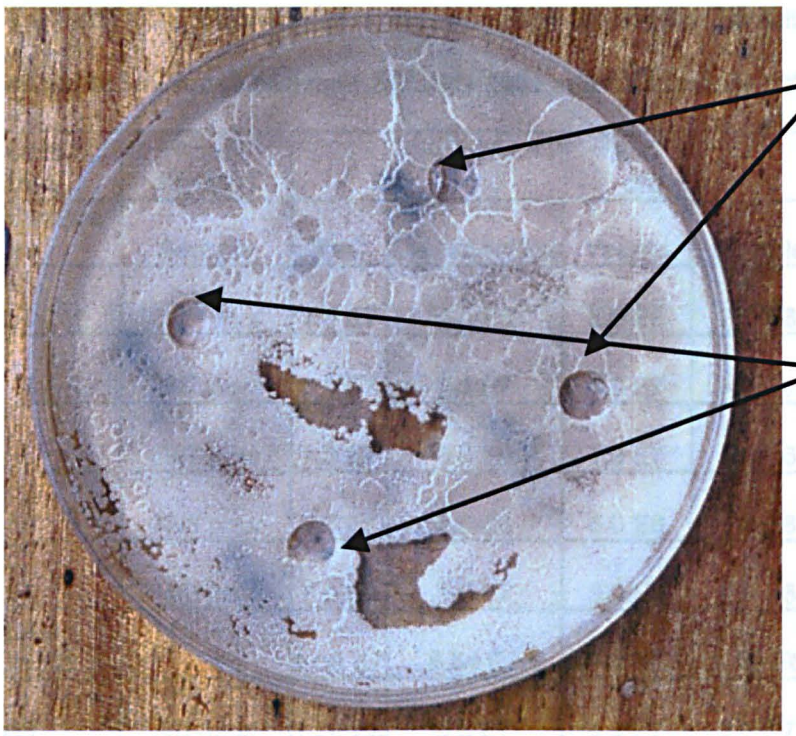


Plate 4.7 **Lawn of *Candida famata* showing no growth of *Penicillium roqueforti***

4.11 Comparison of good and poor quality mature cheeses produced between 23rd July 2001 and 13th August 2001

Several batches of cheeses were sampled on 10th October 2001 and compared as before. The cheeses were produced between 23rd July and 13th August and thus were sampled after 8-12 weeks ripening. The good quality samples exhibited ample blue veining and thus it was impossible to differentiate between blue and white areas. On the other hand the poor quality samples exhibited such a small level of veining that 10g of blue area could not be retrieved, even out of 2 kg samples of cheese and as such only the white areas were tested. It was decided to test the yeast count under anaerobic incubation at the same time as the standard yeast count was performed so that a more direct comparison could be made, with results both coming from the same 10g sub-sample. The pH of the samples was also measured in order to compare good and bad cheeses, particularly since samples 57 and 58 were made on the same day.

Table 4.15 Results of tests on good and poor quality, mature (8-12 weeks) cheeses produced between 23rd July 2001 and 13th August 2001

Date of test	10.10.01	cfu g^{-1}					
Sample no	Sample	TVC	*Lactobacillus*	Yeasts	Moulds	Anaerobic yeast	pH
53	23/7 poor	1.0 E7	4.0 E6	5.0 E6	2.0 E6	5.0E6	4.50
54	28/7 poor	7.0 E7	7.1 E7	1.9 E7	<1 E6	7.2 E7	4.61
55	29/7 poor	1.2 E7	8.0 E6	1.3 E7	1.0 E6	2.0 E6	4.66
56	02/8 poor	6.7 E7	2.0 E6	6.0 E6	1.0 E6	3.0 E6	4.51
57	05/8 poor	7.0 E6	1.0 E7	3.0 E6	2.0 E6	5.0 E6	4.61
Geo mean		2.08 E7	8.54 E7	7.40 E6	1.29 E6	6.41 E6	4.58
58	5/8 good	1.24 E8	9.3 E7	4.4 E7	2.7 E7	1.36 E8	5.80
59	7/8 good	2.0 E6	4.7 E7	1.8 E7	8.0 E6	1.4 E7	5.91
60	8/8 good	3.0 E6	5.0 E6	6.0 E6	1.2 E7	5.0 E6	5.52
61	10/8 good	1.09 E8	8.2 E7	5.5 E7	6.0 E6	9.6 E7	5.61
62	13/8 good	4.6 E7	2.8 E7	4.8 E7	7.0 E6	3.5 E7	5.80
Geo mean		2.06 E7	3.47 E7	2.63 E7	1.02 E7	3.17 E7	5.73
2 sample t-test (P)	Poor vs. good	0.45	0.17	0.04	0.03	0.20	<0.001

The results (Table 4.15) show that there was a highly significant difference (P = <0.001) between the pH of the two sets of samples with the poor cheeses always exhibiting pH values of around 4.6 and good cheeses around 5.7. These pH values reflect the findings of Madkor *et al.* (1987b) and the unpublished results of Hough (2001, pers. comm.) which showed that the pH of Stilton curd decreases to approximately 4.5 at salting and then increases as a result of proteolysis to approximately 6.0 at the end of ripening. Zarmpoutis *et al.* (1996) noted similar changes in pH in Chetwynd blue, an Irish farmhouse blue cheese. It can be assumed that the poorly blued cheeses had not undergone proteolysis and thus the pH stayed at the young curd values. These pH results reflected the findings of experiment 4.6, but were lower than those in other samples of poor cheeses (Sections 4.4, 4.5, 4.6 and

4.10). Since sample 53 was ripened for 12 weeks it is unlikely that this low pH is attributable to the age of the cheese. Comparison of the pH with the mould count (Figure 4.5) shows that this difference might be due to growth of the mould rather than any other factor (r^2=0.48).

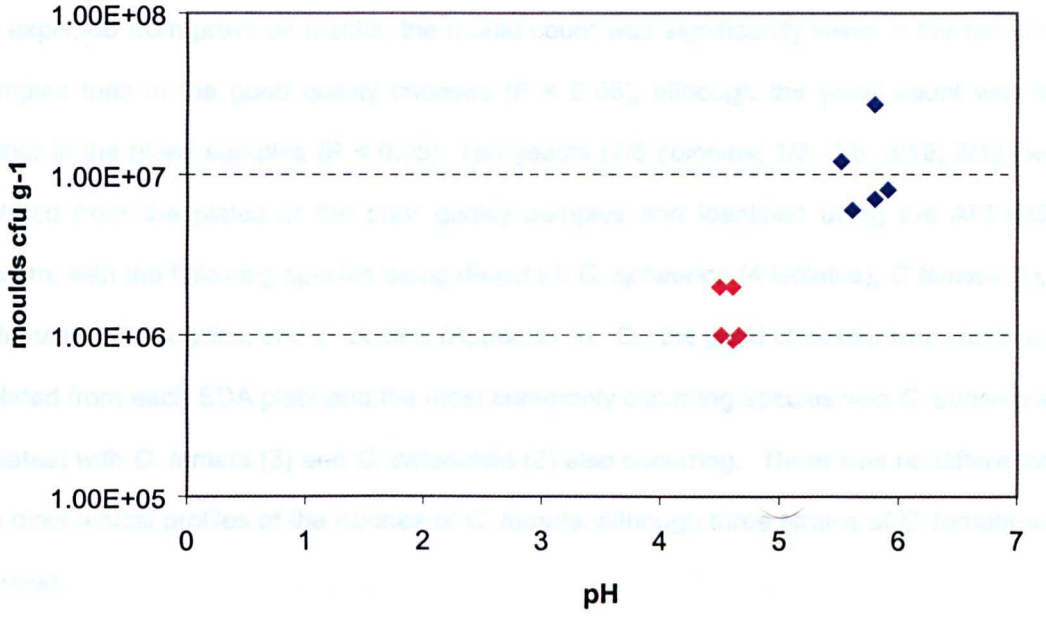


Figure 4.5: Comparison of mould growth with pH of Stilton cheese

The TVC of the poor and good quality cheeses were extremely close (P>0.05), although samples 57 (poor) and 58 (good), manufactured on the same day, showed a wide variation. Given that the raw milk is pumped into silos prior to pasteurisation there is potential for the milk to be identical in origin. However, it may be that one of the cheeses originated from a vat made using the previous night's milk, which may have had a higher bacterial count. It was noted in Chapter 3 that stored milk produced a slightly higher, although still low, count of lactobacilli in the pasteurised milk, so if sample 57 had been made from stored milk then the TLC might be greater than that in sample 58. It was further hypothesised that stored milk could result in down-graded product, but if storage results in increased levels of microorganisms prior to pasteurisation and subsequent survival of pasteurisation, one would expect higher TVC and lactobacilli counts in the product. If the use of stored milk does lead to lower quality product, then it seems more likely that it is due to the production of heat

stable antimicrobial substances, which are able to survive the pasteurisation process. Niku-Paavola *et al.* (1999) demonstrated the heat resistance of anti-microbial substances produced by *Lb. plantarum* VTT E-78076 and thus it is possible that similar substances could be important in milk for cheese manufacture.

As expected from previous results, the mould count was significantly lower in the non-blued samples than in the good quality cheeses ($P < 0.05$), although the yeast count was also higher in the blued samples ($P < 0.05$). Ten yeasts (2/5 colonies; 1/3; 2/6; 3/19; 2/13) were isolated from the plates of the poor quality samples and identified using the API ID32C system, with the following species being detected: *C. sphaerica* (4 isolates), *C famata* (3), *C. catenulata, C. lipolytica,* and *C. boidinii* (Appendix 3). On the good cheeses, two yeasts were isolated from each SDA plate and the most commonly occurring species was *C. sphaerica* (5 isolates) with *C. famata* (3) and *C. catenulata* (2) also occurring. There was no difference in the biochemical profiles of the isolates of *C. famata*, although three strains of *C. famata* were isolated.

There was little difference between the aerobic and anaerobic yeast counts, as may be expected from previous results, although there was a difference in the aerobic yeast count, which was lower ($P < 0.05$) in the poor cheeses than in the good ones. In previous experiments there have been greater numbers of yeasts in poor cheeses than in good cheeses, so this seems to suggest that the number of yeasts present has little influence on the ability of the blue mould to develop and that it is a number of factors that affect this quality. These factors may include the species of yeast present, but equally the salt content and the available oxygen may have an influence. *C. sphaerica, C. famata* and *C. lipolytica* were isolated from the anaerobically incubated plates in equal amounts, whilst *C. boidinii* and *C. catenulata* were not detected on these plates.

Identification of the lactobacilli was conducted as in previous experiments (Appendix 4) and the good cheese was found to contain only *Lb. plantarum*, whilst this was not isolated from the samples of poor quality cheese. In these samples (53-57) *Lb. brevis* and *Lb. paracasei*

subsp. *paracasei* were isolated. The heterofermentative species *Lb. brevis* was isolated frequently during the course of this study, both from good and poor quality cheeses, although it occurred more frequently in poor quality cheeses. Only one strain of this species was isolated during the current experiment and it was markedly different in its biochemical capabilities from those strains previously isolated. Such variation in strains may well give rise to flavour differences between cheeses, since the fermentation of different sugars may produce varying flavour compounds. The strain isolated during the current experiment utilised a very limited number of substrates and it may be that this ability to grow using fewer substrates enabled its survival. *Lb. paracasei* subsp. *paracasei* (an homofermentative species) had not been isolated previously during this study.

The two strains of *Lb. plantarum* isolated during this experiment were different from those isolated in previous experiments and exhibited the ability to utilise a wide range of substrates. It is possible that if 100% of the colonies occurring on these samples had been tested, a wider range of strains would have been isolated and these might have included other strains detected in previous experiments. The cheeses sampled during the current experiment ranged in age from 8-12 weeks and the predominance of *Lb. plantarum*, particularly in the good cheeses might relate to the maturity of the cheese and the growth of *P. roqueforti*, since *Lb. plantarum* may have competed more successfully for the substrates

4.12 Summary of results

Table 4.16 shows a summary of the mean results in order to compare different batches. For the purpose of calculating the geometric mean all samples of less than 1 at the lowest dilution have been converted to 0.9 at that dilution.

Table 4.16 Summary of results of viable counts

				cfu g^{-1}				
Sample	Table no.	DOM	Age (weeks)	TVC	TLC	Yeasts	Moulds	pH
DVI starter – good cheese	4.2	12/98	9	1.9 E7	6.1 E7	>3 E6	>3 E6	-
DVI starter – good cheese blue	4.11	04/99	8	1.6 E9	6.8 E7	2.83 E6	2.59 E7	-
DVI starter – good cheese white	4.11	04/99	8	2.7 E8	1.21 E8	6 E6	8 E6	-
DVI starter – good cheese white	4.13	12/99	8	2.85 E7	1.98 E7	1.10 E7	6.51 E6	5.55
DVI starter – good cheese blue	4.13	12/99	8	4.87 E7	4.56 E7	1.44 E7	2.82 E7	5.95
DVI starter – good cheese	4.13	12/99	8	5.5 E7	7.6 E7	5 E6	4.1 E7	6.10
DVI starter – good	4.14	04/01	12	7.25 E7	1.78 E6	1.35 E7	1.14 E7	5.75
DVI starter – good	4.15	7,8/01	8-12	2.06 E7	3.47 E7	2.63 E7	1.02 E7	5.73
Mean good cheese DVI starter				**6.83 E7**	**3.91 E7**	**8.50 E6**	**1.28 E7**	
DVI starter – poor cheese	4.2	12/98	9	1 E6	1.8 E7	7.2 E5	>3 E6	-
DVI starter – poor cheese blue	4.3	12/98	9	1.11 E8	8.02 E7	1.21 E7	3.67 E7	-
DVI starter – poor cheese white	4.3	12/98	9	9.67 E7	5.99 E7	1.51 E7	5.38 E6	-
DVI starter – poor cheese blue	4.4	04/99	9	1.15 E8	1.36 E8	6.93 E6	1.99 E7	-
DVI starter – poor cheese white	4.4	04/99	9	2.51 E6	2.38 E7	6.48 E6	9.49 E5	-

				cfu g^{-1}				
Sample	Table no.	DOM	Age (weeks)	TVC	TLC	Yeasts	Moulds	pH
DVI starter – poor cheese	4.9	04/00	12	1.18 E7	4.52 E7	1.93 E7	1.00 E6	4.79
Organic cheese – poor	4.6	08/99	12	4.53 E7	4.55 E7	4.80 E7	1.41 E6	5.10
DVI starter – poor cheese	4.14	04/01	12	2.96 E7	1.02 E7	2.72 E7	1.95 E6	5.29
DVI starter - poor cheese	4.15	7,8/01	8-12	2.08 E7	8.54 E7	7.40 E6	1.29 E6	4.58
DVI starter - poor cheese, white	4.8	09/99	12	2.83 E6	2.43 E7	9.49 E5	2.83 E6	5.74
DVI starter - poor cheese, blue	4.8	09/99	12	1.35 E7	3.2 E7	1.52 E7	2.69 E7	5.73
Mean poor cheese – DVI starter				**2.15 E7**	**5.15 E7**	**9.16 E6**	**2.6 E6**	
Bulk starter - good cheese	4.2	12/98	9	<1 E6	4.4 E8	>3 E6	>3 E6	-
Bulk starter – good cheese, blue	4.12	04/99	8	1.69 E8	1.84 E8	2 E6	3.5 E7	-
Bulk starter – good cheese white	4.12	04/99	8	3.83 E6	9.22 E7	1.82 E6	< 1 E6	-
Mean bulk starter good cheese				**8.35 E6**	**1.95 E8**	**2.22 E6**	**4.56 E6**	

DOM = Date of manufacture

Graphical representation of the results of Table 4.16, the mean results of individual experiments, enables the relationships between data to be demonstrated more clearly, but should be read with Table 4.17, which is a statistical analysis of the data.

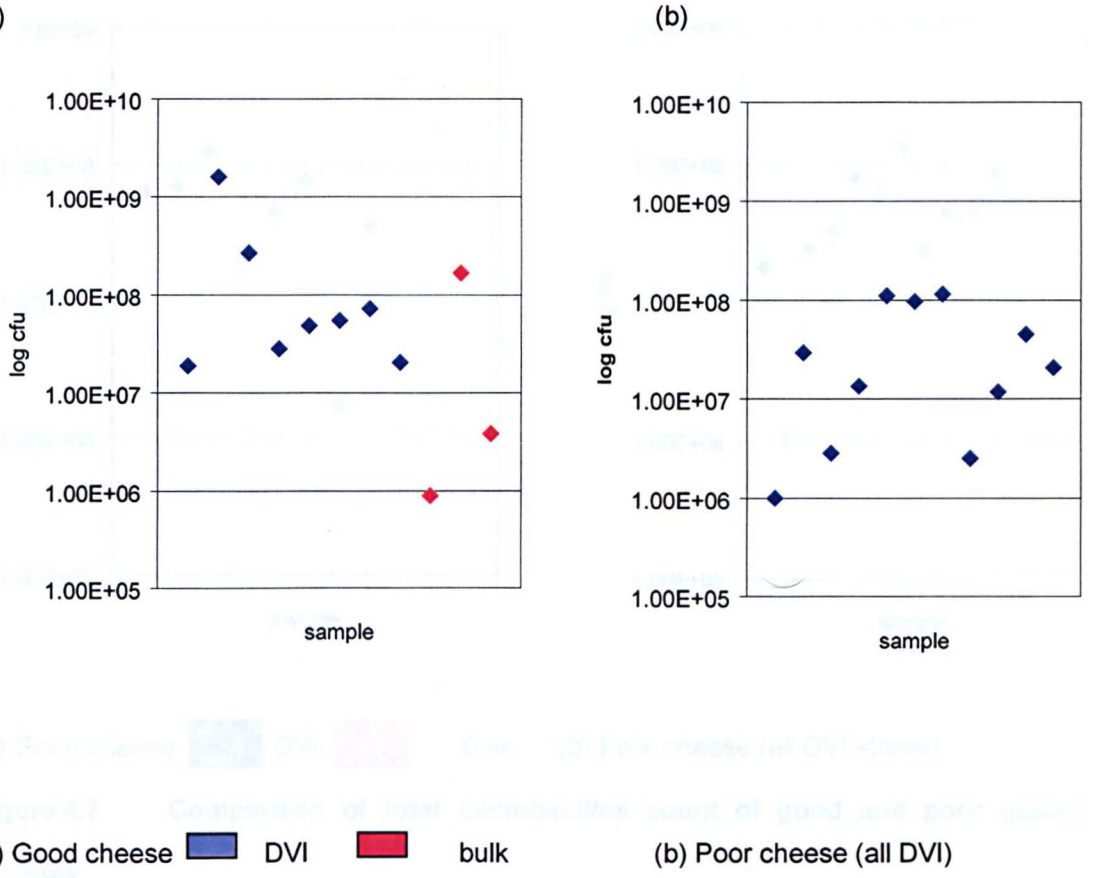


Figure 4.6 Comparison of TVC of good and poor quality Stilton cheese

The graphs in Figure 4.6 a) and b) show the mean TVC of all the cheeses tested and demonstrate that there was some difference between the good and the poor samples. The good cheese show more consistent results, particularly if the samples of cheese made with bulk starter (shown in red) are ignored (since these were a minority of samples during the study and might be different from those produced using DVI starters). It appears that the TVC of mature blue Stilton is likely to be in the region of 10^6 to 10^9 irrespective of whether or not the quality of the cheese, in terms of blue veining, is acceptable, but that the values tend towards the lower end of the range in poor quality cheeses and may be more variable. The TVC of good cheeses was significantly different ($P < 0.05$) from that of poor cheeses (Table

4.17) and Figure 4.6 indicates that cheeses with a TVC of less than 10^7 are likely to be of a poorer quality than those with a higher count.

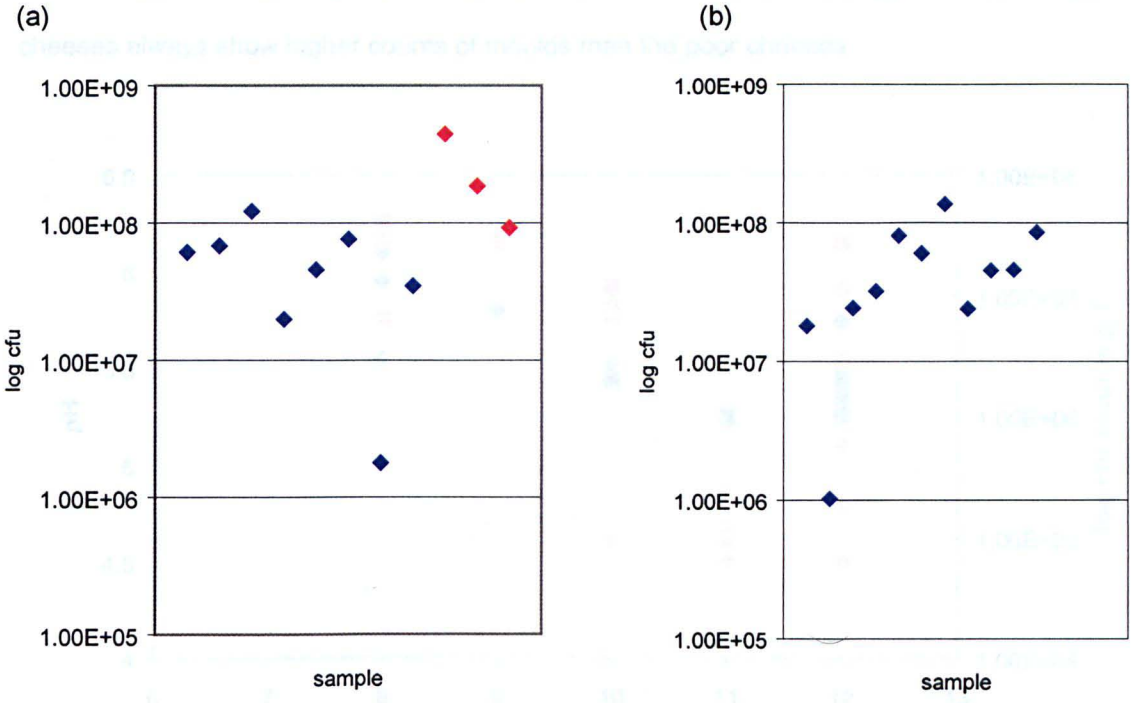


Figure 4.7 Comparison of total *Lactobacillus* count of good and poor quality cheeses

The results in Figure 4.7 a) and b) show that the TLC varied very little between good and poor quality cheeses and suggest that the quality of cheeses, with regard to amount of blue veining, is not related to the number of lactobacilli present, although the species represented in samples may influence blue vein development. This study did not investigate the potential for individual species of lactobacilli to produce anti-microbial substances effective against strains of *P. roqueforti*, which might be the subject of a future study. However, the study did determine the commonly occurring lactobacilli within samples, which is discussed more fully in Section 4.13.

When pH and total viable mould counts are plotted on the same graph (Figure 4.8) it can be seen that there is no relationship between age and pH, since higher pH values were

achieved on some 8 week old cheeses than on those ripened for 12 weeks, irrespective of the quality of the cheeses. Similarly there appears to be no relationship between the total viable mould count and age (when restricted to 8-12 weeks of ripening), although the good cheeses always show higher counts of moulds than the poor cheeses.

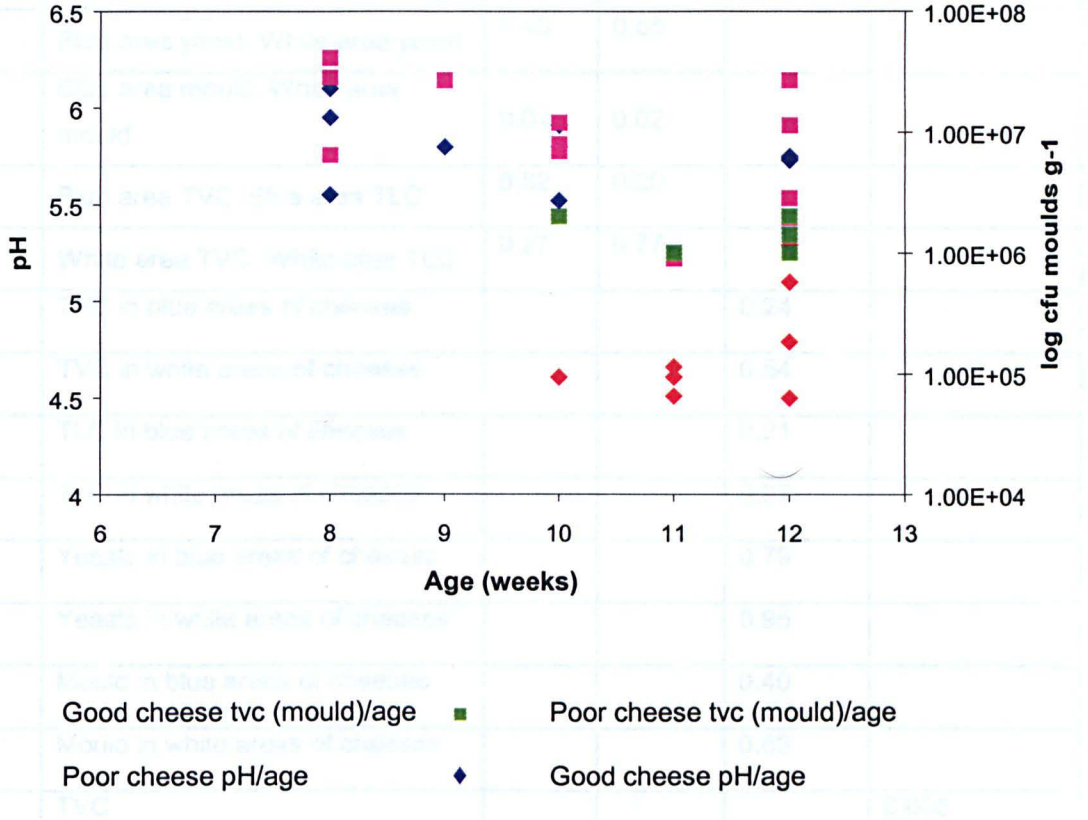


Figure 4.8 Comparison of pH and total viable mould count with age of cheese

Statistical analysis (Table 4.17) was performed on the raw data rather than the mean counts, in order to give more meaningful results. Table 4.17 compares the TVC (row 1), TLC (row 2), yeasts (row 3) and moulds (row 4) in the blue and the white areas of poor cheeses (column 2) and in that of good cheeses (column 3). Column 4 represents a comparison of similar areas of good and poor cheeses in order to determine whether or not there was a significant difference in any of these. For example, TVC (row 5) is compared between blue areas of good and poor cheeses. Column 5 compares cheeses where blue and white areas were undifferentiated, either due to luxuriant growth of *P. roqueforti,* or insufficient blue veining.

Table 4.17 2 sample T-test comparison of all data

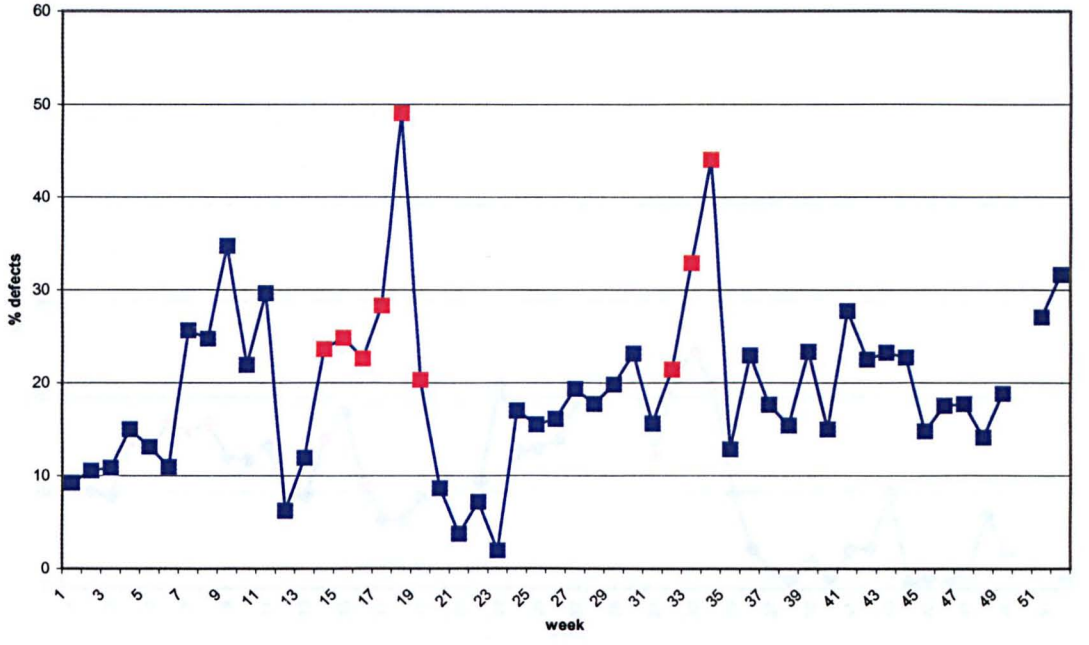
Row	Comparison	Poor cheese	Good cheese	Good: Poor	Good: Poor (composite samples)
1	Blue area TVC: White area TVC	0.02	0.14		
2	Blue area TLC: White area TLC	0.03	0.36		
3	Blue area yeast: White area yeast	0.43	0.55		
4	Blue area mould: White area mould	0.04	0.02		
5	Blue area TVC: Blue area TLC	0.82	0.20		
6	White area TVC: White area TLC	0.27	0.77		
7	TVC in blue areas of cheeses			0.24	
8	TVC in white areas of cheeses			0.54	
9	TLC in blue areas of cheeses			0.21	
10	TLC in white areas of cheeses			0.97	
11	Yeasts in blue areas of cheeses			0.79	
12	Yeasts in white areas of cheeses			0.95	
13	Mould in blue areas of cheeses			0.40	
14	Mould in white areas of cheeses			0.69	
15	TVC				0.005
16	TLC				0.29
17	Yeasts				0.34
18	Moulds				0.03

Comparison of all of the results of this study suggests that there is little difference in the microflora of the blue and white areas of good cheese, except in the number of moulds counted. However, in poor cheeses the blue areas differ from the white areas in terms of TVC, TLC and moulds (rows 1, 2 and 4), with higher counts of each of these in the blue areas than in the white. When samples were differentiated between blue and white areas there was little difference between good and poor cheeses in terms of counts of individual

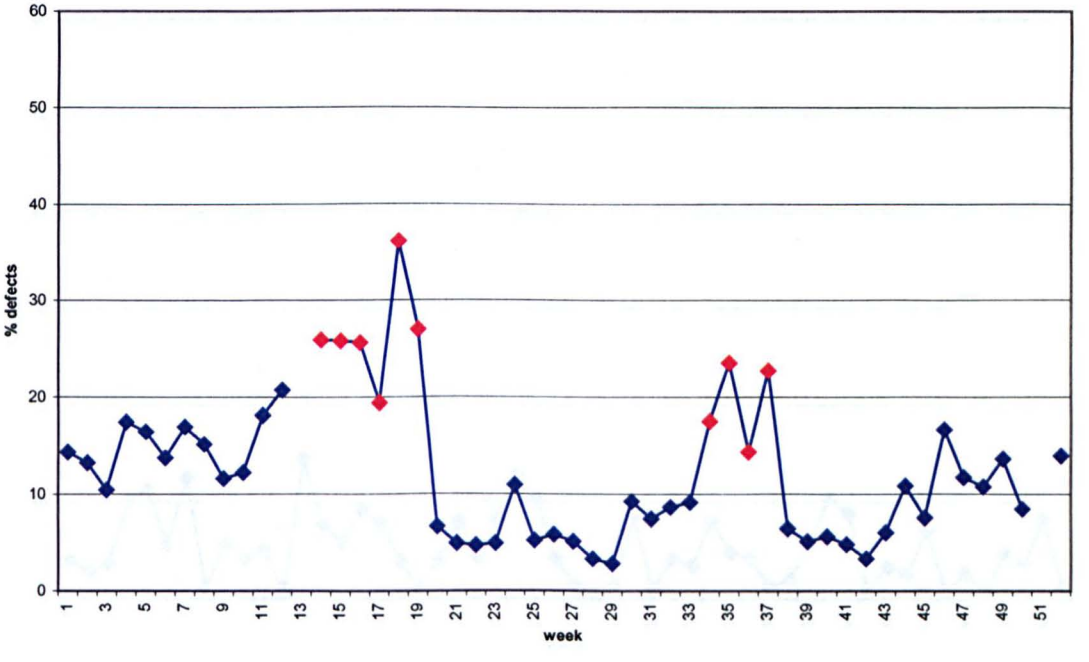
areas (rows 7-14), that is in the blue areas there was little difference between the TVC of good and poor cheeses and this was also true for the white areas (Table 4.17).

Comparison of the samples where there was no differentiation between blue and white areas (row 15) shows a highly significant difference in the TVC of good and poor cheeses with higher counts being obtained in good cheeses. The TLC (row 16) did not vary significantly between good and poor cheeses, which may suggest that there was greater survival of the starter bacteria in the good cheeses, since the TVC could include some lactobacilli, as well as starter bacteria. In the experiment using poor cheeses, described in Section 4.4, both cocci and rods were detected, by Gram staining colonies from TVC plates, although in Section 4.10 only cocci and yeasts were noted on samples of poor cheeses. That is, the TVC may include a number of groups of microorganisms and the detection of higher TVC in good cheeses, combined with no significant difference in TLC might indicate that this difference is due to the presence of larger numbers of starter bacteria.

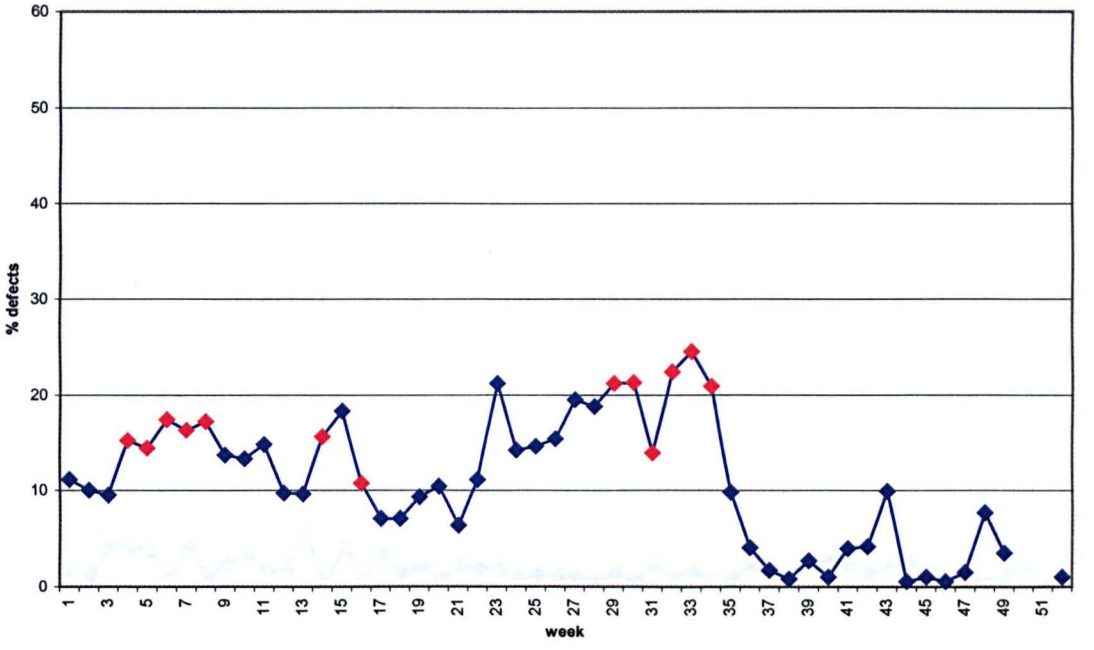
Of the poor quality (defective) samples tested during this study, a large proportion appear to have been manufactured during April. Examination of the percentage defects during the period 1995-2000 (Figure 4.9 a to f) suggests that weeks 13-21 (or thereabouts) may be a significant time for the production of defective product at this dairy, as on each graph there appears to be an increase in the percentage of defective cheeses at some point in this period. Similarly, a higher proportion of defects tended to occur during weeks 32-36 (August-September). However, a general decrease in the number of defective cheeses occurred during this period, with the yearly averages reducing from 19.63% in 1995 to 1.8% in 1999. This implies that these risk periods are now less significant, but the year 2000 (Figure 4.9 f) still shows a small rise during weeks 11-15 (end March to mid April) and weeks 27 – 29 (July). The results displayed in Figure 4.9 are shown as a percentage of total production during the period 1995-2000. Gaps occur where no data were available.



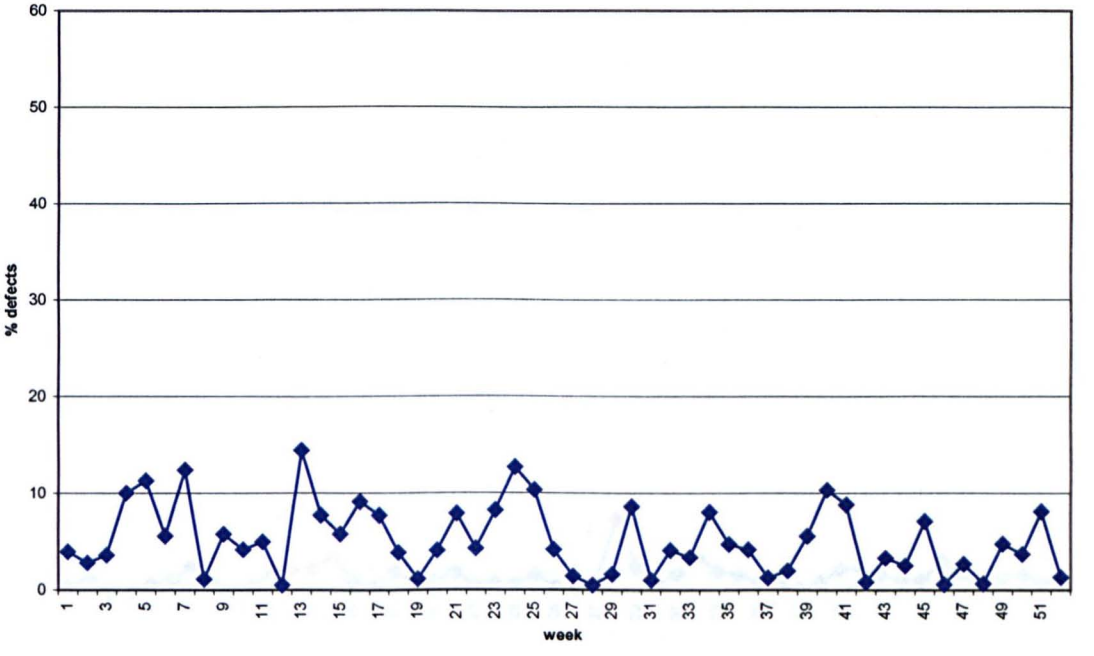
a) 1995



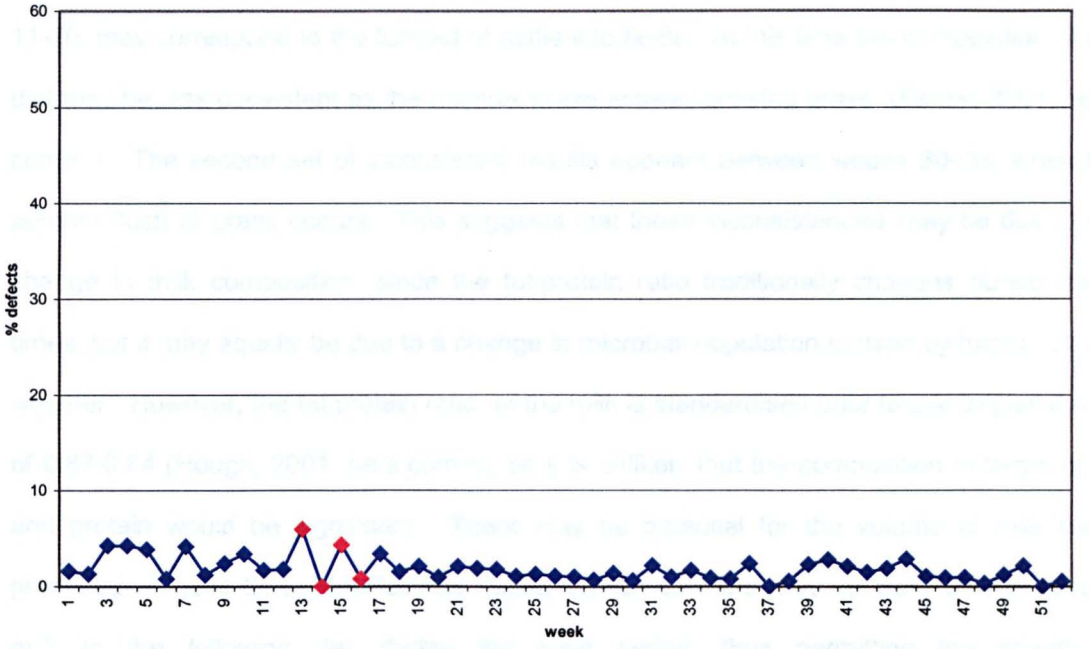
b) 1996



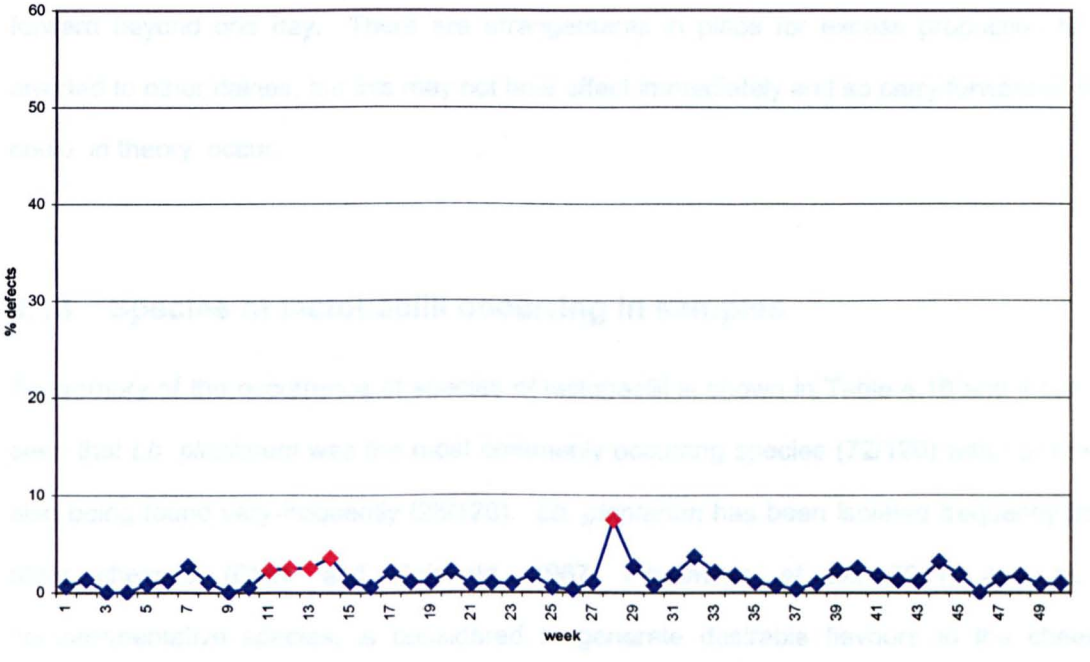
c) 1997



d) 1998



e) 1999



f) 2000

■ apparent loss of control of quality

Figure 4.9 Defective cheeses from the main plant studied.

The reduced consistency in quality during the early part of the year, particularly during weeks 11-20, may correspond to the turnout of cattle into fields. At this time the composition of the diet may be less consistent as the animals graze actively growing grass (Saxby, 2001, pers. comm.). The second set of inconsistent results appears between weeks 30–35, when the autumn flush of grass occurs. This suggests that these inconsistencies may be due to the change in milk composition, since the fat:protein ratio traditionally changes during these times, but it may equally be due to a change in microbial population caused by turnout or wet weather. However, the fat:protein ratio of the milk is standardised prior to use to give a ratio of 0.87:0.84 (Hough, 2001, pers.comm), so it is unlikely that the composition in terms of fat and protein would be significant. There may be potential for the volume of milk being produced at these times to affect the quality insofar as there may be carry over of excess milk to the following day during the flush period, thus permitting the growth of microorganisms in the raw milk. Potentially excess milk could be carried forward on a daily basis for several days although stock rotation should ensure that the same milk is not carried forward beyond one day. There are arrangements in place for excess production to be diverted to other dairies, but this may not take effect immediately and so carry forward of milk could, in theory, occur.

4.13 Species of lactobacilli occurring in samples

A summary of the occurrence of species of lactobacilli is shown in Table 4.18 and it can be seen that *Lb. plantarum* was the most commonly occurring species (72/120) with *Lb. brevis* also being found very frequently (28/120). *Lb. plantarum* has been isolated frequently from other cheeses (Clark and Reinbold, 1967; Fitzsimons *et al.*, 2001) and as a homofermentative species, is considered to generate desirable flavours to the cheese. However, *Lb. brevis* is a heterofermentative species and its presence is generally considered to be undesirable in cheeses since it results in the production of fruity flavours (Laleye *et al.*, 1990).

Table 4.18 Summary of species of lactobacilli detected during the study

Sample	*Lb. plantarum*	*Lb. brevis*	*Lb. curvatus*	*Lb. casei*	*Lb. paracasei* subsp. *paracasei*
LV 191923 poor	6/8	2/8			
LV 201924 poor	3/6	3/6			
40 poor	4/6	2/6			
41 poor	3/4	1/4			
14-16 good	3/9		3/9	3/9	
17-22 good	4/12	4/12	4/12		
31-39 good	6/18	6/18	6/18		
42-52 good and poor	33/33				
53-57 poor		10/14			4/14
58-62 good	10/10				
Total	72/120	28/120	13/120	3/120	4/120

The presence of *Lb. brevis* mostly coincided with the poor quality cheeses (28/38), although where it was detected in good cheeses both *Lb. plantarum* and *Lb. curvatus* were also present. It may be that in those cheeses where all three species were detected, the growth of the heterofermentative species (*Lb. brevis*) was limited by competition from the homofermentative species (*Lb. plantarum, Lb. curvatus* and *Lb. paracasei*) and thus the production of undesirable compounds was lessened. Comparison of the biochemical profiles of the lactobacilli isolated (Appendix 4) reveals differences in the fermentable substrates within species. These are summarised in Table 4.19.

Table 4.19 Assimilation pattern for strains of lactobacilli identified during the study

Substrate	*Lb. plantarum*	*Lb. curvatus*	*Lb. brevis*
Glycerol	6/14	0/3	0/4
Erythritol	0/14	0/3	0/4
D-Arabinose	0/14	0/3	0/4
L-Arabinose	1/14	0/3	2/4
Ribose	13/14	3/3	4/4
D-Xylose	1/14	0/3	3/4
L-Xylose	0/14	0/3	0/4
Adonitol	0/14	0/3	0/4
β methyl-xyloside	0/14	0/3	0/4
Galactose	14/14	3/3	4/4
D-Glucose	14/14	3/3	4/4
D-Fructose	14/14	3/3	4/4
D-Mannose	14/14	3/3	1/4
L-Sorbose	0/14	0/3	0/4
Rhamnose	2/14	0/3	0/4
Dulcitol	0/14	0/3	0/4
Inositol	0/14	0/3	0/4
Mannitol	14/14	3/3	2/4
Sorbitol	5/14	0/3	0/4
α Methyl-D-mannoside	3/14	0/3	0/4
α Methyl-D-glucoside	0/14	0/3	2/4
N acetyl glucosamine	14/14	3/3	4/4
Amygdaline	14/14	0/3	1/4
Arbutine	8/14	0/3	1/4
Aesculin	2/14	0/3	0/4
Salicine	8/14	2/3	3/4
Cellobiose	10/14	1/3	1/4
Maltose	14/14	3/3	4/4
Lactose	14/14	3/3	4/4
Melibiose	8/14	0/3	2/4
Saccharose	8/14	3/3	1/4
Trehalose	8/14	3/3	1/4
Inuline	0/14	0/3	0/4

Substrate	*Lb. plantarum*	*Lb. curvatus*	*Lb. brevis*
Melezitose	6/14	3/3	0/4
D-Raffinose	5/14	0/3	0/4
Amidon	1/14	0/3	0/4
Glycogene	0/14	0/3	0/4
Xylitol	0/14	0/3	0/4
β Gentiobiose	12/14	0/3	1/4
D-Turanose	2/14	3/3	0/4
D-Lyxose	0/14	0/3	0/4
D-Tagatose	0/14	3/3	0/4
D-Fucose	0/14	0/3	0/4
L-Fucose	0/14	0/3	0/4
D-Arabitol	4/14	0/3	1/4
L-Arabitol	0/14	0/3	0/4
Gluconate	7/14	0/3	4/4
2 ceto-gluconate	0/14	0/3	0/4
5 ceto-gluconate	1/14	0/3	3/4

Fourteen strains of *Lb. plantarum* were apparent according to the assimilation patterns. Since such a wide range of strains was present in the cheeses it is to be expected that the species would predominate the microflora, as if one strain died out another may be able to continue growth. The presence of a range of strains, which exhibit the ability to utilise different substrates may give rise to subtle flavour differences between batches of cheese.

Only three strains of *Lb. curvatus* were noted during the study and these strains had few differences in their assimilation patterns, differing in only two substrates, salicine and celobiose. The species was only detected in samples of good quality cheese.

When the isolates from samples 31-39 are compared with those from the cheeses made with bulk starter (17-22), greater differences can be seen in fermentable substrates: the *Lb. brevis* varied in 10 substrates, whilst the *Lb. plantarum* varied in 9. This could potentially yield a product with a very different flavour profile from that made with the DVI starter. Since lactobacilli are not included in the starters, this poses a question as to why the strains of

lactobacilli occurring in cheese made with bulk starter were so different from those occurring in cheeses made with DVI starter.

As discussed previously (Section 4.11), the isolates of *Lb. brevis* detected in the poor quality cheeses (53-57) showed very different profiles from any of those found in the good quality cheeses, which also may indicate that the strain could be responsible for some undesirable qualities in these cheeses. Some of the metabolites of these substrates may be responsible for inhibition of the blue mould and this is an area that should be the subject of a future study.

4.14 Inhibition tests

In order to attempt to replicate the inhibition noted using the stab technique (Section 4.4), yeast colonies were selected and tested against *P. roqueforti* using the same method. Yeasts were isolated and confirmed as *C. famata* prior to conducting the experiments. The isolate used was profile number 6166676 (Appendix 3) which gave no inhibition of *P. roqueforti* when tested on SDA with 1% w/v NaCl addition. Since NaCl has been shown to be stimulatory to *P. roqueforti* at low concentrations (such as 1% w/v) and restrictive at concentrations of >3% w/v (Godinho and Fox, 1981; Lopez-Diaz *et al.*, 1996), it was decided to test the potential for inhibition in high salt conditions. Salt in moisture concentrations of 5.5% w/v are typical of Stilton cheese (Table 1.3), although salt is added to the curd at a rate of 2-2.5% w/v. SDA was prepared with NaCl added at a rate of 6% w/v and poured into petri dishes. After allowing the agar to set *C. famata* was streaked onto the agar and *P. roqueforti* was crossed streaked at 90°, whilst not allowing the two to come into contact. At the same time further plates were prepared, one set containing *P. roqueforti* only, as a control to compare growth and another with two streaks of *P. roqueforti*, of which one was prepared immediately and one prepared after 30 minutes incubation at ambient temperature, to allow the *C. famata* limited time to establish.

Plates 4.8 a) and b) show that there was a reduction in growth of *P. roqueforti* at 6% w/v NaCl when *C. famata* was present. Allowing the yeast a short time (30 minutes) to establish, affected the growth of the mould very slightly (Plate 4.8a), but the colony diameter on the plate without *C. famata* was significantly greater than that on the plates containing *C. famata*. After 5 days incubation under anaerobic conditions, there was ample growth of *C. famata* but very limited growth of *P. roqueforti*, whilst 10 days of incubation resulted in some growth of *P. roqueforti* (colony diameter 1mm) on the plates containing *C. famata* and luxuriant growth (colony diameter 5 cm) on the plate containing *P. roqueforti* only.

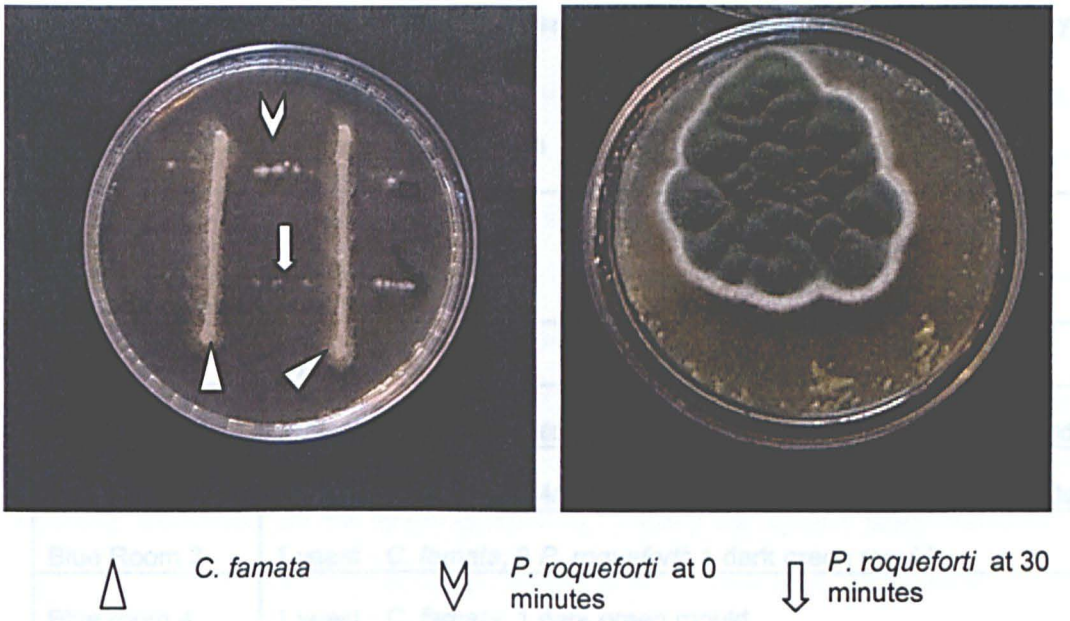


Plate 4.8 Growth of *P. roqueforti* on SDA + 6% NaCl w/v, a) in the presence of *C. famata* and b) in the absence of *C. famata* after incubation for 10 days at 22°C

(Photographed using Kodak MX digital camera)

In practice, *C. famata* may have sufficient time to become established in the curd before proliferation of the blue veins is expected.

4.15 Examination of aerial contamination using exposure plates

In order to determine the presence of yeasts in the environment, exposure plates were taken in five ripening rooms plus the vat room of the dairy, during July 2001. The results (Table 4.20) show that in two rooms, the hastener (where the hoops containing curd fresh from the vat are stored at 21°C for five days) and the white room (the next stage of maturing, with a temperature of approximately 10°C), five or more yeasts were detected, whereas in all other rooms no more than one yeast was found. These were identified using the API ID32C system as before and were identified as *C. famata* (in the white room) and *C. catenulata* (only) in the hastener. The profiles of these can be seen in Appendix 3, where they are compared with previous isolates.

Table 4.20 Results of exposure plates

Sampling Point	Results
Vat room	1 *P. roqueforti*
Hastener	5 yeasts – *C. catenulata* (5/5); 18 *P. roqueforti*; 1 dark green mould
White room	6 yeasts – *C. famata* (4/6) *C. catenulata* (2/6); 3 dark green moulds
Blue Room 3	1 yeast - *C. famata*; 8 *P. roqueforti*; 1 dark green mould
Blue room 4	1 yeast - *C. famata*; 1 dark green mould
Blue room 6	8 *P. roqueforti*; 3 dark green moulds

Isolates of neither species demonstrated inhibition of the *P. roqueforti* either on SDA, SDA plus milk, SDA plus 1% NaCl or SDA plus 1% NaCl and milk. Comparison of the profiles of these yeasts (Appendix 3) suggests that the *C. famata* isolates were, in fact, different sub-species from those which demonstrated anti-microbial activity previously. It seems that several sub-species of the yeast exist in the dairy and that these exhibit different properties. It also seems likely that the sub-species that exhibited anti-mycotic activity during this study

may not be endemic to the dairy, or, if it is then it is eliminated during the atmospheric sanitisation process.

The presence of *C. catenulata* (Table 4.20) may be significant to the quality (or keeping quality) of the product since Nichol and Harden (1993) suggested that this species was responsible for the production of enzymic browning in mould ripened cheeses. In this study, high levels of the enzyme o-diphenol oxidase were detected when the yeast was grown in tryptic soy broth and Gorgonzola type cheeses exhibiting browning were found to contain high levels of the species in brown areas. It may also be significant that in the study by Nichol and Harden (1993) no *P. roqueforti* was detected in the areas of the cheeses where browning occurred. However, the methods used in the current study were not able to detect any inhibition of the mould by *C. catenulata.* This could be due to variation in the strains of the yeast in the two studies since in the current study strain type was not determined. Browning is not uncommon in blue cheeses and was noted in some, but not all, samples in the current study when they were maintained under refrigeration for long periods (in the region of 6 months), which is a longer period than would be normal for such cheese. Stilton cheese is normally marketed after 8-12 weeks of ripening and a shelf life of 2-4 weeks is expected, depending on the length of ripening. During the current study, samples were retained under refrigeration for extended periods and browning sometimes occurred in these samples. It seems likely that strains of yeast that are able to cause browning (such as *C. catenulata* (Nichol and Harden, 1993) or *Yarrowia lipolytica* (Nichol and Harden, 1993; Carreira *et al.*, 1998)) may occur as environmental contaminants but deep cleaning reduces them to a level that is unlikely to cause spoilage.

It is notable that *C. sphaerica* was not isolated on the exposure plates, despite its frequent isolation in samples of cheese during this study. This species is capable of fermenting lactose, producing the enzyme β-galactosidase (Kaminarides and Laskos, 1992) and therefore is likely to be able to grow actively in dairy products, but the source of this species in cheeses tested during this study is not known. It could be postulated that the species is found as a result of contamination from equipment or from personnel.

To summarise the investigation into the microflora of mature Blue Stilton cheese, manufactured at a single dairy, there is variation in the species of lactobacilli present, although *Lb. plantarum* appears to be the most commonly occurring species. Other homo- and heterofermentative species of lactobacilli were found and may have significance in the quality of the end product. However, a number of species of yeast belonging to the genus *Candida* were isolated from a number of cheeses, in particular from those cheeses exhibiting poor blue veining. Within such species, several strains appeared to be present, with these exhibiting differences in their fermentation characteristics. In addition, some of the strains of *C. famata* demonstrated an ability to inhibit the growth of *P. roqueforti* when grown on SDA. Other species of the yeast that were isolated included *C. catenulata* and *C. lipolytica* (the asporogenous form of *Y. lipolytica*), both of which have been implicated as the cause of enzymic browning in blue cheeses.

CHAPTER 5

5 Results of headspace analysis of Stilton Cheese

In collaboration with Christian Salles (Food Science, University of Nottingham) a sample of good quality cheese was compared with that of poor quality cheese using GC-MS olfactometry, in order to compare the volatile compounds found in the headspace. In addition, comparison of those volatile compounds with aromas noted at defined peaks, could potentially be linked to the microflora of the samples.

Samples 53-62 were tested, the microbiological results of which are shown in Table 4.15. Table 5.1 shows the results of the headspace analysis of the good quality cheeses whilst Table 5.2 shows that of poor quality cheeses. The retention times of defined odours noted during olfactometry are placed at a position approximating to the retention times noted in the mass spectrophotometry, in order to give some idea of the possible compounds causing the odour. The measure of certainty, shown in column three, is indicated by the size of the number. Confidence in the results is increased with increasing number and values of less than 900 indicate a lack of confidence in the identification. The intensity of the odour, shown in column six, is on a scale of 0-5 with 5 indicating a very strong odour.

Table 5.1 Results of headspace analysis of good quality, mature Stilton cheese

Retention time (min)	Compound	Measure of certainty	Retention time (min)	Odour	Scale of intensity
2.05	dimethylsulphide	937	1.3	Cheese, sulphur	4
2.15	dichloromethane	945	2.3	Milky	0.5
2.52	2-methyl propanol	942			
2.92	cyclopentane	953			
2.66	butan-2-al	982			
3.84	Hexane	957	3.5	Sweaty	1
3.89	trichloromethane	973			
5.11	3-methyl butanal *	928	5.0	Bad odour	3
5.54	2-methyl butanal	952			
6.06	cyclohexane	979			
6.63	3-methyl butan-2-one or diacetyl	880			
7.73	Hept-1-ene?	959			
8.01	pentanol	904			
8.42	heptane	987			
9.88	dimethylsuphide	977			
10.37	3-methyl butanol	985	10.1	Milky, cheese	1
10.5	2-methyl butanol *	964	10.6	Fruity/sweaty	1
10.7	Heptane?				
11.88	2- propenylidene cyclobutene	974			
12.44	3-hydroxy butan-2-one (acetoin) *	888			
13.88	hexanal	958	14.4	Rubber	0.5
15.67	octane	966			
21.49	Heptan-2-one	976	21.4	Blue cheese	3.0

Retention time (min)	Compound	Measure of certainty	Retention time (min)	Odour	Scale of intensity
			25.1	Sulphide, cabbage	0.5
25.81	3,5-dimethyl butanethioate	945	25.5	Cowshed	4
26.51	3,7-dimethyl-1,3,6-octatriene	956	26.4	Cowshed	2.5
27.2	dimethyltrisulphide	854	27.2	Sulphur	2

* compound produced by lactococci and/or leuconostocs (Mauriello *et al.*, 2001)

The results of headspace analysis shows that the predominant odours in the sample of good quality cheese tested were "cheese, sulphur" and a "cowshed" odour. The initial strong cheese odour was released before any volatile compounds, although this odour was detected at a point close to the peak for dimethylsulphide, which might have been responsible for the sulphur note. However, a second peak for dimethylsulphide was seen at a retention time of 9.88 minutes and no aroma was associated with this peak. The cowshed odour, on the other hand, was released at two distinct points, at retention times of 25.5 and 26.4 minutes. They were close to the peaks associated with 2,3-dimethyl butanethioate (25.81 minutes) and 3,7- dimethyl-1,3,6-octatriene (26.51 minutes).

The next most intense odour was that of blue cheese, which was noted at a retention time of 21.4 minutes, almost exactly that of heptan-2-one. Schwartz and Boyd (1963) and Schwartz and Parks (1963) found that heptanone-2 (heptan-2-one) was the most common methyl ketone produced during blue cheese ripening and was attributable to metabolic activities of *P. roqueforti.* Madkor *et al.* (1987) also found that heptan-2-one was the most abundant methyl ketone in mature blue Stilton cheese, with concentrations ranging from 28.49 – 42.75 μmol/10g dry cheese. This study also found longer chain methyl ketones (C_9-C_{15}), although in lesser quantities, and perhaps future studies should continue the chromatography and olfactometry for a longer period to allow detection of these products. Heptan-2-one and nonan-2-one are considered to be responsible for the typical flavour of blue cheeses (Madkor

et al., 1987) and they originate from the action of *P. roqueforti* on their respective fatty acids (Madkor *et al.*, 1987; Schwartz and Boyd, 1963).

An off-odour, or "bad odour" was also detected at the same level of intensity as the blue cheese odour described above. This odour was produced after 5 minutes retention time and the nearest peak observed during GC-MS was at 5.11 minutes. This peak was 3-methyl butanal, a compound produced by citrate positive strains of lactococci and noted to give malty odours (Mauriello *et al.*, 2001). It is interesting that this was described by the analyst as a "bad odour" and it would have been useful for this to have been replicated using several different analysts, in order to gain more than one description of the odour. Aromas of moderate intensity (2-2.5) were noted at the end of the process and were very close to the peaks of 3,7-dimethyl-1,3,6-octatriene (described as a cowshed odour) and dimethyl trisulphide (described as sulphurous).

When the plots of the GC-MS are reviewed (Appendix 5) it can be seen that the peaks at 9.88 minutes (dimethyl sulphide) and 10.37 minutes (3-methylbutanol) were quite large, but the nearest detectable aroma was after 10.1 minutes and only had an intensity of 1, with a milky or cheesy aroma. This reflects the ability of very small amounts of some volatile chemicals to produce intense aromas, whilst others need far greater amounts to be detected by the human olfactory system.

Other aromas detected were of low intensity and were variously described as "milky" (0.5 intensity and with the nearest peak being that of dichloromethane), sweaty (1, hexane), fruity or sweaty (1, and with the nearest peak being that of 2-methyl butanol), rubber (0.5, hexanol) and sulphide or cabbage. The latter aroma was detected after 25.1 minutes, but there was no peak that could easily be related to this odour. It is interesting to note the description of the aroma associated with 2-methyl butanol as fruity or sweaty, since Mauriello *et al.* (2001)

described the aroma of this compound as being similar to whisky. It is possible that this was simply a reflection of the subjectivity of organoleptic assessment methods.

A number of the volatile compounds detected were identified as being metabolites of the action of lactococci and leuconostocs (Mauriello *et al.,* 2001). It is likely that the occurrence of these compounds in the Stilton cheese under test is as a result of these starter bacteria. These compounds are highlighted by an asterisk in Tables 5.1 and 5.2.

When the results of the good cheese are compared with those of the poor quality cheeses, it can be seen that the volatile compounds vary between the two samples. A compound, which was either acetone or 1-methoxy-2-propanone, was detected at a retention time of 1.70 minutes. Neither compound was detected during analysis of the good cheese, nor was acetone detected in mature Stilton cheese in earlier studies (Madkor *et al.*, 1987).

Table 5.2 Results of headspace analysis of mature, poor quality, Stilton cheese

Retention time (min)	Compound	Measure of certainty	Retention time (min)	Odour	Scale of intensity
1.70	1-methoxy-2-propanone **or** acetone	909	1.1	solvent	1.5
2.20	dichloromethane	829			
2.58	Compound not identified		2.4	Burned	2
2.96	Compound not identified		2.9	Buttery/ creamy	1
3.36	Compound not identified				
3.90	Hexane	892			
4.79	2-methyl-1-propanol	948	4.3	Rubber	1.5
5.20	pentanal	903	5.1	"Bad" odour	3.5
5.60	Compound not identified		5.4	Processed cheese	1
6.04	2-methylpentene	910	5.8	Unidentifiable	0.5
6.71	Unidentified mixture				
9.08	3-methyl butanol	933	8.8	Solvent	1
10.43	2-propylidene cyclobutene	964	10.4	Fruity	1
11.22	hexan-2-one	932			
11.99	Compound not identified				
12.42	Compound not identified				
12.54	Octane	906			
12.71	oct-4-ene	952	14.2	Fruity	1
14.58	Ethylbenzene *	919	14.4	Putrid	2
14.95	1,4-dimethylbenzene	960			
15.21	3-methylbutanol acetate	907	15.2	Fruity	0.5
15.55	heptan-2-one + another compound	917	15.6	Blue cheese	4.5
16.33	Compound not identified				

Retention time (min)	Compound	Measure of certainty	Retention time (min)	Odour	Scale of intensity
18.03	3,7-dimethyl-1,3,6-octatriene	938			
18.57	dimethyl trisulphide	881	18.6	Putrid	2.5
19.30	octen-3-one		19.1	Mushroom	0.5
19.40	octan-3-one	907			
19.50	4-methyl heptan-2-one	879			
20.66	decane	930			
21.19	2-ethylhexanol	944			
21.50	limonene	940			
22.10	3-methylbutyl-2-methylpropanoate	968	21.9	Unidentifiable	1
22.80	8-nonen-2-one	942			
23.27	nonan-2-one	918			
27.50	6-ethyl-2-methyldecane	940	27.3	Putrid	1.5
			31.7	Sour	0.5

* compound produced by lactococci and/or leuconostocs (Mauriello *et al.*, 2001)

Compounds occurring in samples of both good and poor quality cheese

The results indicate that there were few volatile compounds common to both samples of cheese. This may be because the samples exhibited different quality characteristics, but equally, because only one of each type of cheese was tested, it is not possible to know whether individual samples of similar quality cheeses would exhibit similar results. Without further samples it can only be assumed that the differences are due to the result of variation in the microflora, leading to the presence of differing metabolites.

In the good quality cheese three compounds that are known to be produced by lactococci and leuconostocs, ie starter bacteria, were identified and, in addition, a compound which

might have been diacetyl, also produced by starter bacteria, was detected. However, in the poor quality cheese only one such compound was detected. This may be due to reduced metabolism by the starter bacteria in the poor cheeses or possibly degradation of these compounds by other microorganisms. However, it was suggested in Chapter 4 that the starter bacteria may be actively growing in mature, good, cheeses when the pH of the cheese is increased due to growth of the *Penicillium*. If this was so then the presence, in the good cheeses, of compounds resulting from starter bacteria metabolism may be due to this activity.

Table 5.2 shows that there were more fruity flavours evident in the poor quality cheeses, which may be as a result of the growth of *Lb. brevis*, since this species was isolated from samples 53-57. Lalaye *et al.* (1990) found that cheddar cheeses made with added heterofermentative lactobacilli, including *Lb. brevis*, were of a poorer quality than control cheese, manufactured without added lactobacilli and consistently had fruity flavours.

The most intense aroma in the poor quality cheese was that of blue cheese, and again this was detected at the same time as heptan-2-one. However, in this sample another, unidentified, compound was detected alongside heptan-2-one and this also may have contributed to this blue cheese aroma. The intensity of the blue cheese flavour associated with this compound was greater in this sample than in that of the good cheese, yet the assessor was the same person for both samples. This suggests that the additional compound did indeed contribute to the flavour and examination of the chromatogram shows that the peak on the poor cheese was larger than that on the good cheese, which would support this theory. However, there is no way of distinguishing the unidentified compound from the heptan-2-one, so it is not possible to ascertain whether there was more or less heptan-2-one in one or other sample, nor to be certain that the unknown did have a blue cheese aroma.

Other ketones were identified in both good and poor quality cheeses, although most of these were not associated with aromas. Gallois and Langlois (1990) noted that methyl ketones

accounted for about half of the odourous profile of 5 samples of blue cheeses (3 Roquefort, Bleus des Causses, Bleu d'Auvergne), although these were less common in the current study. It has also been suggested that methyl ketones are the significant flavour producers in blue cheeses (Madkor *et al.*, 1987), although the results of this study indicate that whilst they produce a significant blue cheese odour, other compounds may produce more intense flavours. Martelli (1989) also found that other compounds were as important as the methyl ketones in producing flavours in Gorgonzola cheese. Specifically, this study found that alcohols contributed flavours of importance equal to those of the methyl ketones.

An unidentifiable aroma, with an intensity of 1, was detected after a retention time of 21.9 minutes. This might have resulted from 3-methylbutyl-2-methylpropanoate, detected at 22.10 minutes, but also, at 21.50 minutes, the compound limonene was detected. This compound is a cyclic monoterpene and is responsible for the typical odour of lemon oil (Lehninger, 1981). A literature search revealed no evidence of the production of limonene by lactic acid bacteria. However, Chalier and Crouzet (1993) noted the presence of limonene as a by-product of germinating spores of *P. roqueforti* during cultivation in a synthetic medium. This could indicate that its presence in this sample was due to the presence of the species and that spore germination was in progress. Additionally, however, the compound was produced by a soil pseudomonad during metabolism of α- and β-pinene (Yoo *et al.*, 2001). Its presence in this sample possibly indicates contamination by soil pseudomonads, which could only indicate post-process contamination. It seems more likely, however, that its presence is linked to the growth of *P. roqueforti,* although it was not detected in the sample of good quality cheese.

Limonene was not identified during analysis of volatile odorous compounds in French blue cheeses, although a number of unidentified sesquiterpenes were detected during this study (Gallois and Langlois, 1990). These compounds did not yield aroma during this study, although during the current study an unidentifiable odour was noted between the peak for limonene and 3-methylbutyl-2-methylpropanoate.

Sulphur compounds were detected in both good and poor quality cheeses, although in the latter only dimethyl trisulphide was detected, whilst in the former dimethyl sulphide was also identified. Gallois and Langois (1990) found a range of sulphur compounds in blue cheeses (Roquefort, Blue des Causses and Blue d'Auvergne) using GC-MS, including dimethyl sulphide, hydrogen sulphide, dimethyl disulphide and methyl sulphide. These authors concluded that dimethyl sulphide contributed to the aroma of the cheese and the current study supports this view, since a cheesy aroma was detected in the good cheese at the point at which this compound was detected.

Table 5.3 compares the numbers of volatile compounds as groups of chemicals and it can be seen that the good cheese exhibits a greater number of ketones, aldehydes, sulphur compounds and, to a lesser extent, alcohols. It could be suggested that these are associated with the flavour of good quality blue cheese, whilst an increase in ketenes and miscellaneous compounds, including esters, may result in the production of off-flavours. However, on the basis of such a small sample this cannot be asserted and further investigations are required into this area.

Table 5.3 Comparison of volatile compounds produced in good and poor quality Stilton cheese

	Good cheese	Poor cheese
Ketones	3	7
Ketanes	8	4
Ketenes	2	5
Alcohols	4	3
Aldehydes	4	1
Sulphur compounds	3	1
Miscellaneous	2	5
Unidentified	-	8

Microbiological analysis of the two samples, discussed in section 4.11, revealed the presence of several species of yeast. Both good and poor cheeses were shown to contain *Candida sphaerica, C. famata* and *C. catenulata* whilst, additionally, in the poor cheeses *C. lipolytica* and *C. boidinii* were isolated. *C. lipolytica* is a strongly lipolytic species (Van den Tempel and Kakobsen, 1998) and may be associated with the production of some of the volatile compounds noted during this experiment.

Candida sphaerica and *C. famata* are known to ferment lactose, although the latter species exhibits less activity in this than does the former. *C. sphaerica* is also known to be a prodigious gas producer and therefore may contribute towards the open structure of the cheese. However, there is little documentary evidence as to the production of metabolites of lipid catabolism by these species. In future studies this could be an area for investigation, in order to determine whether the levels of individual species of yeast are significant in producing off-odours in the product and to determine exactly what volatile compounds can be produced by these species.

The results of this experiment have clearly demonstrated a significant difference in the range of volatile compounds present in good quality Stilton cheese, exhibiting ample growth of *P. roqueforti*, as compared with poor quality cheese which had few blue veins. However, both cheeses contained heptan-2-one, the principle methyl ketone resulting from growth of the fungus, indicating that some mycelial growth had taken place in both samples. This relates to the mould counts which were of the order of 10^6 g^{-1} in the poor cheese samples (53-57) and 10^7 g^{-1} in the good cheese samples (58-62). The difference in profile of volatile compounds must be due to other factors within these samples and could be related to the species of lactobacilli, but also to the yeasts present.

CHAPTER 6

6 The effect of production site on microflora

The majority of the study was carried out on cheeses from one dairy, so in order to compare the numbers of microorganisms and the main species of lactobacilli and yeasts present across a wider range of manufacturers, samples were taken from three other Stilton producers (dairies two to four). The same tests were carried out, determining TVC, TLC, yeasts and moulds and again selected colonies from the MRS plates were checked for catalase activity, Gram stained and examined microscopically, before being identified using API 50CHL test strips. Yeasts were also selected from the yeast and mould plates and were isolated and identified using the API ID32C test strips. The cheeses tested were all from first grade, good quality, mature (8-10 weeks old) cheese and samples from dairies 2 and 3 had such confluent growth of the blue mould that it was not possible to differentiate between blue and white areas. Samples were taken in January and Table 6.1 shows the results of duplicate samples of individual cheeses and includes the mean results of good cheeses from dairy one, which was used for the previous studies.

Table 6.1 Results of tests on cheeses from three creameries

	cfu g^{-1}				
Dairy No	**TVC**	***Lactobacillus***	**Yeasts**	**Moulds**	**pH**
2	1.17 E8	1.27 E8	4 E6	3.1 E7	7.02
3	2.28 E8	1.06 E8	1 E6	1.9 E7	7.45
4 blue	3.8 E7	7.5 E7	3 E6	2.2 E7	6.18
4 white	6.2 E7	5 E7	1.3 E7	3 E6	5.67
Mean results of good cheeses from Dairy 1	**6.83 E7**	**3.91 E7**	**8.50 E6**	**1.28 E7**	

It can be seen that dairy 2 had a slightly higher TVC than was generally achieved from the cheese of dairy 1 and the TLC was very similar to this count. The yeast count was fairly low although of the same order as samples from dairy 1, whilst the mould count was as expected for a good quality cheese. Ten percent of colonies from the MRS plates were selected and confirmed as lactobacilli and were identified as predominantly *Lb. plantarum*, with *Lb. brevis* also present. These species were often found on the samples from dairy one, although *Lb. brevis* was only detected once on good quality cheese and occurred more frequently on poor quality cheeses. The four yeast colonies were identified, using the API ID32C system, as *C. sphaerica* and *C. catenulata*, species which also occurred frequently in experiments detailed in Chapter 4. It is potentially significant that there was evidence of browning on the cheese from this dairy, appearing as a deep brown area of about $1cm^2$. After storage for a further two weeks post-testing, this brown area had spread to the entire cheese. It is possible that this was due to the action of *C. catenulata.*

Dairy 3 had a similarly high TVC to the other samples and the TLC was also high, although it was about 50% less than the TVC, a ratio similar to that in the samples from dairy 1. The yeast count was low and the only colony detected was identified as *C. sphaerica*, whilst the mould count was fairly high. Only *Lb. plantarum* was identified from a 10% sample of colonies growing on the MRS agar, and the colony appearance of the samples included both circular and elliptical colonies. The age of this cheese was not known as it was purchased from a retailer, but the presence of only one species of lactobacilli may be related to the age of the sample as other species could have died out during maturation. However, since 100% sampling of colonies was not carried out, there is potential for other species to have been undetected, despite sampling taking in a spread of colony characteristics.

Samples from cheese made at dairy 4 had TVC and TLC of the same order as dairy 1. The TLC in the white area exceeded the TVC but not significantly, whilst that in the blue area was slightly less than the TVC. The yeast count in the white area exceeded that in the blue area, whilst the reverse was true of the mould count, which reflected the findings of earlier experiments on cheese from dairy one. The number of moulds recovered from the white

area was very similar to that noted in experiments detailed in Chapter 4 and probably reflected the results of sporulation. With regard to the lactobacilli, approximately 65% of colonies on both the white and the blue samples were ellipsoidal, with the remainder being circular and both types of colony were selected and tested using API 50 CHL and found to be *Lb. plantarum*. This species appeared to predominate on the blue area, whilst on the white area *Lb. pentosus* was found in addition to *Lb. plantarum* (although there was no difference in colony appearance of the two species). Of the 10% sample of colonies (5), three colonies were *Lb. plantarum* and two were *Lb. pentosus*. The three yeast colonies from the blue area were identified as *C. sphaerica*, whilst in the white area four colonies were isolated and identified as *C. sphaerica* and *C. catenulata* in equal proportions.

6.1 Conclusions

Lb. plantarum appeared on samples of all cheeses and appeared to be the most significant species of *Lactobacillus* in mature Stilton cheese. Other species occurred in association with *Lb. plantarum* but these appeared to vary between the dairies and were never found as frequently. However, there is potential for these less frequently occurring species to be significant in the contribution of flavour and thus character of individual dairies. There is also the possibility of heterofermentative lactobacilli such as *Lb. brevis* becoming too numerous and thus producing too much gas.

Carbon dioxide production is undesirable in cheeses such as Cheddar as it lends an open curd structure, but this is not generally considered to be a problem in Stilton and the inclusion of *Ln. mesenteroides* subsp. *cremoris* in the starter is designed to increase curd openness through gas production and hence aid the development of the veins. However, excess carbon dioxide was implicated in the inhibition of *P. roqueforti* when 360 cheeses manufactured using cultures containing 25% of heterofermentative organisms were found to be completely deficient in blue veins with resultant loss of product quality (Hough, 2001, unpublished). Hence, excessive growth of heterofermentative lactobacilli, coupled with gas producing starters, may result in loss of blue vein development. However, in experiments detailed in Chapter 4, *P. roqueforti* was shown to grow and sporulate, albeit slowly, when

incubated under anaerobic conditions and the process of piercing the cheese should enable gaseous exchange to occur. It seems likely that the cause of defective blueing is the presence of inhibitory metabolites other than CO_2.

The cheeses tested in this experiment were all mature cheeses, as were those cheeses tested in previous experiments. However, since all samples were purchased from a retailer rather than being sampled at the manufacturing site, the actual age of the samples is not known. Nonetheless, Stilton is normally ready for market at 8 weeks of age and generally has four weeks shelf life, so it is reasonable to assume that the samples were between 8 and 12 weeks of age. It is possible that *Lb. plantarum* becomes the dominant species during ripening and thus the presence of several species of lactobacilli may indicate less mature cheese. Fitzsimons *et al.* (2001) found a mixture of *Lb. paracasei, Lb. plantarum, Lb. rhamnosus* and unidentified strains of lactobacilli in Cheddar cheese up to 6 weeks of maturation and thereafter only found *Lb. paracasei* strains, which may suggest that one species predominates amongst the lactobacilli as ripening progresses. *Lb. paracasei* was not detected during this experiment, although it was isolated once from cheeses manufactured at dairy one. However, Clark and Reinbold (1967) found that *Lb. casei* and *Lb. plantarum* were the most commonly occurring lactobacilli in 967 cheeses tested.

All moulds found on samples from these three dairies exhibited the typical appearance of *P. roqueforti* (blue-green colonies with a white margin and producing conidia) and the numbers found were very similar in all samples, other than the white area samples from dairy 4, which gave lower numbers, although these were of the same order as in the white areas from cheeses made by dairy 1. The pH results reflected this, with the pH of the white area being lower than that of the blue area. However, the pH of the white area, at 5.67, indicated that proteolysis had taken place although to a less extent than in the blue areas. In young cheeses, sampled before proteolysis had commenced to any significant extent, the pH values found during this study and during studies by others (Madkor *et al.*, 1987b; Zarmpoutis *et al.*, 1996) were in the region of 4.8, so it is clear that metabolic end products

had raised the pH in the cheeses tested. In addition to this the texture of cheeses used in this experiment was soft and creamy, further indicating proteolysis.

The yeast counts were low in samples from all three dairies, although they were slightly higher in the blue area of cheeses from dairy 3. The predominant yeast species appeared to be *C. sphaerica*, as was the case in cheeses made in dairy 1. Since these samples were all from good quality cheeses exhibiting ample development of blue veins, inhibition testing was not carried out using any species of yeast isolated. The presence of *C. sphaerica* in the brine used for salting of Feta cheese was noted in a study by Kaminarides and Laskos (1992). Additionally, it was reported that the strain found was capable of high production of the β-galactosidase enzyme and was also able to produce CO_2 in large quantities. This latter characteristic may be noteworthy in terms of the quality of Stilton cheese, since it may, particularly in the presence of heterofermentative lactobacilli, give rise to levels of CO_2 that could be considered to be inhibitory, or alternatively could beneficially assist the open structure. However, Van den Tempel and Jakobsen (1998) found that *C. sphaerica* appeared only rarely in Danablu cheese, with *C. famata* and *C. catenulata* being the predominant species.

The results of this experiment suggest that whilst there are similarities in cheeses produced at different dairies, there are also differences. The number of microorganisms in terms of total count, lactobacilli and yeasts and moulds, is of the same order irrespective of dairy of origin, but the species occurring within these groups varies to some extent. The predominant *Lactobacillus* occurring in Blue Stilton cheese appears to be *Lb. plantarum* and the predominant yeast, *C. sphaerica*, but other species occur to a lesser extent and may be significant in lending unique characteristics to cheeses.

CHAPTER 7

7 General Discussion and Conclusion

Blue Stilton cheese is manufactured using a mixed starter culture containing *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis* var. *diacetylactis* and *Ln. mesenteroides* subsp. *cremoris,* with *P. roqueforti* added as a secondary starter. Stilton cheeses vary in appearance, flavour and texture, between dairies and between batches from the same dairy. This study aimed to investigate the potential relationships between microorganisms growing in mature Blue Stilton cheese and to determine whether these may affect the microflora and thus the organoleptic and visual qualities of the cheese.

7.1 The microflora of Blue Stilton during ripening

Part of this study followed a vat of milk during the cheese manufacturing and ripening process, with cheeses being sampled over a period of eight weeks. During this time the microflora changed insofar as the selected groups of microorganisms were recovered in differing numbers over the ripening period.

Initially, in the pasteurised milk, very low numbers of lactobacilli were recovered. Since the milk was sampled prior to addition of the starter (and members of this genus are not included in the starter), it is likely that these were a result of either post-pasteurisation contamination or survival of the pasteurisation progress. Jordon and Cogan (1999) suggested that *Lb. paracasei* DPC2103 was able to withstand pasteurisation temperatures and it seems reasonable to suggest that other strains and species of lactobacilli may also be heat tolerant. The only species of *Lactobacillus* recovered from the milk samples was *Lb. acidophilus,* although this species was not detected in further samples. Other species of lactobacilli may, however, have been present in the milk, but in such small numbers as to be undetectable in the volumes tested. Further studies on this aspect might try using the membrane filtration method, using 100 ml of milk, to see whether or not this results in detection of a wider range of species.

Within 24 hours the lactobacilli population had reached numbers of the order of 10^2 cfu g^{-1}, some of which may have been due to contamination from equipment and personnel and some through survival of pasteurisation, coupled with subsequent growth. Fitzsimons *et al.* (2001) found initial counts of lactobacilli of approximately 10^2 cfu g^{-1} in Cheddar cheese, although this study did not report the levels found in milk used for the manufacturing process. Other studies (Reddy and Marth, 1995; Lues *et al.*, 1999) have detected lactobacilli in significant numbers from the beginning of ripening, although Haque *et al.* (1997) did not detect the presence of lactobacilli in Cheddar cheese until two months of ripening when they were found occurring at levels of about 10^2 g^{-1}.

Initial counts of yeasts were very low, but during the ripening period the number increased to around 10^7 cfu g^{-1}, a level that was found in mature cheeses throughout the remainder of the study. Other studies have shown higher initial numbers of yeasts (Fleet and Mian 1986; Gobbetti *et al.*, 1997; Lues *et al.*, 1999) and, in some cases, the numbers declined during ripening, although van den Tempel and Jakobsen (1998) noted an increase in the yeast count during ripening in Danablu cheese. The low initial counts found during the current study may be due to pasteurisation of the milk and, since all Stilton cheese is made from pasteurised milk it is likely that initial counts would be similar in cheeses produced at other dairies. The yeast counts in mature cheeses produced at four creameries were found to be very similar during this study.

Prior to addition of the fungal starter, there was no detectable mould count in the pasteurised milk (<1 cfu g^{-1}), although after addition of the starter, *P. roqueforti* was detected at a level of 2.3×10^3 cfu g^{-1}. This level remained constant in the curd until day 5 of ripening and only reached the levels found in the final product after around 5 weeks of ripening. This suggests that limited growth took place between inoculation and piercing and that this growth was largely mycelial, with sporulation mainly occurring after piercing. Given the open curd structure of Blue Stilton, it could perhaps be expected that some (albeit limited) sporulation would occur prior to piercing. The purpose of piercing the cheese is to enable gaseous

exchange and hence sporulation, but the results of this study indicate that this is initiated prior to piercing. This may not be the case in close textured cheese as there may be insufficient oxygen within the curd to permit sporulation.

7.2 The microflora of mature Blue Stilton

The main area of this study was the investigation of a number of mature cheeses, in order to determine the number and range of microorganisms present. Initially, samples were tested for the presence of coliform organisms, in addition to the tests discussed in 7.1. However, these organisms were not found in any sample (1-16) and the test was dropped from further samples.

7.2.1 Lactobacilli

Throughout the main part of this study, lactobacilli were detected in mature cheese at levels ranging from $10^7 - 10^9$ g^{-1}. This level is higher than that found by Gobbetti *et al.* (1997) in a study of Gorgonzola cheese, when levels of mesophilic lactobacilli were always between 10^5 and 10^6 g^{-1} during days 1 to 86 of ripening. It is also higher than that found during a study by Lues *et al.* (1999), where levels of around $10^5 g^{-1}$ were detected in mature (96 days) Cheddar cheese. However, Fitzsimons *et al.* (2001) found lactobacilli occurring in Cheddar cheese, after 8 weeks of storage, in similar numbers to those found in this study. In none of these studies were lactobacilli added to the milk and so their incidence was as a result of post-process contamination or survival of heat treatment, where applied. Stilton cheese seems to be very similar to other types of cheese, both blue and non-blue, in terms of the growth of lactobacilli and these microorganisms become a significant part of the microflora of the mature cheese.

It is possible that *Lb. plantarum* becomes the dominant species of *Lactobacillus* in Stilton cheese during ripening, since only *Lb. acidophilus* was isolated from a milk sample, whilst the species *Lb. brevis, Lb. curvatus* and *Lb. plantarum* were isolated from a number of samples, although the latter species was most commonly isolated from cheese samples

during this study. These results agree with those of Naylor and Sharpe (1958a), who found that English Cheddar was dominated by *Lb. plantarum* and *Lb. brevis* and with Clark and Reinbold (1967), who found that *Lb. plantarum* and *Lb. casei* were the most commonly occurring lactobacilli in Cheddar cheese. However, Fitzsimons *et al.* (2001) demonstrated that *Lb. paracasei, Lb. plantarum* and *Lb. rhamnosus* dominated the microflora of Cheddar cheese up to six weeks after manufacture, although they found that thereafter *Lb. paracasei* was most commonly found.

Several strains of *Lb. plantarum, Lb. curvatus* and *Lb. brevis* were isolated from samples during the course of this study. A total of 14 strains of *Lb. plantarum* were isolated from 72 isolates of the species and these strains differed in their ability to utilise a range of substrates. The utilisation of different substrates may well result in the production of diverse metabolites and some of these may be flavour compounds. This suggests that cheeses from different batches could exhibit very different flavour profiles. This was demonstrated to some extent by the analysis of volatile compounds, discussed in Chapter 5, but only two cheeses, from different batches, were tested and this area should be investigated in more detail in future studies.

Stilton cheese is ready for market at about 8 weeks of age and is generally given a shelf life of about 4 weeks, although cheese may be marketed at 12 weeks, depending on the requirements of customers. Cheddar is normally graded at 6 weeks, but may be matured for considerably longer depending on the degree of maturity and depth of flavour required. The presence of a mix of species of lactobacilli in the cheeses tested during the current study may be due to the age of the samples (between 8 and 12 weeks), but may also be due to the growth of the blue mould, *P. roqueforti*, which may produce metabolites that are useable as energy sources for a range of lactobacilli. However, if *Lb. plantarum* does become the dominant species, as the results suggest (since it was isolated more frequently and was the only species isolated from samples 58-62), this could be due to the presence of growth factors which the species is able to utilise more readily than other species.

Although many studies have shown a reduction in the number of species of lactobacilli present in cheese during maturing, there seem to be few suggestions as to the reason for this. Fitzsimons *et al.* (2001) suggested that the reason for the dominance of *Lb. paracasei* over *Lb. plantarum* and *Lb. rhamnosus* was the inability of the latter two species to utilise the same energy source as the former. It may equally be possible that during the growth of *P. roqueforti,* suitable energy sources are made available to *Lb. plantarum.* Samples 53-57 were found to contain only *Lb. brevis* and *Lb. paracasei* subsp. *paracasei,* with the former occurring more frequently than the latter, despite the fact that these cheese were made at a similar time (and in one case, on the same day) as samples 58-62. Samples 53-57 exhibited very little development of blue veins, whereas samples 58-62 had widespread blue veining. This suggests that the sporulation of *P. roqueforti* may have an effect on the growth of *Lb. plantarum*, which may be due to the production of energy sources for the species.

Lb. plantarum and *Lb. curvatus* are homofermentative species and, as such, are considered to produce acceptable flavours in cheese. However, the heterofermentative species, which include *Lb. brevis*, are generally considered undesirable since they may result in an open curd structure and off flavours (Puchades *et al.*, 1989; Laleye *et al*, 1990). Open curd is not considered to be a problem in Stilton cheese and, in fact, is actively encouraged (by the addition of *Ln. mesenteroides* subsp. *cremoris* in the starter) in order to assist the development of veins within the cheese. However, *Lb. brevis* may produce other metabolites which could result in the development of off flavours in the cheese. This was reflected during examination of the volatile compounds in Stilton cheese, using headspace analysis by GC-MS olfactometry (Chapter 5). In the cheeses exhibiting little development of blue veins, where *Lb. brevis* was the most commonly isolated species (and *Lb. plantarum* was not isolated), several fruity aromas were detected. In the good quality cheese tested by the same method, from which only *Lb. plantarum* and not *Lb. brevis*, was isolated, these fruity aromas were not detected. It is concluded that the fruity flavours noted in the poor quality cheese were as a result of metabolic activity of *Lb. brevis.* This supports the findings of Laleye *et al.* (1990) who found that Cheddar cheese manufactured with added heterofermentative lactobacilli consistently had fruity flavours.

Fitzsimons *et al.* (2001) also demonstrated lactobacilli counts of the order or 10^7 to 10^8 in Cheddar cheeses of ten or more weeks, which stabilised at 10^8 after 13 weeks. It may be significant that the lactobacilli counts reached this level sooner in the Stilton cheeses sampled during the current study. This may be due to the lower pH achieved in Stilton at the beginning of ripening, when the pH is typically around 4.5, whereas in cheeses such as Cheddar the pH at the beginning of ripening is nearer to 5.4. This lower pH could encourage the growth of the more acid tolerant species of lactobacilli and restrict the growth of the lactococci, resulting in faster development of the lactobacilli than in Cheddar type cheeses.

It appears that the numbers of microorganisms in good quality cheeses is of the same order irrespective of creamery of origin, with total viable counts of 10^7 to 10^8, *Lactobacillus* counts of 10^7 to 10^8, yeasts in the order of 10^6 and moulds of 10^7. There appears to be some variation in the species of lactobacilli present, though, with dairy one mainly yielding *Lb. plantarum, Lb. brevis, Lb. curvatus* and *Lb. paracasei* subsp. *paracasei*; dairy two, *Lb. plantarum* and *Lb. brevis*; dairy three, *Lb. plantarum* only and dairy four *Lb. plantarum* and *Lb. pentosus*. However, far more extensive testing was carried out on cheeses from dairy one and samples from this dairy included both good (first grade) and poor (second grade) cheeses. This resulted in a greater chance of isolation of both desirable and undesirable species from these samples. It is, however, clear from the results of individual cheeses that each cheese has a unique microflora, although *Lb. plantarum* seems to be common to all samples.

7.2.2 Yeast species

The number of yeasts detected in mature Stilton cheeses varied from $10^6 - 10^8$ g^{-1} and the most commonly occurring species were *C. famata* and *C. sphaerica*, with *C. catenulata, C. lipolytica, C. dubliniensis* and *C. boidinii* occurring less frequently. This level of yeasts is much higher than that reported by Reddy and Marth (1995), who noted levels of around 10^1 - 10^2 g^{-1} in Cheddar cheese up to 36 weeks of ripening, although Lues *et al.* (1999) detected

around 10^6 g^{-1} at the start of ripening of Cheddar cheese and 10^4 g^{-1} at the end. Gobbetti *et al.* (1997) studied the microbiology of Gorgonzola cheese, an Italian variety of blue cheese and detected counts of yeasts ranging from 10^5 g^{-1} at day 1 of ripening to approximately 10^6 g^{-1} at 36 days and thereafter. These results are similar to those of the current study and suggest that there may be a greater number of yeasts existing in blue cheeses.

The presence of *C. sphaerica* may be explained by the ability of the species to ferment lactose (Barnett *et al.*, 2000). The species showed high production of β-galactosidase during a study by Kaminarides and Laskos (1992) and could, therefore, be expected as a normal part of the dairy microflora. Similarly, strains of *C. famata* are also able to ferment lactose, although this is often a weak reaction (Barnett *et al.*, 2000). Lactose is a growth substrate for both of these species, whilst *C. catenulata, C. lipolytica, C. dubliniensis* and *C. boidinii* are unable either to ferment or utilise lactose as a carbon source. The growth of these species in mature Stilton cheese must be due to the presence of one or more other growth factors, which may be metabolites of another microorganism. *C. sphaerica* is a strong CO_2 producer (Groniem, 1968) and thus large numbers of the species may result in open curd or blown cheeses.

C. famata has been isolated from a wide range of dairy products including the brine of feta cheese (Kaminarides and Laskos, 1992), Roquefort cheese (Devoyod and Sponem, 1970) and cheddar and cottage cheeses (Fleet and Mian, 1987). The yeast has also been found in raw and pasteurised milk samples from a number of dairies (Fleet and Mian, 1987; Fleet 1990; Van den Tempel and Jakobsen, 1998). A study by Van den Tempel and Jakobsen (1998) found that *C. famata* became the dominant yeast after 28 days storage of Danablu cheese and reached levels of 10^5 – 10^9 at this stage. It has been shown that *C. famata* is salt tolerant (Kaminarides and Laskos, 1992) and during the current study, the yeast was found to be able to grow well at 6% w/v NaCl, whereas *P. roqueforti* grew less luxuriantly at this concentration than at lower concentrations of NaCl. Thus, if poor distribution of salt, or alternatively over-salting, occurs in cheeses, there is potential for the predominance of *C. famata* over *P. roqueforti.*

C. famata has been shown to demonstrate anti-microbial activity against *P. digitatum* (D'hallewin *et al.*, 1999), which indicates that the species is capable of producing anti-mycotic substances. Arras *et al.* (1998) also showed that strains 21D, 22D and 30F of *C. famata* were able to reduce infection of artificially wounded citrus fruits with *P. italicum*. In the current study, there was some evidence of the ability of *C. famata* to inhibit the growth of *P. roqueforti*, although the mechanism for this inhibition was not determined. Future studies should investigate this area further, in order to determine the mechanism of inhibition, which may be due to the production of an anti-microbial substance, but may also be due partly to competition for a substrate.

Species of *Candida*, which included *C. famata* and *C. catenulata* were found to cause enzymic browning in mould ripened cheese in a study by Nichol and Harden (1993). In this study, Gorgonzola style cheeses were tested and three species of *Candida* were isolated from brown areas of the cheeses which were subsequently found to produce browning on milk agar. Two strains of *C. famata* were detected, although only one strain produced browning on milk agar. However, Carreira *et al.* (1998) tested strains of *C. catenulata* during a study of surface discolouration of ewes' cheese and did not find evidence that it caused browning. Browning is a relatively common defect in blue cheeses and the isolation of *C. famata* along with *C. lipolytica* and *C. catenulata* in the current study suggests a risk to the quality of the cheese. Increased levels of the species may accentuate this risk and routine monitoring of cheeses may be worth consideration by manufacturers.

The incidence of yeasts in the curd may be due to environmental contamination, although when exposure plates were used to determine aerial contamination only *C. catenulata* and *C. famata* were detected and yeasts were not detected in the vat room, where the milk is exposed to the atmosphere for approximately 20 hours. The more common isolation of *C. famata* in cheese samples probably relates to its ability to utilise lactose, which *C. catenulata* is unable to utilise (Barnett, 2000). However, the most commonly occurring species during the study, was *C. sphaerica* which was not isolated from exposure plates. It seems likely

that this species entered as a post-process contaminant via equipment. The species is able to ferment lactose and therefore can grow actively in dairy products and could, potentially, survive in poorly cleaned equipment. However, further testing of aerial contamination could yield the species and this area should be checked in further studies, by repeated use of exposure plates and identification of the species isolated.

7.2.3 Levels of *P. roqueforti* in mature cheese

Generally, *P. roqueforti* was detected at levels of around $10^6 - 10^8$ g^{-1}, although the presence of high levels of the species on plate counts did not necessarily mean that the cheese would exhibit luxuriant blue veining. Those cheeses that were down-graded because of a deficiency of blue veins still exhibited counts of around 10^6 g^{-1}.

7.3 Comparison of good and poor quality cheeses

A large part of this study was devoted to the comparison of the microflora of good and poor quality cheeses, as determined by the extent of development of the blue veins. The production of cheese deficient in blue veins is of major importance to manufacturers, since these cheese cannot be retailed and either have to be used as a food ingredient or may be wasted.

7.3.1 Comparison of pH

The results of this study have shown that the pH of Stilton cheese increases in response to the growth of the mould, *P. roqueforti*. During ripening of normal, good quality cheeses, which exhibit ample blue veining, the pH increases from around 4.5 to values in the range 5.5 – 6.1 (depending on whether the pH is measured in the blue area or between the veins). However, in cheeses exhibiting poor development of the blue veins, this value only reaches levels ranging from 4.5 – 5.7. This increase in pH is a result of the proteolytic action of *P. roqueforti* and was noted during other studies (Madkor *et al.*, 1987b; Zarmpoutis *et al.*, 1996; Gobbetti *et al.*, 1997).

The study showed that there was always an increase in pH during ripening, but that in the poorly blued cheeses this was generally less marked than in the good cheeses. Proteolysis in those cheeses exhibiting poor development of the blue veins was probably due to growth of the mould, *P. roqueforti*, even though sporulation did not occur, since the counts of the species always showed an increase over the initial inoculum. However, lactobacilli have also been shown to contribute to proteolysis (Muehlenkamp-Ulate and Warthesen, 1999) and therefore may have contributed to the increase in pH observed in poorly blued samples.

7.3.2 Comparison of the microflora of cheeses exhibiting luxuriant blue veining, with those yielding few blue veins

The results of this study suggest that there is a relationship between the quality of the cheese in terms of proliferation of blue veins and the presence of other species of microorganisms. Statistical analysis showed a significant difference in the TVC of good cheese as compared with poor cheeses, although little difference in the lactobacilli counts. However, the incidence of the heterofermentative species *Lb. brevis* was greater in poor quality cheeses than in good cheeses and in the latter *Lb. brevis* was usually accompanied by a greater number of homofermentative species, including *Lb. plantarum, Lb. curvatus, Lb. casei* and *Lb. paracasei* subsp. *paracasei*.

There was also a significant difference in the level of *P. roqueforti* recovered from good and poor cheeses, which may have been due to inhibition of the species by other microorganisms. There may also have been inhibition of the species as a result of close texture in the cheeses and investigations suggested that locally high levels of NaCl could have influenced growth. Further studies could investigate the combined effects of a_w and Eh on the growth of *P. roqueforti*.

7.3.3 The potential for inhibition of *P. roqueforti* by other microorganisms

A major concern of manufacturers of Blue Stilton is the incidence of cheeses which exhibit insufficient growth of *P. roqueforti* and thus blue veins. The reasons for this are unknown but it was felt that it could be due to the presence of an anti-microbial agent. In samples 23-26, 40 and 41 (which had few blue veins) *C. famata* was the most commonly isolated species of yeast and was tested for potential inhibition of *P. roqueforti.* Zones of clearing were noted around stabs of the yeast species when incubated under anaerobic conditions. This might be because the yeast was stimulated to produce anti-microbial substances in these conditions, or equally, because *P. roqueforti* was unable to flourish as well in such an atmosphere and was, therefore, more susceptible to competition from other species.

Further experiments using strains of *C. famata* isolated from other samples of Stilton did not repeat the inhibition described above. However, one strain did demonstrate evidence of anti-microbial activity, but only when the medium contained 6% NaCl. A control plate, using only *P. roqueforti,* showed that the addition of 6% NaCl did not prevent growth of the species, but when *C. famata* was cross-streaked, growth of *P. roqueforti* was restricted. These experiments were also conducted under both aerobic and anaerobic conditions and, as before, inhibition only occurred in samples incubated anaerobically. This inhibition could be due to the increased level of NaCl affecting the ability of the mould to compete with other organisms, but could be due to the production of anti-microbial substances by the yeast, which could be stimulated by adverse growth conditions.

The ability of *C. famata* to inhibit the growth of other species of *Penicillium* (*P. italicum, P. digitatum*) has been demonstrated by others (Arras *et al.*, 1998; D'hallewin *et al.*, 1999) and the results of the current study may indicate a wider ability. In addition, however, the anti-microbial capabilities of lactic acid bacteria are well documented (Lindgren and Dobrogosz, 1990; Gourama and Bullerman, 1995; Adams and Nicolaides, 1997; Niku-Paavola *et al.*, 1999) and individual species may also contribute to poor growth of *P. roqueforti.* This possibility should be investigated further in future studies on blue cheese.

7.4 Comparison of the microflora of blue areas with that of white areas

Several cheeses were sampled so that the blue veined areas could be differentiated from white areas. This could not be carried out on all samples as in some cases blue veining was confluent and in others, so deficient that insufficient was available for sub-sampling. Additionally, cryosectioning and subsequent staining was used to examine a section across a blue vein in sample 28.

In poor quality cheeses, significant differences were noted in the TVC, TLC and mould count, between the blue and white areas. There was no significant difference in the yeast count of these areas. The difference in mould count could be anticipated given the presence or absence of blue veins. However, the differences in TVC and TLC are less predictable. In good cheeses there was no difference in TVC and TLC between blue and white areas and this leads to the question "is this cause or effect?". In the samples exhibiting few blue veins, both the TVC and the TLC were lower in the white areas than in the blue areas, which may have been due to the increased pH of the blue areas. The higher pH of the areas of blue veining may have enabled continued growth of both starter and non-starter bacteria and the results of cryogenic sectioning may support this, since there was evidence of both cocci and rods in the areas close to the blue veins. Curd particles appeared to be surrounded by rods, presumably lactobacilli, which would suggest that these could grow equally well in blue or white areas and other studies have demonstrated the growth of lactobacilli in non-blue cheeses (Naylor and Sharpe, 1958b; Clark and Reinbold, 1967; Lues *et al.*, 1999; Fitzsimons *et al.*, 2001). However, the increased pH, or other factors such as production of growth factors, in the blue areas may further encourage growth of members of this genus.

Fitzsimons *et al.* (2001) found that cores of cheddar cheese taken from the same cheese gave slight variations in the species of lactobacilli, indicating that there may be variations in the distribution of species within blocks of cheese. They proposed that this might be due to

the ability of species to survive localised salt in moisture conditions or temperature differentials during maturing. It is likely that such variation would also occur in blue cheeses and the pH and other changes brought about by growth of *P. roqueforti* could increase this variability. The results of biochemical checks on samples 31-39, shown in Appendix 4, showed that *Lb. plantarum* was the most commonly occurring species of *Lactobacillus* in the blue veined areas, whilst in the areas between the blue veins, the white areas of curd, *Lb. curvatus* and *Lb. brevis* were isolated, although *Lb. plantarum* was not. This may support the theory discussed in section 7.2.1, that *Lb. plantarum* becomes the dominant species in Stilton during maturation. It also supports the hypothesis that *Lb. plantarum* is the most common species of *Lactobacillus* in good quality, blue Stilton, exhibiting luxuriant growth of the mould *P. roqueforti.*

The influence of the species of lactobacilli isolated during this study, on the growth of *P. roqueforti* should be investigated further and may form the basis of future studies. However, of equal importance is the influence of *P. roqueforti* on the growth of individual species of lactobacilli, since this study has not been able to identify whether the relationship between proliferation of blue veins and occurrence of *Lb. plantarum* is due to inhibition of *P. roqueforti* by lactobacilli, or production of growth factors for *Lb. plantarum* by *P. roqueforti.*

7.5 Volatile compounds in mature Stilton cheese

A sample of cheese exhibiting luxuriant growth of *P. roqueforti* was compared with a poor quality cheese that showed few blue veins, using GC-MS olfactometry. As discussed in section 7.2.1 *Lb. brevis* was the most commonly occurring species of lactobacilli in the latter sample and it was clear from the results of this experiment that there were significant differences, not only in the volatile compounds in the two samples, but also in the aroma profiles. It is likely that these differences were attributable to a combination of the growth of different species of lactobacilli and the variation in proliferation of *P. roqueforti.*

The lipolytic activities of *P. roqueforti* have been investigated during a number of studies (Schwartz and Boyd, 1963; Schwartz and Parks, 1963; Madkor *et al.,* 1987a) and this study

also found methyl ketones, presumably originating from growth of the species, in both good and poor quality cheeses. However, fewer methyl ketones were found in the good cheese than in the poor cheese, although this does not mean that there were less in terms of the volume of these compounds and milky or cheesy aromas were noted more frequently in the good cheese than in the poor quality sample.

It seems likely that the production of fruity flavours in the poor quality cheeses was attributable to the presence of *Lb. brevis*, but whilst studies have linked fruity flavours to the growth of this heterofermentative species, there is little evidence to suggest what compounds the species may yield during growth in cheese. This area requires further investigation in order to determine the importance of the species to the quality of the cheese. In addition, such a study could investigate the critical level of the species and the significance of the ratio of the species to other, homofermentative, species. However, it is clear from the results of this study, that the range and combination of species of lactobacilli influences the flavour of the cheese and as such will be a major factor in the difference in flavour profiles of cheeses manufactured at individual creameries.

7.6 Summary of recommendations and conclusions

This study has shown that the microflora of blue Stilton cheese is similar to other cheeses in that lactobacilli form a significant part of the microflora of the mature cheese, despite the fact that they are not used in the starter cultures. Their range of species of *Lactobacillus* present in the cheese might be closely linked to the growth of *P. roqueforti*. Furthermore, the presence of a range of species and strains could affect the flavour of the cheese through the production of volatile compounds. This relationship should be investigated further in order to determine the effects on product quality and, additionally, the sources of lactobacilli in the product should be investigated. In addition, future studies should examine the potential for species of lactobacilli to inhibit the growth of *P. roqueforti*.

The volatile compounds produced during fermentation are vitally important to the quality of the final product and the investigation into the range of compounds and the associated aroma profile should be investigated further. In this study, only one cheese of each type was sampled and it is recommended that cheeses produced over an extended period should be tested. The study indicated that there may be critical times of the year in terms of the quality of product and by examining these compounds and aromas over such a period the existence (or not) of critical periods could be confirmed. The reason for any variability could then be investigated.

The study also showed that the levels of yeasts in Stilton cheese increased rapidly from a low threshold post pasteurisation. It seems likely that blue cheeses tend towards higher levels of yeasts than Cheddar types and this might be linked to the growth of *P. roqueforti*. The importance of some species of yeast to the quality of the product has also been identified during this study, with possible inhibition of *P. roqueforti* by *C. famata* being noted. This is, potentially, a significant result of the study and further investigations should attempt to characterise the conditions necessary for such inhibition and the mechanisms involved. In addition, the study has detected the presence of other species of *Candida* which may cause browning in the product, thus reducing the shelf life.

The study has given information on the microorganisms within Stilton cheese, a product that has little published literature, but many questions have been raised, which need further investigation.

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APPENDIX 1

Appendix 1 - Media formulae

M.R.S Agar (de Man, Rogosa, Sharpe) (Oxoid CM361)

	$g\ l^{-1}$
Peptone	10.0
"Lab Lemco" Powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	$1ml\ l^{-1}$
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05
Agar	10.0

pH 6.2 ±0.2

Milk Agar (Oxoid CM21)

	$g\ l^{-1}$
Peptone	5.0
Yeast extract	3.0
Milk solids (equivalent to 10 ml fresh milk)	1.0
Agar No 3 (Oxoid L13)	15

Maximum Recovery Diluent (Oxoid CM 733)

	$g\ l^{-1}$
Peptone	1.0
Sodium chloride	8.5
pH 7.0 ± 0.2	

Rose Bengal Chloramphenicol Agar (Oxoid CM 549)

	$g\ l^{-1}$
Mycological peptone	5.0
Glucose	10.0
Dipotassium phosphate	1.0
Magnesium sulphate	0.5
Rose-Bengal	0.05
Agar	15.5
pH 7.2±0.2	
Chloramphenicol supplement	50mg

Sabouraud Dextrose Agar (CM 41)

	$g\ l^{-1}$
Mycological peptone	10.0
Glucose	40.0
Agar	15.0
pH 5.6 ± 0.2	

APPENDIX 2

Appendix 2 - Results of investigations into the variations in the pH of Blue Stilton cheese

White Areas	Blue areas
5.45	6.54
4.99	6.60
5.65	6.68
5.50	6.81
5.70	6.48
5.45	5.80
5.20	6.10
5.30	5.63
5.60	5.63
5.70	5.90
6.42	7.02
6.45	6.53
5.90	6.99
5.81	6.13
6.43	6.32
5.91	6.99
5.99	6.16
5.84	6.25
5.74	6.28
5.71	6.32
Mean 5.74	Mean 6.36
SD 0.389	SD 0.426

APPENDIX 3

Appendix 3 - Comparison of identification characteristics of yeast species isolated during this study

API ID 32 C

Date and ID number	5777150131	5577350132	7.2001 7370150135	10.10.01 5775150137	10.10.01 5775150137	5577350135	7265140131
Sample source	FF1945	FF1945	White room	P5580	P5576	Cropwell	Colston
Substrate							
Galactose	+	+	+	+	+	+	+
Actidione	-	-	+	-	-	-	+
Sucrose	+	+	+	+	+	+	+
N-acetyl-glucosamine	+	+	+	+	+	+	-
DL-lactate	+	-	+	+	+	-	+
L-arabinose	+	+	-	+	+	+	-
Cellobiose	+	+	+	+	+	+	-
Raffinose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Trehalose	+	+	-	+	+	+	+
2 aceto-gluconate	+	+	-	-	-	+	-
α-methyl-D-glucoside	+	+	-	+	+	+	+
Sorbitol	+	+	+	+	+	+	+
D-xylose	+	+	-	-	-	+	-

Date and ID number	5777150131	5577350132	7.2001 7370150135	10.10.01 5775150137	10.10.01 5775150137	5577350135	7265140131
Ribose	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	-
Rhamnose	-	-	-	-	-	-	-
Palatinose	+	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-
Glucuronate	-	-	-	-	-	-	-
Melezitose	+	+	+	+	+	+	+
Gluconate	-	-	-	-	-	-	-
Levulinate	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-
Glucose	+	-	+	+	+	+	+
Sorbose	-	+	-	+	+	-	-
Glucosamine	-	-	+	+	+	+	-
IDENTIFICATION	*C. famata*	*C. famata*	*C. famata*	*C. famata*	*C. famata*	*C. famata*	*C. sphaerica*
INHIBITION	+		-		+		-

Date and ID number	10.10.01 7261150131	10.10.01 7261140131	10.10.01 7261140130	10.10.01 7261140133	10.01.01 2300111011
Sample source	Poor (P5580)	P5581	P5581 anaerobic	Poor cheeses	P 5581 anaerobic
Substrate					
Galactose	+	+	+	+	-
Actidione	+	+	+	+	+
Sucrose	+	+	+	+	-
N-acetyl-glucosamine	-	-	+	-	+
DL-lactate	+	+	+	+	+
L-arabinose	-	-	-	-	-
Cellobiose	-	-	+	-	-
Raffinose	+	+	+	+	-
Maltose	+	+	+	+	-
Trehalose	+	+	-	+	-
2 aceto-gluconate	-	-	-	-	-
α-methyl-D-glucoside	-	-	-	-	-
Sorbitol	+	+	+	+	+
D-xylose	-	-	-	-	-
Ribose	-	-	-	-	-
Glycerol	+	-	+	-	+
Rhamnose	-	-	-	-	-
Palatinose	+	+	+	+	-

Erythritol	-	-	-	-	+
Melibiose	-	-	-	-	-
Glucuronate	-	-	-	-	-
Melezitose	+	+	+	+	-
Gluconate	-	-	-	-	-
Levulinate	-	-	-	-	-
Mannitol	+	+	+	+	+
Lactose	+	+	+	+	-
Inositol	-	-	-	-	-
Glucose	+	+	-	+	+
Sorbose	-	-	-	+	-
Glucosamine	-	-	-	-	-
IDENTIFICATION	*C. sphaerica*	*C. sphaerica*	*C. sphaerica*	*C. sphaerica*	*C. lipolytica*
INHIBITION	-	-	-	-	

API 20C

Sample	FF1945	July 2000	April 2000	April 2000	April 2000	White room	White room	Deep	Surface
ID number	6776773	6152110	2156733	6177773	2156732	6356773	6356551	6066671	6166676
Substrate									
Glucose	+	+	+	+	+	+	-	+	+
Glycerol	+	+	+	+	-	+	+	+	+
2 aceto-gluconate	+	+	+	+	+	+	+	-	-
L-arabinose	+	-	-	-	-	+	+	-	-
D-xylose	+	-	-	-	-	-	-	-	-
Adonitol	+	+	+	+	+	+	+	-	-
Xylitol	+	-	+	+	-	-	-	+	+
Galactose	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+
α-methyl-D-glucoside	+	-	+	+	+	+	+	+	+
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	-	-
Cellobiose	+	-	+	+	+	+	-	+	+
Lactose	+	-	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Saccharose	+	-	+	+	+	+	-	+	+
Trehalose	+	-	-	+	-	+	+	+	+

Melezitose	+	-	+	+	-	+	+	+	+
Raffinose	+	-	+	+	+	+	-	-	+
IDENTIFICATION	*C. famata*	*C. dubliniensis*	*C. famata*	*C. famata*	*C. famata*	*C. famata*	*C. famata*	*C. sphaerica*	*C. famata*
INHIBITION	+		NT	+	NT	-	-	NO	LTD

APPENDIX 4

Appendix 4 - Comparison of biochemical profiles of lactobacilli

Samples 17-22 Substrate	Good cheeses, bulk starter June 1999	Good cheeses, bulk starter June 1999	Good cheeses, bulk starter June 1999
Glycerol	-	+	-
Erythritol	-	-	-
D-Arabinose	-	-	-
L-Arabinose	-	-	-
Ribose	+	+	+
D-Xylose	-	-	-
L-Xylose	-	-	-
Adonitol	-	-	-
β methyl-xyloside	-	-	-
Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	-	-	-
Rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
Mannitol	+	+	+
Sorbitol	-	-	-
α Methyl-D-mannoside	-	-	-
α Methyl-D-glucoside	-	-	-
N acetyl glucosamine	+	+	+
Amygdaline	-	+	+
Arbutine	-	+	+
Aesculin	-	-	-
Salicine	-	+	+
Cellobiose	-	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	-	+	-
Saccharose	+	+	+
Trehalose	+	+	+
Inuline	-	-	-

Samples 17-22 Substrate	Good cheeses, bulk starter June 1999	Good cheeses, bulk starter June 1999	Good cheeses, bulk starter June 1999
Melezitose	+	+	-
D-Raffinose	-	+	-
Amidon	-	-	-
Glycogene	-	-	-
Xylitol	-	-	-
β Gentiobiose	-	+	+
D-Turanose	+	-	-
D-Lyxose	-	-	-
D-Tagatose	+	-	-
D-Fucose	-	-	-
L-Fucose	-	-	-
D-Arabitol	-	-	+
L-Arabitol	-	-	-
Gluconate	-	-	+
2 ceto-gluconate	-	-	-
5 ceto-gluconate	-	-	-
IDENTIFICATION	*Lb. curvatus*	*Lb. plantarum*	*Lb. brevis*

Samples 27-30 Substrate	LV 191923 elliptical	LV191923 elliptical	LV191923 circular	LV201924 white	LV201924 blue
Glycerol	-	+	+	+	-
Erythritol	-	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	-	-	-	-	-
Ribose	+	+	+	+	+
D-Xylose	-	-	-	-	-
L-Xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β methyl-xyloside	-	-	-	-	-
Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Mannitol	+	+	+	+	+
Sorbitol	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-
N acetyl glucosamine	+	+	+	+	+
Amygdaline	+	+	+	+	+
Arbutine	+	+	+	+	+
Aesculin	-	-	-	-	-
Salicine	+	+	+	+	+
Cellobiose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	-	+	+	+	-
Saccharose	+	+	+	+	+
Trehalose	+	+	+	+	+
Inuline	-	-	-	-	-

Samples 27-30 Substrate	LV 191923 elliptical	LV191923 elliptical	LV191923 circular	LV201924 white	LV201924 blue
Melezitose	-	+	+	+	-
D-Raffinose	-	+	+	+	-
Amidon	-	-	-	-	-
Glycogene	-	-	-	-	-
Xylitol	-	-	-	-	-
β Gentiobiose	+	+	+	+	+
D-Turanose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	+	-	-	-	+
L-Arabitol	-	-	-	-	-
Gluconate	+	-	-	+	+
2 ceto-gluconate	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-
IDENTIFICATION	*Lb. brevis*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. brevis*

Samples 31-39 (Good)	Blue	Blue	White	Blue	White
Substrate					
Glycerol	-	-	-	-	-
Erythritol	-	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	-	-	-	-	+
Ribose	+	+	+	+	+
D-Xylose	-	-	-	+	+
L-Xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β methyl-xyloside	-	-	-	-	-
Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	-	-
L-Sorbose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Mannitol	+	+	+	-	+
Sorbitol	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	+	+
N acetyl glucosamine	+	+	+	+	+
Amygdaline	-	-	-	-	-
Arbutine	-	-	-	-	-
Aesculin	-	-	-	-	-
Salicine	+	+	+	+	+
Cellobiose	-	-	+	-	-
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	-	-	-	+	+
Saccharose	+	+	+	-	-
Trehalose	+	+	+	-	-
Inuline	-	-	-	-	-
Melezitose	+	+	+	-	-
D-Raffinose	-	-	-	-	-
Amidon	-	-	-	-	-

Samples 31-39 (Good)	Blue	Blue	White	Blue	White
Substrate					
Glycogene	-	-	-	-	-
Xylitol	-	-	-	-	-
β Gentiobiose	-	-	-	-	-
D-Turanose	+	+	+	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	+	+	+	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	-	-	-	-	-
L-Arabitol	-	-	-	-	-
Gluconate	-	-	-	+	+
2 ceto-gluconate	-	-	-	-	-
5 ceto-gluconate	-	-	-	+	+
IDENTIFICATION	*Lb. curvatus*	*Lb. curvatus*	*Lb. curvatus*	*Lb. brevis*	*Lb. brevis*

Samples 31-39 (Good)	Blue	Blue	Blue	Blue
Substrate				
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
Ribose	+	+	+	+
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β methyl-xyloside	-	-	-	-
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	+	+
L-Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	+	+	+
Sorbitol	-	-	-	-
α Methyl-D-mannoside	-	-	-	-
α Methyl-D-glucoside	-	-	-	-
N acetyl glucosamine	+	+	+	+
Amygdaline	+	+	+	+
Arbutine	-	-	-	-
Aesculin	-	-	-	-
Salicine	-	-	-	-
Cellobiose	+	-	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Melibiose	-	+	+	+
Saccharose	-	-	-	-
Trehalose	-	-	-	-
Inuline	-	-	-	-
Melezitose	-	-	-	-
D-Raffinose	-	-	-	-
Amidon	-	-	-	-

Samples 31-39 (Good)	Blue	Blue	Blue	Blue
Substrate				
Glycogene	-	-	-	-
Xylitol	-	-	-	-
β Gentiobiose	+	+	+	+
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Gluconate	+	-	+	+
2 ceto-gluconate	-	-	-	-
5 ceto-gluconate	-	-	-	-
IDENTIFICATION	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*

Samples 40-41					
Substrate					
Glycerol	-	-	-	-	-
Erythritol	-	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	-	-	-	-	+
Ribose	+	-	+	+	+
D-Xylose	-	-	-	-	+
L-Xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β methyl-xyloside	-	-	-	-	-
Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	-
L-Sorbose	-	-	-	-	-
Rhamnose	-	-	-	+	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Mannitol	+	+	+	+	-
Sorbitol	-	-	+	+	-
α Methyl-D-mannoside	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-
N acetyl glucosamine	+	+	+	+	+
Amygdaline	+	+	+	+	-
Arbutine	-	-	+	+	-
Aesculin	-	-	-	-	-
Salicine	-	-	+	+	-
Cellobiose	-	-	+	+	-
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	-	-	-	-	-
Saccharose	+	-	+	+	-
Trehalose	-	-	+	+	-
Inuline	-	-	-	-	-
Melezitose	-	-	-	-	-
D-Raffinose	-	-	-	-	-
Amidon	-	-	-	-	-

Samples 40-41					
Substrate					
Glycogene	-	-	-	-	-
Xylitol	-	-	-	-	-
β Gentiobiose	+	-	+	+	-
D-Turanose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	-	-	+	+	-
L-Arabitol	-	-	-	-	-
Gluconate	-	-	+	-	+
2 ceto-gluconate	-	-	-	-	-
5 ceto-gluconate	-	-	+	-	+
IDENTIFICATION	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. brevis*

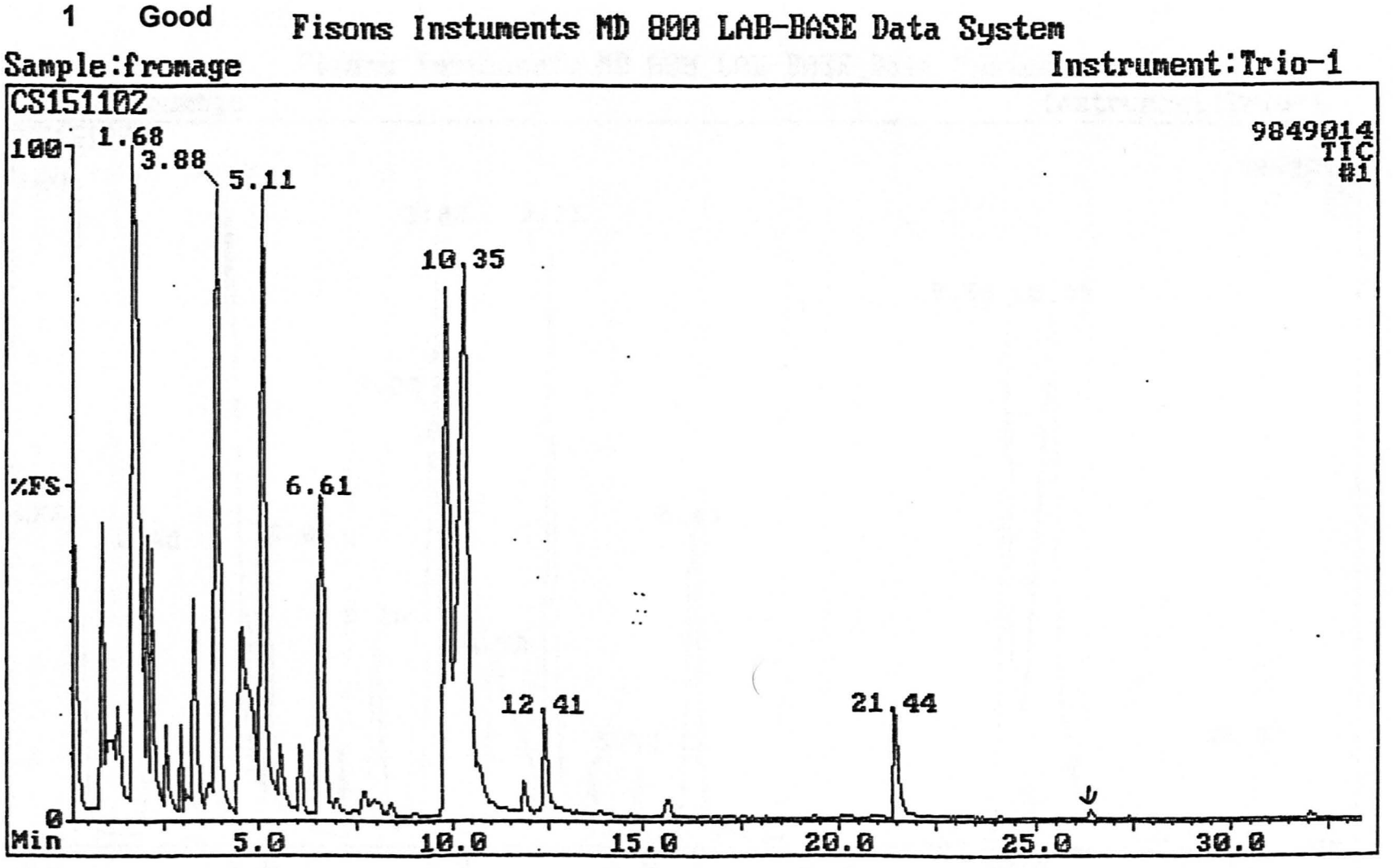
Samples 42-52	Strain 1	Strain 2	Strain 3	Strain 4
Substrate				
Glycerol	+	+	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
Ribose	+	+	+	+
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β methyl-xyloside	-	-	-	-
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	+	+
L-Sorbose	-	-	-	-
Rhamnose	+	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	+	+	+
Sorbitol	+	+	-	-
α Methyl-D-mannoside	+	+	-	-
α Methyl-D-glucoside	-	-	-	-
N acetyl glucosamine	+	+	+	+
Amygdaline	+	+	+	+
Arbutine	+	+	-	-
Aesculin	+	-	-	-
Salicine	+	+	-	-
Cellobiose	+	+	-	-
Maltose	+	+	+	+
Lactose	+	+	+	+
Melibiose	+	+	-	-
Saccharose	+	+	+	+
Trehalose	+	+	-	-
Inuline	-	-	-	-
Melezitose	+	+	-	-
D-Raffinose	+	+	-	-
Amidon	+	-	-	-

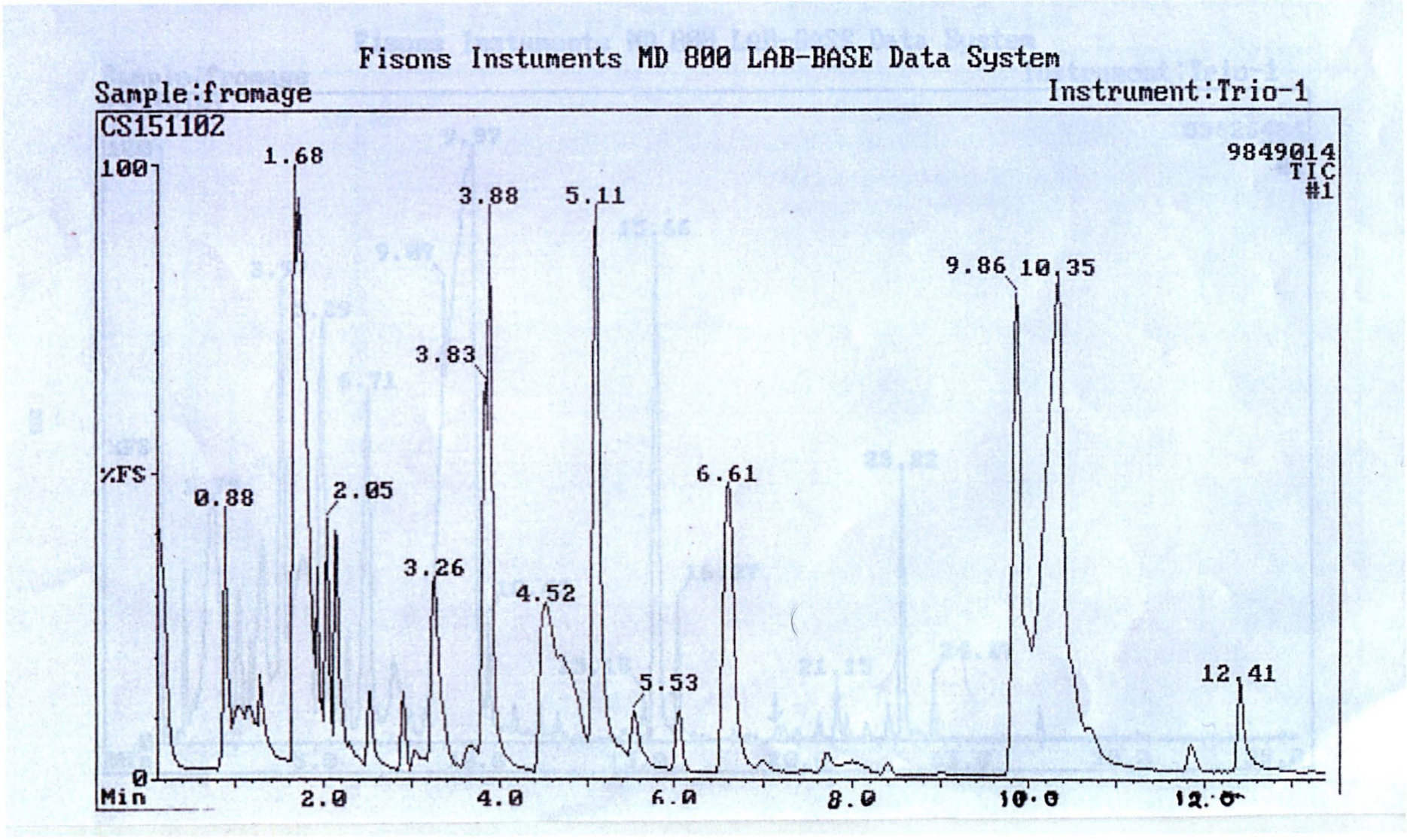
Samples 42-52	Strain 1	Strain 2	Strain 3	Strain 4
Substrate				
Glycogene	-	-	-	-
Xylitol	-	-	-	-
β Gentiobiose	+	+	+	+
D-Turanose	-	+	-	-
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	+	-	-	-
L-Arabitol	-	-	-	-
Gluconate	+	-	-	+
2 ceto-gluconate	-	-	-	-
5 ceto-gluconate	-	-	-	-
IDENTIFICATION	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*

Samples 53-62 Substrate	Good cheeses, 5568 Aug 2001	Good cheeses, 5573	Poor cheeses, 5581 0 Aug 2001	Poor cheeses 5581 E Aug 2001
Glycerol	+	+	-	+
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	+	-
Ribose	+	+	+	+
D-Xylose	+	-	+	-
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β methyl-xyloside	-	-	-	-
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	-	+
L-Sorbose	-	-	-	-
Rhamnose	+	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	+	-	+
Sorbitol	+	+	-	-
α Methyl-D-mannoside	+	+	-	-
α Methyl-D-glucoside	-	-	-	-
N acetyl glucosamine	+	+	+	+
Amygdaline	+	+	-	+
Arbutine	+	+	-	-
Aesculin	+	-	-	-
Salicine	+	+	-	+
Cellobiose	+	+	-	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Melibiose	+	+	-	-
Saccharose	+	+	-	+
Trehalose	+	+	-	+
Inuline	-	-	-	-

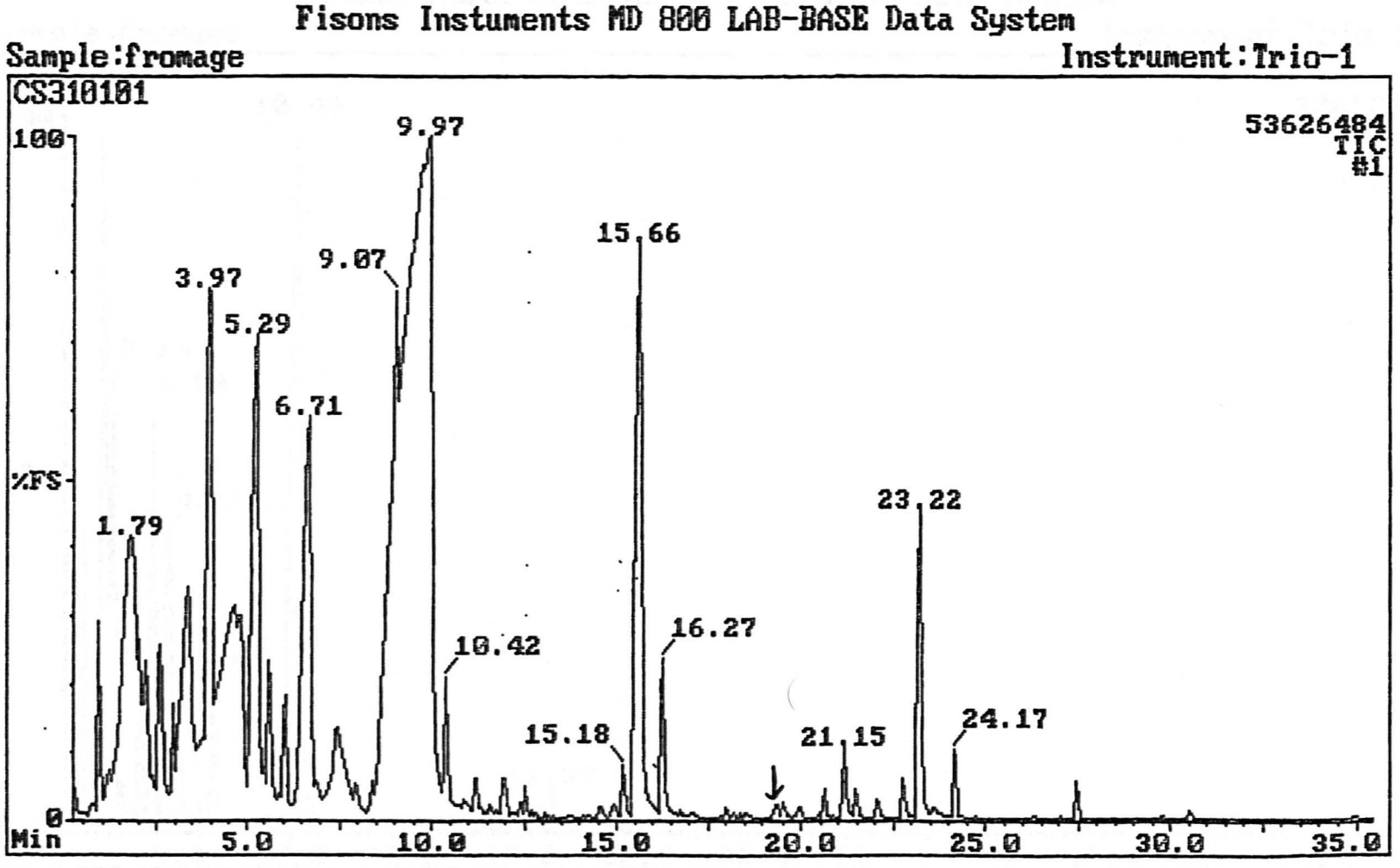
Samples 53-62 Substrate	Good cheeses, 5568 Aug 2001	Good cheeses, 5573	Poor cheeses, 5581 0 Aug 2001	Poor cheeses 5581 E Aug 2001
Melezitose	+	+	-	+
D-Raffinose	+	+	-	-
Amidon	+	-	-	-
Glycogene	-	-	-	-
Xylitol	-	-	-	-
β Gentiobiose	+	+	-	+
D-Turanose	-	+	-	+
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	+
L-Fucose	-	-	-	-
D-Arabitol	+	-	-	-
L-Arabitol	-	-	-	-
Gluconate	+	-	+	-
2 ceto-gluconate	-	-	-	-
5 ceto-gluconate	-	-	+	-
IDENTIFICATION	*Lb. plantarum*	*Lb. plantarum*	*Lb. brevis*	*Lb. paracasei* subsp. *paracasei*

Appendix 5 - Plots from GC-MS of good and poor quality cheeses

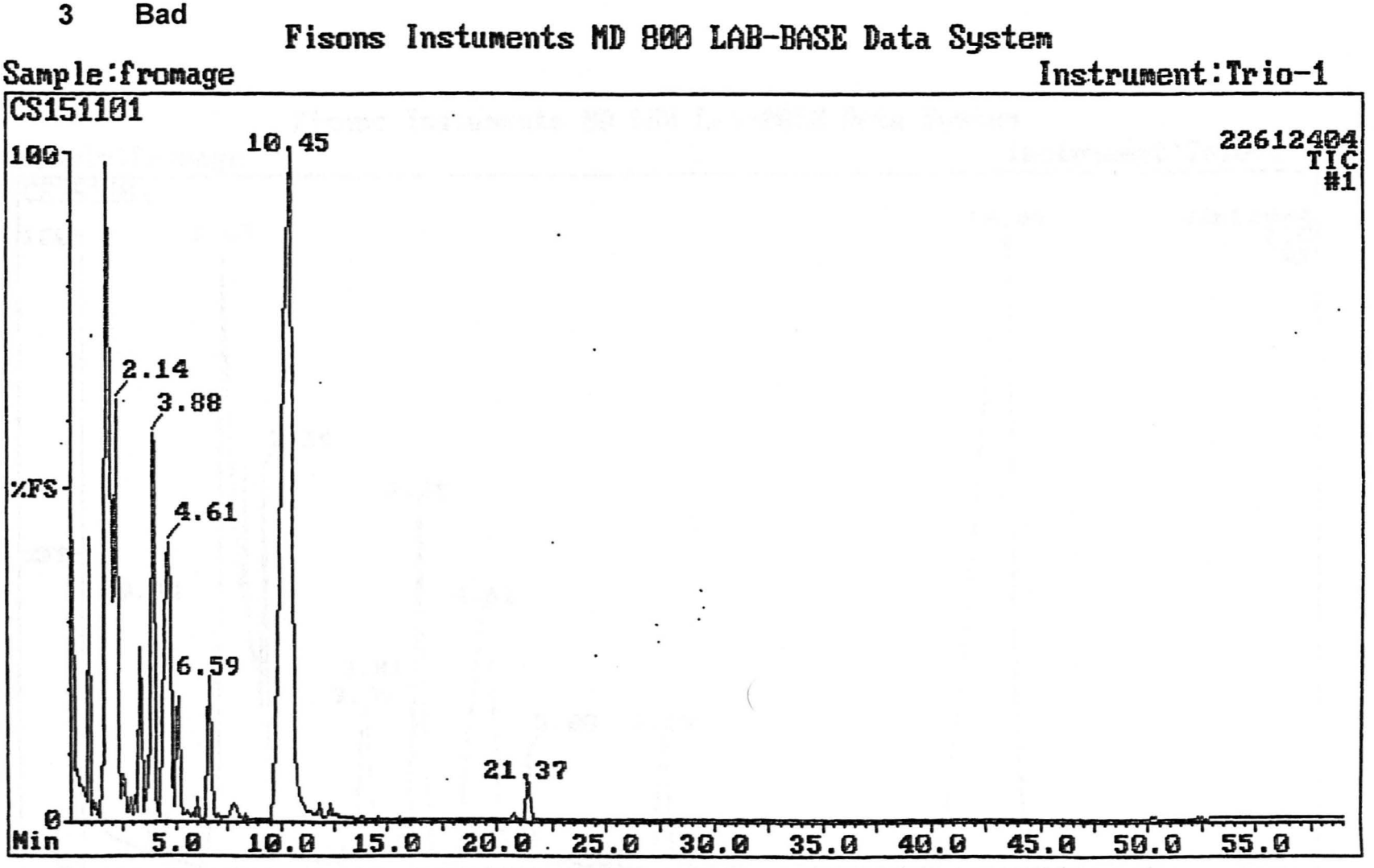

1 Good
Fisons Instuments MD 800 LAB-BASE Data System
Sample:fromage
Instrument:Trio-1
CS151102
9849014
TIC
#1
100
%FS
0
Min
1.68
3.88
5.11
6.61
10.35
12.41
21.44
5.0
10.0
15.0
20.0
25.0
30.0


Fisons Instuments MD 800 LAB-BASE Data System
Sample:fromage
Instrument:Trio-1
CS151102
9849014
TIC
#1
100
%FS
0
Min
1.68
3.88
5.11
9.86
10.35
3.83
0.88
2.05
6.61
3.26
4.52
5.53
12.41
2.0
4.0
6.0
8.0
10.0
12.0

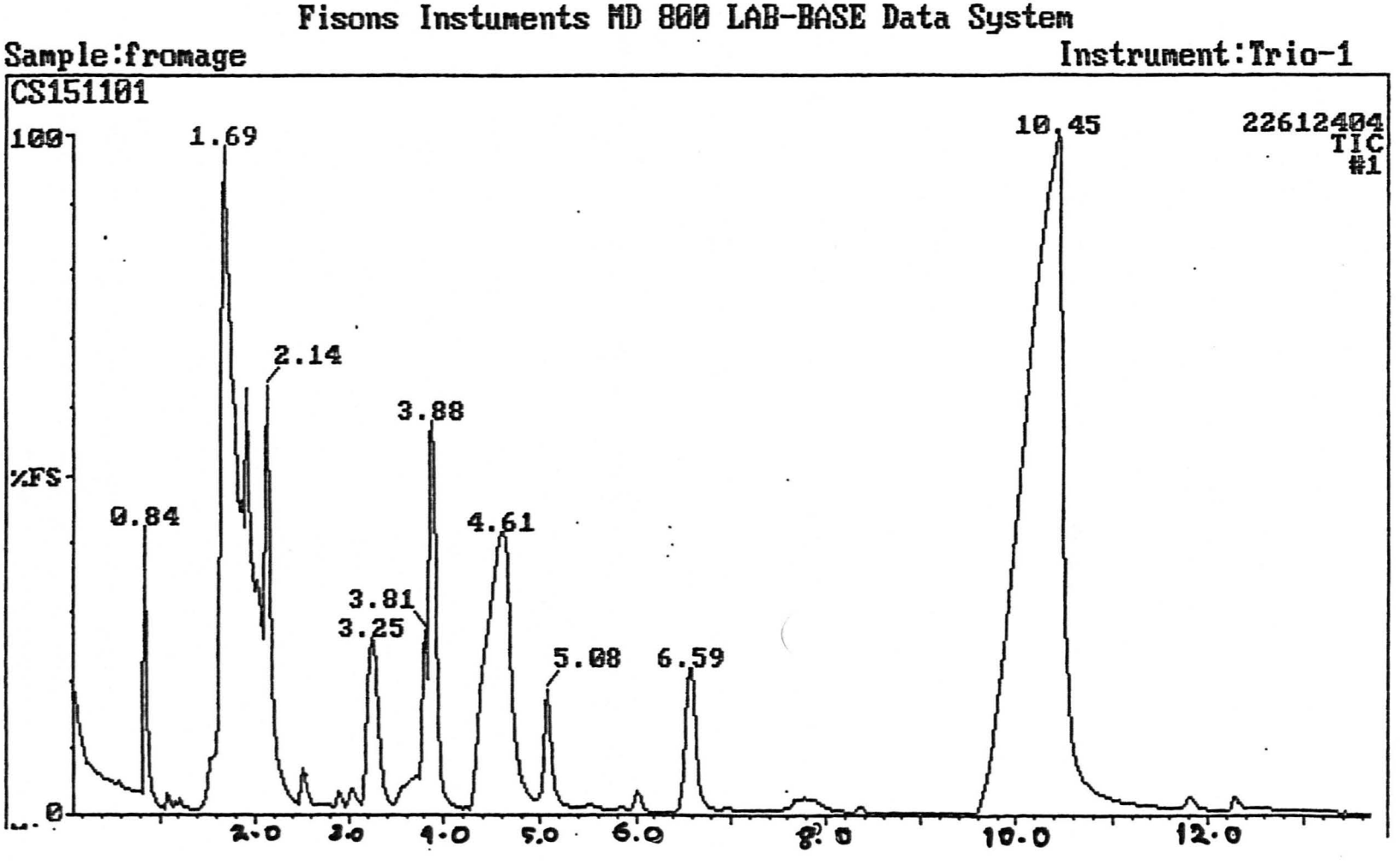
Bad Stilton


Fisons Instuments MD 800 LAB-BASE Data System
Sample:fromage
Instrument:Trio-1
CS310101
53626484
TIC
#1
100
%FS
0
Min
5.0
10.0
15.0
20.0
25.0
30.0
35.0
1.79
3.97
5.29
6.71
9.07
9.97
10.42
15.18
15.66
16.27
21.15
23.22
24.17

3 Bad


Fisons Instuments MD 800 LAB-BASE Data System
Sample:fromage
Instrument:Trio-1
CS151101
22612404
TIC
#1
100
%FS
0
Min
10.45
2.14
3.88
4.61
6.59
21.37
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0

4 Bad


Fisons Instuments MD 800 LAB-BASE Data System
Sample:fromage
Instrument:Trio-1
CS151101
100
%FS
0
1.69
2.14
3.88
0.84
4.61
3.81
3.25
5.08
6.59
10.45
22612404
TIC
#1
2.0
3.0
4.0
5.0
6.0
8.0
10.0
12.0