THE OCCURRENCE, GROWTH AND SURVIVAL OF YEASTS IN MATURED CHEDDAR

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OCCURRENCE, GROWTH AND SURVIVAL OF YEASTS IN MATURED CHEDDAR

by

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Submitted in fulfilment of the requirements

for the degree of

MAGISTER SCIENTIAE

in the

Department of Microbiology and Biochemistry,

Faculty of Natural Sciences,

University of the Orange Free State, Bloemfontein

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March 1999

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"Homes are built on the foundation of wisdom

and understanding. Where there is knowledge,

the rooms are furnished with valuable, beautiful things."

ONE OF KING SOLOMON'S WISE SAYINGS.

This dissertation is dedicated to

HESTER CHARLTON- PERKINS

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons for their contributions to the successful completion of this study:

Prof. B.C. Viljoen, Department of Microbiology and Biochemistry, University of the Orange Free State, for his able guidance in planning and executing this study and for his constructive and able criticism of the dissertation;

Clover S. A, for their financial support;

To wife, for her wonderful support throughout the study;

To my parents, for their love, interest and encouragement, and

Finally, to **God** for His strength throughout the study.

CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

A wide variety of yeasts are associated with different cheese varieties (Deak and Beuchat, 1995; Devoyod, 1990; Fleet, 1990; Fleet, 1992). Depending on the strain properties and the contamination level, the yeasts affect the ripening process positively (de-acidification, aroma substances) or negatively (odour and taste defects) (Eliskases-Lechner and Ginzinger, 1995). Bacteria usually cause fermentation of milk products and are therefore considered to be of major importance during the making of cheese (Cousin, 1982). Yeasts, however, play a significant role in the spoilage of dairy products and the ripening of some cheese varieties due to their physiological characteristics including their ability to progress at low temperatures, low moisture content, elevated salt concentrations and their resistance against physico-chemical stresses of importance in food preservation (Fleet and Mian, 1987; Seiler and Busse, 1990; Warth, 1991; Deak and Beuchat, 1996).

Limited studies have been conducted on the composition of yeast flora of matured Cheddar cheese and their positive and detrimental role during the manufacturing and ripening process. Commercial cultures for the application in Cheddar cheese processing comprise different yeast species (e.g. *Candida valida, Debaryomyces hansenii,* etc.), however, in South Africa these cultures are rarely used. The function of yeasts in Cheddar cheese making, remains only partly explored, due to the lack of preordained studies and research in this area which result in partial, occasional and incomplete information which fail to reflect the significant role of yeasts.

In this study we endeavoured to determine the incidence of yeasts, their interaction with bacteria during processing, and properties which governed their growth and survival in matured Cheddar cheese.

2. LITERATURE REVIEW

2.1 Historical background of Cheddar cheese

Cheese is one of the oldest foods of mankind. It is also referred to in the Old Testament of the Bible. It is related that Jesse said to his son David, "Carry these ten cheeses unto the captain of their thousand and look how they brethren fare" (1 Samuel 17-18). Shobi brought "honey and butter and sheep, and cheese of kine, for David, and for the people that were with him, to eat." (2 Samuel 17-29) Also: "Hast thou not poured me out like milk, and curdled me like cheese?" (Job 10:10).

In Greece 2500 years ago, cheese was a prominent article of food and sold by the Greeks in several Mediterranean countries. The milk of cows, goats, sheep, water buffalo and other animals has been used for cheese making (Wilster, 1964). Edible cheese, 2000 years old, was found in 1948 in a tomb in the region of Siberia (Wilster, 1964). In 1974 some Russians found a cheese in the permafrost of the Siberian tundra. It was at least 2000 years old and was said to be an unrivalled delicacy (Dairy processing handbook, 1995). Cheddar cheese has its origin in the county Somersetwest in Southwestern England. The name "Cheddar" is taken from the town Cheddar located in that county. The biggest cheese ever made was a Cheddar cheese weighing 15190 kg produced in January 1964 by the Eliscensen Foundation to be exhibited at the World Expo in New York and it took 43 hrs to produce. (Dairy processing handbook, 1995)

2.2 The outline of mature Cheddar cheese processing

Cheese of various types, is produced in several stages according to principles that have been worked out by years of experimentation. Each type of cheese has its specific production formula, often with a local touch. Cheese-making is the process by which liquid milk from female domestic animals is transformed, first into a gel by the action of rennet, then by physical and

microbial action. During the ripening period, lactic acid is produced by means of lactic acid bacteria, casein and fat are metabolized, and little by little, the cheese takes on its own particular, characteristic taste and flavour (Devoyod, 1990). The main stages of production of matured Cheddar cheese are illustrated schematically in Fig. 1.

2.2.1 Milk used for cheese making

The milk from any mammal can, in theory, be transformed into a cheese-like product, but for purely practical reasons, milk from domesticated animals has always dominated production. The chemical composition of the more important milks used for cheese-making are shown in Table 1.

The vagaries of the local country side play an important role in which kind of milk to be used for cheese-making. Cow milk, for instance is more readily available in lowland areas, whereas mountain tribes have relied on sheep or goats as the sources of raw material (Robinson, 1995). Cow milk is normally used for cheese-making, but in some countries milk from other mammals is used to produce certain varieties of cheese. Sheep milk is used for the making of Roquefort cheese, goat milk for many varieties of cheese in Italy and Greece and buffalo milk is used in India and Egypt (Robinson 1981). The composition of milk depends on many factors which include; a) Breed (strain of the breed and breeding policy), b) Feeding routines of the animals (nutritional value of foods), c) Stage of lactation (number of previous lactations, d) Health of the animal (physical conformation of the animal), e) Management of the herd, f) Intervals between milkings, g) Climate (Geographical region) and h) Time of the year (spring, low quality; summer, normal quality and autumn, high quality), (Scott, 1986).

For the manufacturing of quality cheese, the raw milk should be of good general bacteriological quality to avoid undesirable fermentations and enzymic reactions, and should be free from inhibitory substances, such as residual antibiotics, which interfere with the growth of the starter bacteria. Milk is cooled down to 4°C at the farm and stored in refrigerated bulk tanks. It is collected in refrigerated or isolated road tankers and transported to the

Table 1. Chemical compositions of milks from the more important species of mammal, (g/100g liquid milk), (reproduced from Tamime and Robinson, 1985).

Type	Water	Fat	Protein	Lactose	Ash	Calcium
Buffalo	82.1	8.0	4.2	4.9	0.8	
Camel	87.1	4.2	3.7	4.1	0.9	
Cow	87.6	3.8	3.3	4.7	0.6	0.08
Goat	87.0	4.5	3.3	4.6	0.6	0.95
Mare	89.0	1.5	2.6	6.2	0.7	
Sheep	81.6	7.5	5.6	4.4	0.9	

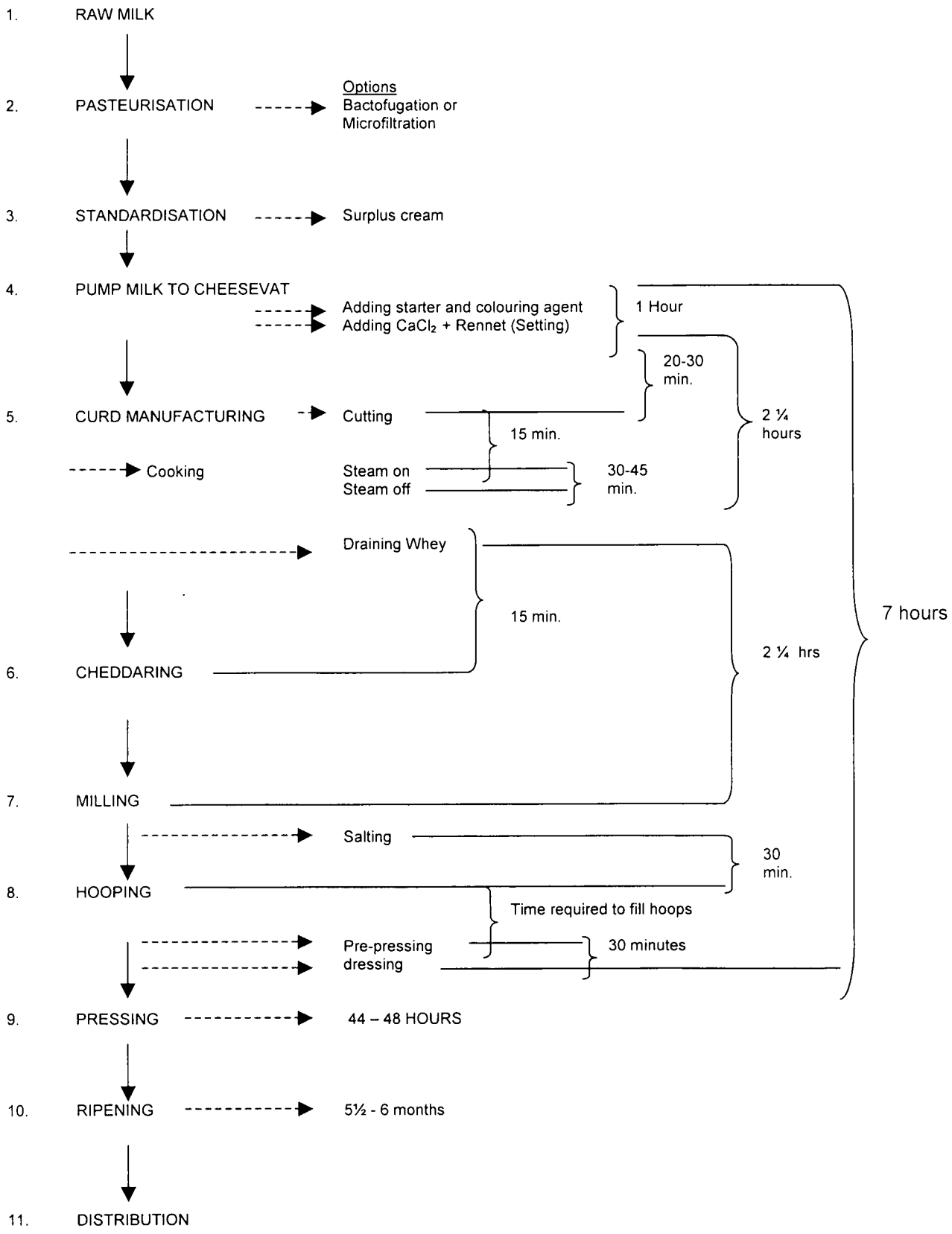

Fig. 1. Schematic illustration of the manufacturing process of mature Cheddar cheese. (Wilster, 1964; Dairy processing handbook, 1995).

cheese factory where it is stored in silos at 2 - 4°C for a maximum period of 72 hrs, before it is pasteurised for cheese-making.

To achieve a quality uniform cheese in large commercial plants, the manufacturing process must be as consistent as possible. The first requirement is uniformity of the raw milk. By bulking the milk in a silo, differences in the milk composition from various suppliers can be evened out (Fox, 1993b). For Cheddar cheese varieties the casein/fat ratio must be between 0.67 and 0.72 (Fox, 1993b). The higher the fat content in the cheese milk the more difficult it is to remove moisture from the rennet coagulum under the same manufacturing conditions, since the presence of fat interferes mechanically with the syneresis process (Fox, 1993b).

2.2.2 Fermentation of the milk

2.2.2.1 The normal microbial flora of cheese milk

When raw milk arrives at the dairy plant, the total bacterial counts are between 10^3 –10^7 cfu/ml, depending on the levels of hygiene at the farms. The microorganisms present consist of psychrotrophs, mostly *Pseudomonas, Aeromonas, Alcaligenes*, a small number of lactic acid bacteria, spore-forming Gram-positive rods, coryneform bacteria, micrococci and coliforms (Robinson, 1981). Of these, only the psychrotrophs will multiply during transport and storage, particularly if the temperature in the insulated tankers and milk silos is allowed to rise. This growth leads to the production of extracellular lipases and proteinases, particularly by pseudomonads, *Achromobacter, Acinetobacter* and *Aeromonas*. Table 2 lists some of the groups of microorganisms which have been found in milk supplies.

For the cheesemaker, the activity of the microorganisms and their reaction in the milk are very important. The activity of the microorganisms in the milk or curd determines the acceptability of a microorganism present whether desired or undesired, or even harmful (e.g. pathogens). Pathologically abnormal milk, like mastitic milk, contains organisms like *Streptococcus agalacteae, S. dysgalactiae, S. uberis* as well as *Staphylococcus aureus* (Scott, 1986). Infected milk may

Table 2. Microorganisms found in raw milk supplies (Reproduced from, Scott, 1986).

Group	Micro organism	Comments (mainly contaminants, expect N= Normal)
	Streptococcus bovis	Thermoduric
	Streptococcus feacalis	Thermoduric
	Streptococcus thermophilus	Thermoduric
	Streptococcus lactis	N
	Streptococcus cremoris	N
	Streptococcus citrovorus	N
	Streptococcus agalactiae	Diseased udder
	Streptococcus dysgalactiae	Diseased udder
	Micrococcus luteus	Thermoduric
	Micrococcus varians	Thermoduric
	Other *Micrococcus* spp.	Various from air contamination
	Staphylococcus aureus	Diseased udder
	Pseudomonas fluorescens	Psychrotrophic
	Pseudomonas putida	Psychrotrophic
	Pseudomonas fragi	Psychrotrophic
	Pseudomonas cepacia	Psychrotrophic
	Pseudomonas aeruginosa	
	Pseudomonas maltophilia	
	Pseudomonas alcaligenes	
	Pseudomonas pseudoalcaligenes	
	Corynebacterium lacticum	
	Corynebacterium bovis	
	Corynebacterium pyrogenes	
Coliforms	*Escherichia coli*	
	Klebsiella freundii	
	Klebsiella cloacae	
	Klebsiella aerogenes	Psychrotrophic
	Enterobacter liquifaceus	Psychrotrophic
Anaerobic spore forming rod bacteria (bacili)	*Bacillus cereus*	Thermoduric
	Bacillus subtilis	Thermoduric
	Bacillus licheniformis	Thermoduric
	Bacillus circulans	Thermoduric
Gram negative rod forms	*Aeromonas hydrophila*	Psychrotrophic
	Alcaligenes viscosus	Psychrotrophic
	Acinetobacter spp.	
	Achromobacter spp.	
Gram positive rod forms	*Brevibacterium spp.*	
	Microbacterium lacticum	
	Arthrobacter spp.	
	Lactobacillus spp.	
	Streptomyces spp.	From cereal food and decaying matter
	Actinomyces bovis	From diseased udder lesions
Yeast	Various	Occasional
Moulds	Various	Occasional

also contain coliform species, *Pseudomonas pyocyanae, Clostridium pyogenes* and even *Mycobacterium tuberculosis* (Scott, 1986). Late lactation milk, usually also contains high bacterial counts. The number of microorganisms depends on the hygiene exercised during the production of the milk, the season of the year, the milk handling and the transportation methods. The reactions induced by various microorganisms during warm weather are illustrated in Table 3, (Scott, 1986).

Pasteurisation of milk for cheese making (72°C/15-17s) greatly reduces the total count of microorganisms in the cheese milk, but their enzymes may survive the heat treatment and give rise to off-flavours in the cheese (Law et al., 1976). Pasteurised cheese milk normally consists of the thermoduric organisms which have survived pasteurisation, namely some corynebacteria, micrococci, enterococci and spores of *Bacillus* and *Clostridium*; post-pasteurisation contaminants such as other micrococci, occasionally coagulase positive staphylococci, coliforms, lactic acid bacteria including lactobacilli, pediococci, leuconostocs, enterococci, (Scott, 1986), moulds and yeasts (Viljoen and Greyling, 1995) may also be present.

2.2.2.2 Starter systems

Starter cultures contribute most to the cheese manufacturing process. Cheesemakers were using starter cultures long before they knew anything about bacteria, which consequently also reflects on their ignorance of acid production during cheese processing (Bester, 1978). Based on experience, the ancient cheesemakers learned that by taking sour milk, the acid forming abilities are transferred. Later on they started to use whey as starter culture by taking some of the previous days cheese whey to inoculate the milk. This method was not very reliable, consequently they had to find an alternative method.

The first research started in the late eighties of the previous century in Germany, Denmark and the United States of America. The first commercial starter was produced by Hansens in the late nineties of the previous century (Davis, 1965). During the past decades several research efforts to improve

Table 3. Reaction expected in cheese milk and by groups of bacteria during warm weather (Reproduced from, Scott, 1986).

Bacterial group	% of reaction by each group growing in warm milk				
	Acidity	Acid and clot	Alkali	Proteolysis	Lipolysis
Streptococci	12	88	Nil	9	8
Micrococci	15	3	2	14	18
Coliforms	60	40	1	Nil	10
Pseudomonads	Nil	Nil	22	85	70
Corynebacteria	2	4	1	30	10
Lactobacilli	0	85	Nil	18	22
Bacilli	4	8	2	55	44

starter culture technology for Cheddar cheese making have been attempted. Some studies have emphasised improvement of body, texture and flavour of the cheese (Cogan, et al., 1991). Others have sought to overcome or minimise problems related to bacteriophage infection (Cogan, et al., 1991). The use of characterised single strain starters has resulted in greater control over cheese flavour and phage infection. These strains have been used successfully in paired rotations and multiple strain blends (Czulah, et al. 1979; Gillies and Curtis 1963; Lawrence et al, 1978; Limsoutin et al, 1977; Martley and Lawrence, 1972; Lawrence and Pearce, 1972).

Traditionally, several different starters were used in the production of cheese. The solution was often based on the whim of the cheese producer rather than on sound scientific principles and starters were transferred numerous times before use, either by the culture supplier or by personnel in the factory. These procedures are likely to change the ratio and numbers of the different strains or species in a culture (Cogan, et al. 1991).

2.2.2.3 Starter cultures

Several microorganisms (bacteria, yeasts, moulds or combinations of these) are employed in the fermentation process of milk during the manufacturing process of cheese, mainly to produce lactic acid from lactose. This imparts a fresh, acid flavour to curd cheeses, assists in the formation of the rennet coagulum, and by causing shrinkage of the curd and moisture expulsion, promotes characteristic texture formation during cheese making (Robinson, 1981). Lactic acid bacteria used as starters in cheese-making include lactococci, leuconostocs and lactobacilli (Table 4). The objective of using starter cultures, is to produce clean- flavoured cheese with a high rate of lactic acid production in the early stages since the development of lactic acid inhibits the growth of undesired contaminants (Scott, 1986).

Basically starters used in cheese-making can be classified into mesophilic cultures with an optimum growth temperature of 30 °C and thermophilic cultures with an optimum growth temperature of 45°C. (Cogan et al., 1991). Table 4 shows the uses of various starters in the dairy industry.

Table 4 New and old names of various starters and their uses (Reproduced from the, The Bulletin of the IDF (263/1991) Cogan et al., 1991).

Type	Old Name	New Name	Product
Mesophillic			
O	*Streptococcus cremoris*	*Lactococcus lactis ssp. cremoris*	Cheddar cheese
	Streptococcus lactis	*Lactococcus lactis ssp. lactis*	Feta cheese Cottage cheese Quarg
L*	*Streptococcus cremoris*	*Lactococcus lactis ssp. cremoris*	Continental cheese (with eyes)
	Streptococcus lactis	*Lactococcus lactis ssp. lactis*	
	Leuconostoc citrovorum	*Leuconostoc mesenteroides ssp. cremoris*	Lactic Butter
	Leuconostoc lactis	*Leuconostoc lactis*	Feta cheese
D**	*Streptococcus cremoris*	*Lactococcus lactis ssp. cremoris*	Lactic Butter
	Streptococcus lactis	*Lactococcus lactis ssp. lactis*	
	Streptococcus diacetylactis	*Cit Lactococci****	
LD	*Streptococcus cremoris*	*Lactococcus lactis ssp. cremoris*	Continental cheese (with eyes)
	Streptococcus lactis	*Lactococcus lactis ssp. lactis*	moulds ripened cheese
	Streptococcus diacetylactis	*Cit Lactococci****	Culture buttermilk
	Leuconostoc citrovorum	*Leuconostoc mesenteroides ssp. cremoris*	Lactic Butter
	Leuconostoc lactis	*Leuconostoc lactis*	
Thermophillic			
	Streptococcus thermophilus	*Streptococcus salivarius ssp. thermophilus*	Yoghurt
	Lactobacillus bulgaricus	*Lactobacillus delbrueckii ssp. bulgaricus*	Mozzarella cheese
	Streptococcus thermophilus	*Streptococcus salivarius ssp. thermophilus*	Emmental cheese
	Lactobacillus helveticus	*Lactobacillus helveticus*	Grana cheese
	Lactobacillus lactis	*Lactobacillus delbrueckii ssp. lactis*	

*L = Leuconostoc, ** D = Diacetylactis and *** CIT * = Abbreviation for citrate which is metabolized to flavour and aroma compounds.

Species of the general streptococci, leuconostocs and lactobacilli are used as combined cultures, or as single strain cultures, or as mixtures of single strain cultures (Scott, 1986). Commercial suppliers of starter cultures have given codes to their own particular cultures, whether single or mixed and can usually supply literature giving details of the culture. Starter bacteria are inhibited by antibiotics (Robinson, 1981), bacteriophage (Cogan and Accolas, 1990) and detergent and disinfectant residues (Robinson, 1981).

2.2.2.4 The application of different methods using starter cultures for manufacturing of mature Cheddar cheese

Currently two kinds of starters are used commercially, Bulk starters or Mass starters and Direct vat starters.

2.2.2.4.1 Bulk starters or Mass starters

Bulk starters or Mass starters include an inoculum of 0,2 – 1,0% of an active culture, with a concentration of 1-5 x 10^9 cells/ml resulting in a final concentration of 1-5 x 10^7 cells/ml. During the inoculation of the cheese milk with the starters, the cheesemakers always have to take precaution to avoid phage contamination. Leenders and Stadhouders (1981) and Lewis (1987) have developed methods of aseptic inoculation of culture tanks. Whole milk, skim milk, 10% reconstituted skim milk powder, milk fortified with various nutritional ingredients or phage inhibitory media can be used for bulk starter production (Cogan et.al., 1991). Phage inhibitory media are mainly used in the US for production of Cheddar and Mozzarella cheese, but are not generally used with mixed strain cultures as they lead to changes in the strain balance (Cogan et al., 1991). Phage inhibitory media are milk based, containing large amounts of phosphates or other salts, which chelate the Ca^{2+} essential for the attachment of most phages to the bacterial cell wall (Cogan et al., 1991). In the absence of free Ca^{2+}, phage is unable to attach to their hosts and consequently cannot invade and destroy the starter bacteria (Collins et.al., 1950; Watanabe and Takesue, 1972; Neve and Teuber, 1991). The media also contain various nutrients to stimulate growth (Collins et.al., 1950; Watanabe and Takesue, 1972; Neve and Teuber, 1991).

In most cheese factories, bulk starter production occurs in enclosed vats of volumes between 1000 and 5000 l. The medium is heated to 85 – 90°C inside the tank before it is cooled to an inoculation temperature of ± 42°C for thermophilic cultures and ± 21°C for mesophilic cultures. Sterile air enters the tank as it cools, while a positive air pressure is maintained during the subsequent incubation.

2.2.2.4.2 Direct vat starters

Direct vat starters comprise 0,00037% dehydrated starter cultures used as inoculum with a concentration of $5 \times 10^{10} - 5 \times 10^{11}$ cells/g which resulting in a final concentration of $2 \times 10^5 - 20 \times 10^5$ cells/ml milk. Direct set cultures were introduced in the late 1960's and are at present available either in a deep frozen or freeze-dried form (Porubcan and Sellars, 1979). These cultures were first developed for Swiss type cheese (Rousseaux et al., 1968).

Direct set cultures have been commercially available from several culture houses for the last 15-20 years but have only been widely accepted in the late 1980's in the UK cheese industry (Table 5) (Stanley , 1996).

Table 5. Growth of DVI market share (UK), (Stanley, 1996):

Year	Estimated % of cheese made with DVI system
1980	<1%
1985	5%
1990	20%
1995	40%

The main advantages of direct set cultures according to Cogan et al. (1991) are the following:

a) The number of strains and their ratio are controlled, minimising the risk of phage infection of the whole culture.

b) Aroma formers and strains contributing to flavour development are known to be present.
c) Genetic variation of starters is minimised because they are not subcultured during production.
d) The risks of phage infection via mother cultures and bulk starter units are eliminated.
e) Performance in the cheese vat is more consistent allowing better control of the final product.

2.2.3 Function of a starter culture

According to Bester (1978), a good starter culture has the following characteristics, namely:

Ability to produce lactic acid

Ability to break down the protein, when applicable.

The main task of the culture is to develop acid in the curd by changing the lactose in the cheese to lactic acid. The acid lowers the pH, which is important in assisting syneresis (contraction of the coagulum accompanied by elimination of whey). Salts of calcium and phosphorus are released, which influence the consistency of the cheese and help to increase the firmness of the curd. The acid producing bacteria also suppress bacteria that survive pasteurisation or recontamination bacteria by utilising the available lactose or due to lactic acid production.

The culture also plays an important role in the formation of flavour (Law and Sharpe, 1977). The ripening process is a combined proteolytic effect where the original enzymes of the milk and those produced by bacteria in the culture, together with rennet enzyme, cause decomposition of the protein.

2.2.4 The microbiological and chemical aspects of mature Cheddar cheese ripening/maturation

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

The bacteriological composition of raw milk, as well as the heat treatment received by milk prior to cheese making, influence the quality of the resultant Cheddar cheese. High psychrotroph populations are likely to cause reduced recovery of milk solids as cheese, higher moisture contents, pasty texture and off-flavours (Cousin, 1982; Fairbaim and Law, 1986; Law et al. 1976 and 1979). Tittsler et al. (1946) reported that the quality of cheese corresponded to the quality of the milk use, especially when milk was of lower grades. Smith et al. (1956) indicated that milk with a high number of bacteria develops into cheese of poor quality, even if the milk was pasteurised before use. Good-quality milks yielded cheeses with higher flavour scores than poor-quality milk (Wilson et al., 1945).

The gradual breakdown of carbohydrates, lipids and protein during ripening is mediated by several agents, including:

a) Residual coagulant
b) Starter bacteria and their enzymes,
c) Non-starter bacteria and their enzymes,
d) Indigenous milk enzymes, especially proteinases, and
e) Secondary inocula with their enzymes.

When various factors such as starter type, level of non-starter lactic acid bacteria and ripening temperature were compared, it was found that ripening temperature is the single most important factor that effects the flavour of matured Cheddar cheese (Daene and Aderson, 1942; Dorn and Dahlberg, 1942; Freeman, 1952; Freeman, 1959; Hansen, 1946; Marquardt, 1943 and Law et al., 1976).

Proteolytic and lipolytic changes in the cheese-ripening are caused by the microflora in cheese; both starter organisms and non-starter organisms surviving pasteurisation and cooking temperatures contribute to the enzymatic activities (Manning et.al., 1976; Sharpe, 1975).

Cheese-making involves three main processes namely, the decomposition of protein, the decomposition of lactose and the decomposition of fat. Changes in cheese-ripening may be divided into two general stages. The first stage includes changes that occur in carbohydrate, fat and protein, resulting in the accumulation of lactic acid, fatty acids and free amino acids. The second stage comprises changes involving the formation of compounds brought about by the action of enzymes primarily from microorganisms on the compounds (Stadhouders and Veringa, 1973; Dulley, 1974; Harvey et.al., 1977; Desmazeud and Gripon, 1977; Turner and Thomas, 1980).

There are mainly two major types of proteolytic agents in cheeses:

a) Coagulating enzymes: rennet or rennet substitutes

b) Proteolytic enzymes of starter cultures: mesophilic and thermophilic lactic acid bacteria, fungal and yeast starters (Desmazeud and Gripon, 1977; Lenoir, 1984).

Rennet is the first proteolytic agent involved in the overall mechanism of casein breakdown in cheeses. Rennet coagulation is a two-stage process, involving the enzymatic formation of para-casein and peptides, and the precipitation of para-casein by Ca^{2+} at temperatures > 20°C.

Alais et.al. (1953) and Nitschmann and Keller (1955) clearly demonstrated that specific proteolysis occurs during the primary phase of rennet action; that this proteolysis is complete before the on set of coagulation; that more than one peptide is produced and that α-casein, rather than β-casein, is the substrate for this specific proteolysis. The coagulation process involves an enzymatic stage. During this stage rennet attacks the Phe_{105} – Met_{106} bond of casein by solubilizing a fraction of this protein (Mercier et.al., 1973; Delfour et.al., 1965; Wake, 1959).

Lactic acid bacteria possess mainly aminopeptidase activities and to a lesser extent endopeptidase activities (Castberg and Morris, 1976; Desmazeaud and Juge, 1976; Exterkate, 1975). These enzymes release amino acids and are therefore responsible for an increase in the amount of NPN and phosphotungstic acid soluble N.

Desmazeaud and Gripon (1977) showed that lactic acid bacteria contribute primarily to the formation of amino acids and short chain peptides, but also to a slight endopeptidase activity which is different from the action of the rennet, since the latter does not produce any amino acids but mainly peptides. This is in contrast to the findings of Green and Forster (1974), who showed that rennet and proteases from lactic acid bacteria exhibit similar patterns of protein breakdown in cheeses. According to Reddy et al. (1984), lactic acid bacteria initially increase in numbers (1,44 X 10^9 cfu/g to 2,88 X 10^{10} cfu/g) within the first 15 days of ripening. After 15 days, their numbers stabilise and remain constant up to the 4^{th} month, followed by a decline in numbers from 7,4 X 10^8 to 0,5 X 10^6 cfu/g after 10 months of ripening. The depletion of nutrients is mainly responsible for the decline in the number of viable bacterial populations (Reiter et.al., 1964; Haines and Harmon, 1973). Proteolytic and lipolytic bacteria, however, increase at a much faster rate in the first 5 months of cheese ripening (Reddy et.al, 1984). Proteolytic bacteria increase in numbers from 1,4 x 10^4 to 3,5 x 10^6 cfu/g and lipolytic bacteria from 3.5 x 10^4 to 8.0 x 10^5 cfu/g followed by a subsequent decrease in numbers up to 10 months of ripening (proteolytic bacteria 1,05 x 10^5 cfu/g and lipolytic bacteria 1,25 x 10^5 cfu/g) (Reddy et al., 1984). Visser (1977) and Monet et al. (1986) confirmed that the starter bacteria attain maximum numbers in Cheddar cheeses at/or shortly after the end of processing followed by a decline in cell numbers. The breakdown mechanism of cheese protein is illustrated schematically in Fig. 2 (Desmazeaud and Gripon, 1977).

The fermentation of lactose is initiated by the enzymes excreted by the lactic acid bacteria. The most significant quantitative change, which takes place in Cheddar cheese after pressing, is the fermentation of lactose (Turner and Thomas, 1980). According to the Dairy processing handbook (1995), lactose is already fermented before the curd is hooped.

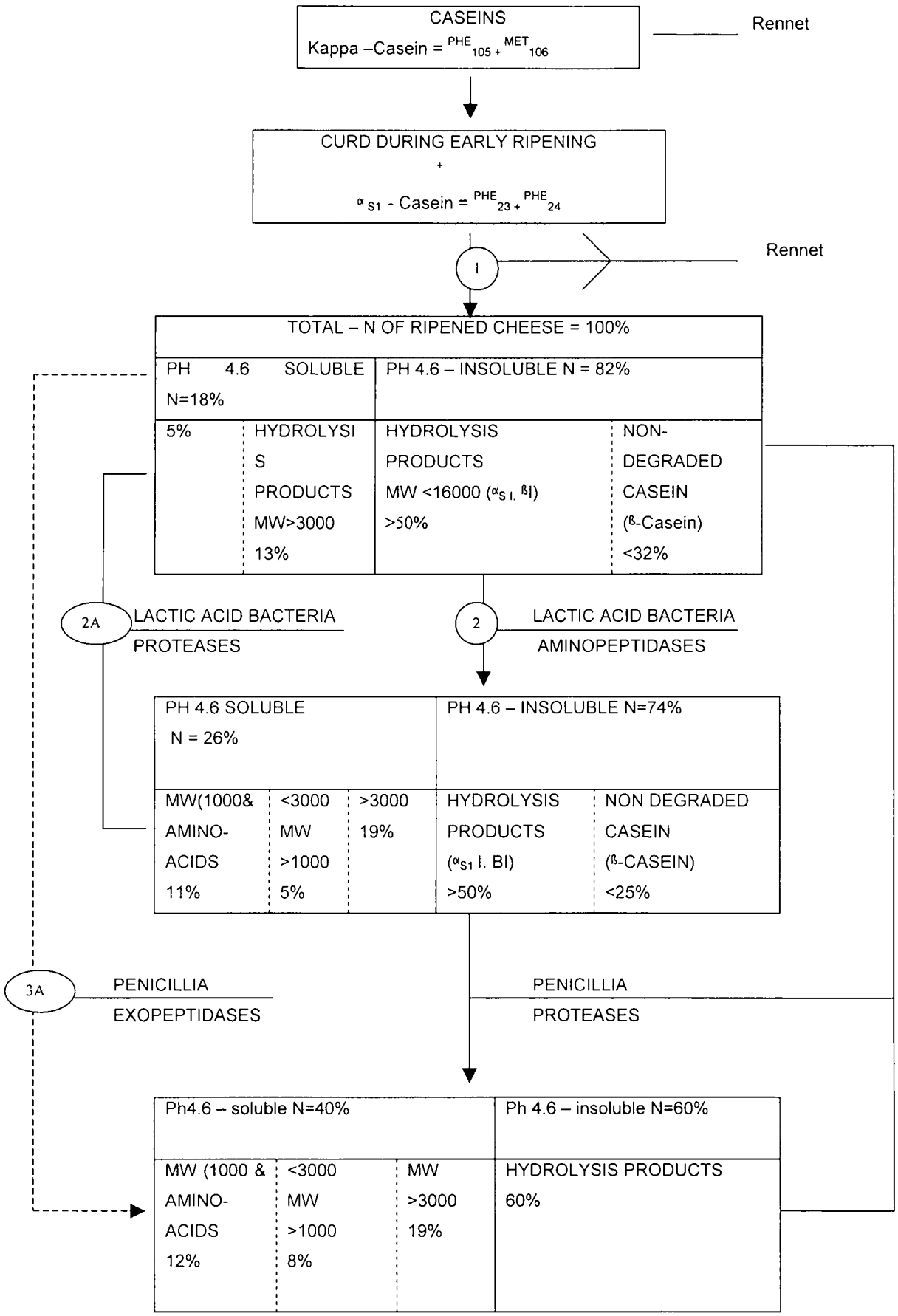

Fig. 2 Schematic breakdown mechanism of cheese protein (Desmazeaud and Gripon 1977).

Harper and Kristoffersen (1956) showed that lactose is completely fermented before the manufacturing process is completed. The metabolic pathways and products derived from lactose and citrate fermentation in cheese are indicated in Fig. 3 (Harper and Kristoffersen, 1956).

Lactose:

Non-starter organisms, which are predominantly pediococci, and starter bacteria (lactic acid bacteria) are mainly responsible for lactose fermentation (Turner and Thomas, 1980). Early work on the lipolytic activity of lactic acid bacteria by Long and Hammer (1937) and Peterson and Johnson (1949) suggested that lactic acid bacteria show some lipolytic activity, but only after prolonged incubation for several months. Stadhouders and Mulder (1958) concluded that lactic acid bacteria produce only small amounts of lipase, if any, and these microorganisms have no influence on fat hydrolysis in cheese ripening. It is later proved that lactic acid bateria is also responsible for fat hydrolysis in cheese, that contribute to the basic flavour in Cheddar cheese (Reddy et al., 1984; Stadhouders and Veringa, 1973).

Oterholm et.al. (1968) tested seventeen strains of lactic acid bacteria, all of the organisms possessed detectable lipolytic activity. Fryer et al. (1967) and Reiter et al. (1964) confirmed these results. The fact that glycerol ester hydrolases are present in these organisms, which account for the predominant bacterial flora in Cheddar cheese suggests, however, that lactic acid bacteria are important in cheese ripening due to the fermentation of lactose, proteolysis and lipolysis. According to Stadhouders and Veringa (1973), fatty acids are also produced during cheese ripening by lactic acid bacteria.

According to Reddy et al. (1984), the lipolytic activity in Cheddar cheese is induced by starter organisms as well as non-starter organisms responsible for the increase in lipolytic activity within the cheese during the ripening period, whereas the lactic acid bacteria contribute to the basic flavour in Cheddar cheese.

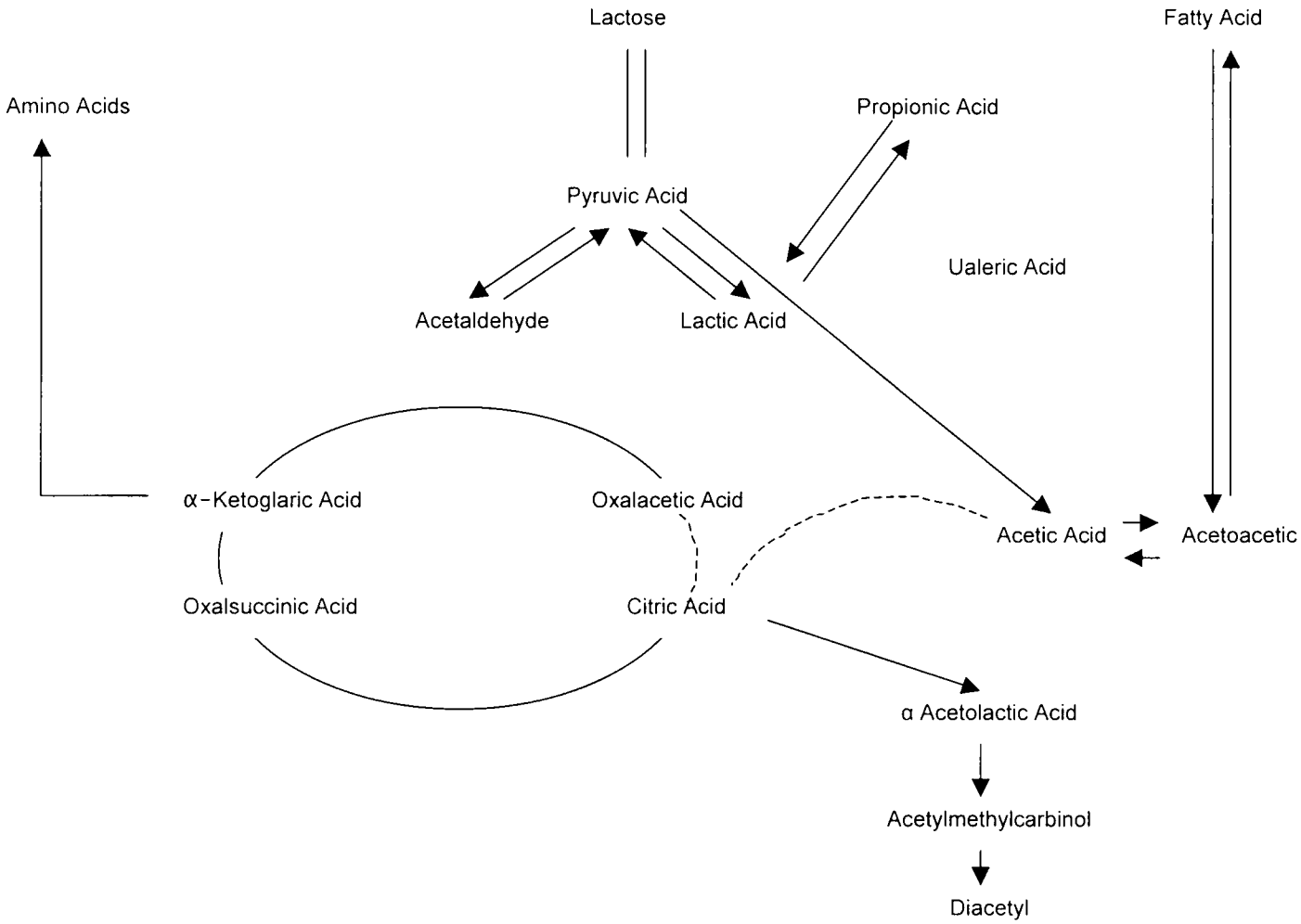

Fig. 3 The Metabolic pathways and products of lactose fermentation in cheese (Haper and Kristoffersen, 1956).

2.2.5. Microbial changes during salting of cheese

The major difficulty in achieving cheese of uniform quality in modern Cheddar cheese plants results from the relatively wide variation in salt in moisture levels that occur in the cheese (Fox, 1993b). The salt in the cheese plays a substantial role in the quality of Cheddar cheese by controlling: (a) the final pH of the cheese, (b) the growth of microorganisms, specifically starter bacteria and undesirable species such as coliforms, staphylococci and clostridia, and (c) the overall flavour and texture of the cheese (Fox, 1993b). Commercial lactic acid cultures are stimulated by low levels of NaCl, but are very strongly inhibited by levels > 2.5% NaCl (Fox, 1993a). *Lactococcus lactis* subsp. *lactis* starters are generally more salt-tolerant than strains of *Lactococcus lactis* subsp. *cremoris* but there is also considerable variation in salt sensitivity between strains of *Lactococcus lactis* subsp. *cremoris* (Fox, 1993a). Mesophilic lactic acid bacteria vary in their tolerance to salt (Robinson, 1981). Salt also controls the rate of proteolysis of caseins by the rennet, plasmin and bacterial proteases.

2.2.6 Standards for quality of mature Cheddar cheese

The quality of Cheddar cheese is characterised by the following properties:

a) Flavour - Fine and highly pleasing characteristic Cheddar cheese flavour showing well developed degrees of flavour sharpness and must be free of any undesirable flavours and odours.

b) Body and texture – A plug drawn from the cheese shall be firm, appear smooth, waxy, compact, close and translucent but may have a few mechanical openings if not large and connecting. Should be free from curdiness and possess a cohesive velvet like texture. May possess not more than one sweet hole per plug but shall be free from other gas holes.

c) Colour – Shall have a uniform, bright attractive appearance, practically free from white lines or seams. May show numerous tiny white specks. May be coloured or uncoloured, but if coloured it should be a medium yellow-orange.

2.3 Yeast spoilage of cheese

Spoilage yeasts are defined as yeasts that produce undesirable changes in foods during the fermentation processes (Deak and Beuchat, 1996; Fleet, 1990; Fleet, 1992). Substantial growth of yeasts may cause undesirable changes due to the production of metabolic products, such as the formation of unnatural odours or flavours, or to metabolic activity causing an increase in pH due to the utilisation of organic acids (Walker, 1977). Yeasts are playing an important role as spoilage organisms in dairy products as well as ripening agents of some cheese varieties (Fleet and Mian, 1987; Seiler and Busse, 1990; Brocklehurst and Lund, 1985).

Excessive growth of *Candida albicans, Geotrichum candidum, Kluyveromyces marxianus, Pichia membranaefaciens, Yarrowia lipolytica, Debaryomyces hansenii, Candida zeylanoides, Cryptococcus albidus* and *Cryptococcos laurentii* can cause undesirable sensory changes, softening of structure, slime formation and blowing of the cheese (Romano et.al., 1989; El-Bassiony et.al., 1980; Brocklehurst and Lund, 1985; Engel, 1986b; Ingram, 1958; Walker and Ayres, 1970; Lenior, 1984; Pitt and Hocking, 1985; Seiler and Busse, 1990; Rohm et.al., 1990; Tudor and Board, 1993). Yeasts can spoil cottage cheese or similar types of unripened soft cheeses, which are particularly prone to spoilage due to higher water contents (Brocklehurst and Lund, 1985; Engel, 1986b; Fleet, 1990). Yeast populations of 10^6 to 10^7 cfu/g frequently develop during refrigerated storage of these products resulting in flavour and odour defects and gassiness (Brocklehurst and Lund, 1985; Engel, 1986a; Guiraud and Galzy, 1976). For the same reason, brined cheeses, such as Feta and Domiati, are prone to yeast spoilage (Haddadin, 1986). Roostita and Fleet (1996) also showed that yeasts can exhibit strong growth in cheese during storage, impacting on their sensory quality and shelf -life.

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

2.4 Occurrence, growth and significance of yeasts in cheese

Yeasts are widely distributed in nature, and as might be expected, are present in cheese. The starter bacteria, usually cause fermentation and are therefore considered to be of major importance during the manufacturing process of Cheddar cheese, as shown in 2.2.4 (The microbiological and chemical aspects of mature Cheddar cheese ripening/maturation) and by Cousin (1982). Yeasts, however, possess the ability to grow under conditions unfavourable to many bacteria, like low temperatures, low pH-values, low water activities and high salt concentrations (Fleet, 1990; Fleet and Mian, 1987; Rohm et.al., 1992; Seiler, 1991; Tudor and Board, 1993) reaching high populations and therefore may contribute substantially to the final product.

The main yeast species found during maturation and retailing include *Debaryomyces hansenii, Kluyveromyces marxianus, Yarrowia lipolytica* and various species of *Candida* (Lenoir, 1984; De Boer and Kuik, 1987; Nooitgedaght and Hartog, 1988; Besancon et.al., 1992; Roostita and Fleet, 1996; Fleet, 1990; Devoyod, 1990). These yeasts play a very important role in the making of cheese due to its ability to produce lipolytic and proteolytic enzymes, the fermentation of residual lactose, the utilisation of lactic acid and autolysis, all of which have an impact on the quality of the final product (Choisy et.al., 1987a and 1987b; Fleet, 1990; Devoyod, 1990).

Nooitgedaght and Hartog (1988) reported yeast counts of $>10^5$ cfu/g in Camembert and Brie cheeses. *Yarrowia lipolytica, Debaryomyces hansenii*, and *Kluyveromyces marxianus* were the most frequently isolated species. Roostita and Fleet (1996) reported yeast counts up to $10^6 - 10^8$ cfu/g, mainly

representatives of the species *Debaryomyces hansenii, Saccharomyces cerevisiae, Candida lipolytica, Candida kefyr* and *Cryptococcos albidus,* in Camembert and Blue-veined cheeses. According to Reddy and Marth (1995), yeast counts of < 300/g and < 100/g for unsalted and salted Cheddar cheeses respectively, were obtained. Prentice and Brown (1983) reported a maximum level of yeasts of 5.0 x 10^3 cfu /g in Cheddar cheese. Yeast levels, however, can rise as high as 10^5 cfu/g without any deleterious effect on the quality of the product (Prentice and Brown 1983). Fleet and Mian (1987) found that almost 50% of Australian Cheddar cheese sampled, contains 10^4-10^6 yeast cells/g. The use of yeast proteases in the maturation of Cheddar cheese was also reported (Grieve, 1982; Grieve et.al., 1983; El-Soda, 1986).

Yamauchi et.al. (1976) reported the possibility of using *Debaryomyces hansenii* as a starter culture, based on the species, proteolytic activity encouraging the survival and growth of lactic acid bacteria. The inclusion of *Debaryomyces hansenii* as part of the starter culture has a dual role by also inhibiting the germination of undesired microorganisms, like *Clostridium butyricum* and *Clostridium tyrobutyricum* in cheese brines (Seiler and Busse, 1990). Fatichenti et.al. (1983) and Deiana et.al. (1984) proposed the inclusion of *Debaryomyces hansenii* as a starter culture for the making of Romano cheese based on its inhibitory effect on the growth of spoilage species and the species, proteolytic activity. Pultost, a special traditional Norwegian cheese, is also manufactured by using a yeast culture of *Candida rugosa* exhibiting proteolytic activity.

Lactose-fermenting yeast species, like *Kluyveromyces marxianus*, contribute to blue type cheeses due to the production of CO_2 causing openings in the curd that helps *Penicillium roquefortii* to grow in the internal fissures. This contributes to the characteristic blue vein appearance of the cheese (Devoyod, 1990; Prooks et.al., 1959). Yeasts also contribute to the ripening of Camembert cheese due to the fermentation of lactose adding to aroma and taste (Lenior 1984). *Kluyveromyces marxianus, Kluyveromyces lactis, Candida versatilis, Debaryomyces hansenii* and *Saccharomyces cerevisiae* are frequently isolated from the inner and outer part of Camembert cheese (Schmidt and Lenoir, 1980). The yeast species present in the soft cheeses

furthermore inhibit the growth of *Mucor; Penicillium roquefortii* and *Penicillium camemberti* responsible for slow development of the mould cultures. Most of the yeasts isolated from Camembert cheese are able to assimilate lactose and lactic acid, and exhibit lipolytic and proteolytic activity. All these characteristics contribute to the development of cheese aroma. The use of yeast species as part of starters for the manufacturing of Camembert cheese, however, is still the exception.

Yeasts also play an important role in the production of feta cheese, being present in the brine of the cheese. *Saccharomyces cerevisiae* and *Candida famata* were the dominant yeasts isolated by Kaminarides and Laskos (1992) responsible for flavour development. In addition, yeasts added to the formation of aroma components, or precursors of aroma (amino acids, fatty acids, esters, etc.) due to their proteolytic, lipolytic and esterifying activities (Lenoir 1984). Furthermore, yeasts excrete vitamins resulting in growth stimulation of other microorganisms, which include the starter cultures (Devoyod and Desmazeaud, 1971; Purko et.al., 1951).

The positive interaction between yeasts and starter cultures and the abilities of yeasts to assist the starter cultures during cheese processing based on proteolytic and lipolytic activities and the production of amines, are well documented for surface ripening cheeses (Besancon et.al., 1992; Grieve et.al., 1983; Hartley and Jezeski, 1954; Kalle et.al., 1976; Kaminarides and Anifantakis, 1989; Lenoir, 1984). It has been mentioned that yeasts improve the quality of numerous cheeses, mainly by their lipolytic activity (Prooks et.al. 1959; Mahmoud et.al. 1979; Masek and Zak 1981). The lipolytic enzymes excreted by *Yarrowia lipolytica* have been added to cheese milk to improve the taste of Cheddar cheese (Forss, 1969) and blue-veined cheeses (Parmelee and Nelson, 1949a and 1949b).

The involvement of yeasts during the processing and maturation of Cheddar cheese is not clear. Studies revealed a lack of specific examination of the yeasts positive role during the manufacturing process. Studies on the biochemical activities of yeasts are still in progress and must be completed, and carried further, in order to understand the potential and positive action of

the organisms in cheese-making. Positive contributions, attributed to yeasts during the ripening, are the fermentation of lactose, assimilation of lactic acid, formation of components or precursors of aroma, stimulation of starter cultures, acceleration of the maturation process and the inhibitory effects against spoilage organisms.

Therefore, yeasts possess the potential to be incorporated as part of the starter cultures for the making of Cheddar cheese. The application of yeasts as starter cultures for the making of Cheddar cheese, however, is currently not widely recognized, mainly due to poor reproducible results, owing to the lack of sufficient knowledge of the yeasts physiology.

2.5 Factors affecting the survival and growth of yeasts in cheese

Cheese is an ideal habitat for the growth and survival of yeasts due to the availability of the necessary nutrients and competitive environmental conditions. Therefore, based on the cheese characteristic nutritional composition, a specific association of yeasts is expected. The main factors affecting the survival and growth of yeasts are the chemical composition of the product, the conditions of storage and the inherent properties of the yeast species present (Fleet, 1992). The intrinsic parameters, which include physical, chemical and structural properties in the nature of foods primarily determine the predominant microbial population in foods (Deak, 1991; Deak and Beuchat, 1996). Water activity, nutrients and acidity are the most important intrinsic factors, while temperature and atmospheric composition are the most important external factors that effect the growth and survival of yeasts (Deak, 1991). Dairy products become contaminated from the environment and only the yeasts that possess the proper physiological attributes to respond to the ecological determinants will survive under the selective pressures exerted by the internal and external environments of the dairy product (Deak, 1991; Deak and Beuchat, 1996).

Interaction among yeasts and other microorganisms also influences the development of microbial colonization and eventually a particular yeast

community will develop. All these dynamic changes are determined by the ecological factors present in dairy products (Deak, 1991; Deak and Beuchat, 1996). Yeasts associated with cheese can be classified in two groups: The first group possesses the characteristics which enable them to survive and reproduce (Deak and Beuchat, 1996). The second group comprises those yeasts which lack these characteristics and this transient yeast community is solely dependent on dissemination for survival.

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CHAPTER 2

THE INCIDENCE OF YEASTS ASSOCIATED WITH MATURED CHEDDAR CHEESE

1. ABSTRACT:

Although there have been many bacteriological surveys of cheese processing and the maturation of cheese, little attention has been given to the composition and distribution of yeast flora of such environments. A study was therefore undertaken with the objective of identifying the principal yeast contaminants associated with matured Cheddar production. A total of 168 yeast strains were isolated from matured Cheddar cheese and in the vicinity of the processing line. Until the identity of the yeasts associated with the maturation of the Cheddar cheese has been established, little headway will be possible in studies which seek to assess the contribution of these organisms to the spoilage of matured cheeses. Once the identity of the yeast flora of cheese had been established, the survey was extended to determine the sources of yeast infection during the processing of the cheese. Although yeast populations were initially low in numbers, they represented a much higher proportion of the total microflora at the time of maturation when the cheeses were stored at low temperatures. Species of the genera *Debaryomyces*, *Dekkera* and *Rhodotorula* were the most frequently isolated yeasts. Other genera encountered were *Trichosporon*, *Candida* and *Torulaspora*.

The results obtained showed that the yeasts played a substantial role in the deterioration as well as the ripening of mature cheese mainly due to the environmental storage conditions at low temperatures, low water activity and low pH values.

2. INTRODUCTION

Yeasts are an important component of the microflora of many cheese varieties causing spoilage due to their fermentative abilities (Fleet, 1991; Fleet and Mian, 1987; Seiler and Busse, 1990). Lactose fermentation during maturation of cheese resulting in over-ripening could lead to an increase in acidity, gassiness, fruity flavours, as well as softening of the product texture (Fleet, 1991; Jakobsen and Narvhus, 1996).

Little consideration, however, has been given to the importance of yeasts in dairy products contributing positively to the final product. Yeasts play a substantial role in cheese manufacturing due to their ability to progress at low pH values, temperatures and water activity, as well as elevated salt concentrations (Fleet and Mian, 1987; Seiler and Busse, 1990). The possibility of using *Debaryomyces hansenii* and *Yarrowia lipolytica* as starter cultures are proposed since these species stimulate the lipolytic and proteolytic activities (Fleet, 1991; Devoyod, 1990) during the ripening of cheese and utilize the lactic acid and acetic acid (Fleet, 1991; Devoyod, 1990) present. The utilization of organic acids consequently results in an increase in pH, stimulating the growth of the starter cultures and assuring uniformity (Fleet, 1991, Devoyod, 1990). Yeasts also contribute to the formation of precursors of aroma components such as amino acids, fatty acids and esters, and stimulate the growth of the starter cultures due to the excretion of growth factors like B-vitamins, pantothenic acid, niacin, riboflavin and biotin (Purko et al., 1951; Lenoir, 1984).

The types of yeasts capable of growth in cheeses, after establishing themselves as part of the normal microflora, have not been studied in detail (Viljoen and Greyling, 1995; Welthagen and Viljoen, 1998). Furthermore, no reference was found describing the extent to which these species survive and progress in matured Cheddar. The prolonged period of maturation provides an enhanced selective environment in matured cheeses due to the lower moisture contents. Consequently, these cheeses are likely to support only a few microbial species. This paper reports on the frequency of occurrence and types of yeast species

found in South African matured Cheddar. In addition, the sources of yeast contamination in the cheese factory are also identified.

3. MATERIALS AND METHODS

3.1 Matured Cheddar Cheese Manufacture

Matured Cheddar cheese was manufactured at a commercial cheese factory in the Orange Free State in South Africa. The procedure for cheese-making was carried out as described by Kosikowski (1977).

3.2 Sampling Methods And Selection Of Isolates

Environmental samples were taken from all surfaces in duplicate by RODAC (Favero et al. 1968) Yeast Extract Glucose Chloramphenicol Agar (YGC) (Merck, Darmstadt, Germany; pH 6.6). Working surfaces samples included stainless steel packaging tables, the cheddaring vats, cheesevats, aprons and hands of the workers. Aseptic samples were taken from all ingredients (rennet, salt, colouring agent, calcium chloride and starter culture) added during production. Product samples, included the cheese curd before and after salting of the product manufactured on the day of sampling. Cheese and curd samples were taken on all occasions in triplicate, representing the initial count after setting, the middle stage of cheese cheddaring and at the end of cheddaring. The means of the replicates accounted for a single cheese sample as shown in Fig. 1.

Liquid (rennet, calcium chloride, raw and pasteurized milk and colouring agent) samples were diluted in sterile quarter strength Ringers solution. For all solid (curd, cheese and salt) samples, 20g portions were placed in 180ml sterile quarter strength Ringers solution, homogenized in a Colworth 400 stomacher (London UK) for 2 min and the liquid portion diluted. Further decimal dilutions were carried out as required for microbiological assays and plated in triplicate by

the spread plate technique onto Yeast Extract Glucose Chloramphenicol (Merck) agar. Inoculated agar plates were incubated for 120h at 25°C and counted. Individual counts for each type were calculated. All yeast colonies were isolated from the highest dilutions on plates containing YGC agar. Yeast extract glucose agar plates with chloramphenicol were strategically placed within the cheese plant and used for air sampling with an exposing time of 15 minutes. Yeast isolates were subcultured on Yeast extract Malt extract agar (YM) and incubated for 120h at 25°C for control of purity by colony morphology and microscopy. The pure cultures were stored at 4°C on (YM) agar during the period of investigation.

3.3 Characterization Of Yeast Isolates

The representative yeast isolates were identified by using the methods described by Kreger-van Rij (1984) and the computerized identification system of Barnett et al. (1990). Each isolate was inoculated into 6 sugar fermentation media, 32 carbon source assimilation media and vitamin free medium (Van der Walt and Yarrow, 1984). Additional tests performed included growth at 37°C, in 50% D-glucose medium, urea hydrolysis, splitting of arbutin, 0,01% and 0,1% cycloheximide and staining of 4-weeks-old cultures with Diazonium Blue B salt reagent (Van der Walt and Hopsu-Havu, 1976). Assimilation of nitrogen compounds, as performed by means of the auxanographic method (Lodder and Kreger-van Rij, 1952), was also included.

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

4. RESULTS AND DISCUSSION

4.1 Microbial Enumeration

Cheeses from 6 separate days production were examined over a period of six months. Bulkstarter inoculation of the cheese was applied during the first 3 days of production whereas Direct vat inoculation starters were applied during the next 3 days production. Six cheeses were selected from each days production accumulated in a total of 36 cheeses to represent the beginning, middle and end of each cheese vat manufacture. The cheeses were stored under normal (8°C – 11°C) environmental conditions and examined at 48 hrs, 12 days, 30 days and thereafter at 30 day intervals for the remaining 6 months.

A total of 167 yeast strains were isolated during the 6 days of mature Cheddar manufacturing from a single cheese factory. The individual yeast strains obtained from different sources and the number of yeast strains of each species isolated are shown in Table 1.

The use of bulk starter inoculation of the cheese, the hands and the cutting equipment contributed mainly to yeast contamination yielding counts as high as 720 cfu /16 cm^2 and 496 cfu/16 cm^2 respectively. By implementing direct vat inoculation, the yeast populations were generally much lower. The hands and aprons were the major sources of contamination after pasteurization, 21 cfu/16 cm^2 were found on the hands and 28 cfu/16 cm^2 were found on the aprons (Table 2). Low numbers of yeasts were isolated from the plates exposed to the air for 15 min during cheese making and the use of bulk starters. The highest yeast counts were observed near the drainvats, represented by 2 – 12 cfu/ 15 min. When DVI starters were used, the number of yeasts isolated was much lower represented by the highest counts in the vicinity of the picking area yielding 3 cfu/15 min. In contrast to previous findings (Welthagen and Viljoen, 1998), yeasts were also found in the rennet reaching counts of 10 cfu/ml when the cheeses were manufactured with bulk starters. When DVI starters were used, higher numbers

Table 1 Yeasts associated with matured Cheddar manufacturing and sources of contamination

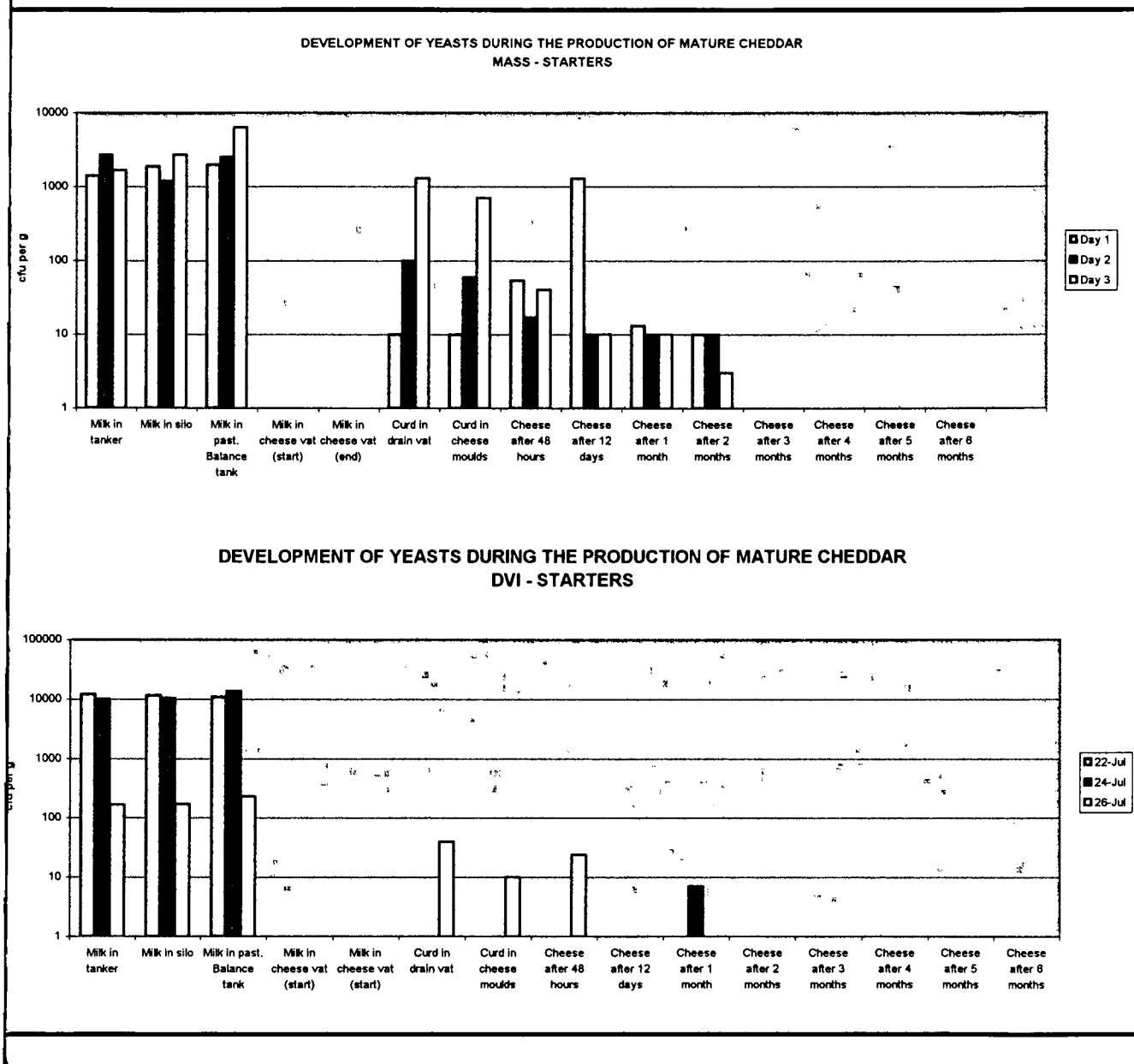
		SOURCES OF CONTAMINATION							
ISOLATES	**PERCENTAGES OF YEAST STRAINS**	**RAW MILK**	**EQUIP-MENT**	**AIR**	**RENNET**	**HANDS**	**APRONS**	**CURD**	**CHEESE**
Candida rugosa	2.99 %	1	1						3
Candida versatilis	8.38 %	4	1	3		1			5
Debaryomyces hansenii	28.74 %	19	5	6	3	4	2	5	4
Debaryomyces vanrijiae	2.4 %			1		2			1
Dekkera anomala	1.8 %		1						2
Dekkera bruxellensis	1.8 %		1						2
Dekkera custersiana	19.16 %	9	12	1		3		2	5
Rhodotorula glutinis	1.2 %			1				1	
Rhodotorula minuta	2.99%	1				1			3
Rhodotorula mucilaginosa	11.98 %	5	4	5		3		1	2
Toruluspora delbrueckii	3.59 %	3			1	1			1
Trichosporon beigelii	14.97 %	2	4		1	1	1	1	15

of yeasts were found in the rennet yielding counts of 2.2 x 10^2 cfu/ml. Calcium chloride was also responsible for yeast contamination yielding counts of 3.0 x 10^2 cfu/ml. Based on the results obtained regarding the environmental sources of yeast contamination, we clearly demonstrated the frequent occurrence of contaminating yeasts during Cheddar making in this factory. The lower number of yeast contamination during the use of DVI starters was due to a higher focus on hygiene, during the manufacturing of cheese with DVI starters. The number of yeast populations, however, may vary between plants and even between consecutive days within the same plant. The variation in the salt concentration (Seiler and Busse, 1990), temperature (Davenport, 1980), accidental occurrence of contaminating yeasts (Fleet, 1991), hygienic standards, efficiency of pasteurization (Fleet and Mian, 1987) and the initial yeast flora within the raw milk, all contribute to the progression of microbial loads. Despite the high frequency of occurrences of yeasts from environmental sources, yeasts are generally heat sensitive (Fleet, 1990). Therefore, yeasts contributing to the final product are considered as post-pasteurization contaminants. The development of yeasts during matured Cheddar making using Mass and DVI starters is shown in Fig 1.

a) **Mass Starters**:

Yeast counts as high as 6.3 x 10^3 cfu/ml were observed in the raw milk before pasteurization. No yeasts, however, were present after pasteurization indicating that mainly post-pasteurization yeast contaminants contribute to the final product.

Maximum yeast counts of 1.31 x 10^3 cfu/g in the cheese for a single cheese vat were achieved after 12 days followed by a rapid decline in numbers. No yeasts were present after 3 months (Fig. 1). Variable yeast counts of the three cheeses representing the beginning, middle and end of a specific cheesevat, ranging between 2.1 x 10^1 cfu/g to 2.86 x 10^3 cfu/g, were obtained. There is no proper explanation for the lack of consistency in yeast counts, being present in high numbers in one cheese, and absent in another, despite being manufactured under similar conditions.



. 1 Development of yeasts in matured Cheddar cheese

Yeast counts as high as 10^7 and 10^8 cfu/g have been reported in cheese (Koburger, 1971; Hup and Stadhouers, 1972; Koburger and Farhat, 1975; Henson et al., 1982; Brodsky et al., 1982; Williams, 1986; Jarvis and Shapton, 1986; Banks and Board, 1987), however, the yeast counts never exceeded 10^4 cfu/g in this study. The number of yeasts increased during the salting process of the curd, whereas the total bacteria count decreased indicating the necessity to control yeast progression during the production process. Excessive growth of yeast during processing may result in the inhibition of the starter cultures due to competition for available carbohydrates (Welthagen and Viljoen, 1998).

b) **DVI Starters:**

The initial yeast populations were much higher in the raw milk during the use of DVI starters, reaching counts as high as 1.32×10^4 cfu/g. Despite the initial high count of yeasts, the yeast numbers present in the curd and cheese were much lower compared with the use of mass-starters (Fig 1). The lower number of yeasts within the cheese is attributed to the reduced number of yeasts originating as environmental contaminants after pasteurization (Table 1). The higher tempo of growth of the DVI starters in the draining vats resulting in faster acid production may also contribute to the reduced number of yeasts due to enhanced competitiveness based on carbohydrate utilization.

4.2 Yeast Identification

Twelve yeast species belonging to the genera *Candida, Debaryomyces, Dekkera*, *Rhodotorula, Torulaspora* and *Trichosporon* were identified. The number of yeast strains (and the type) of each species isolated from the different sources is shown in Table 1.

Debaryomyces hansenii was the most frequently isolated yeast species, present in a wide range of sources (Table 1) within the cheese factory, including the raw milk, equipment, air, rennet, hands, aprons, curd and the final product. The species represented 28,74 % of the total number of yeast species isolated during the manufacturing process of matured Cheddar. Welthagen and Viljoen (1998)

Table 2 Surface and air samples of yeast colonies per 16 cm^2 during mature Cheddar cheese processing

Samples (surfaces and air)	Yeast colonies					
	Day 1 Mass starters	Day 2 Mass starters	Day 3 Mass starters	Day 4 DVI starters	Day 5 DVI starters	Day 6 DVI starters
Sampling bottles						
Stirring equipment				16		
Thermometer				40		
Milk reception pipe				20		
Line before evaporator					20	
Evaporator		400				
Lines after evaporator						
Before cooler						
After cooler					14	
Silo panels	10		34	70	20	
Silo in and outlet			78	12		
Silo lid						
Silo				6		8
Pasteurizer balance tank						
Before pasteurizer						
After pasteurizer						4
Cheesevat						
Cheesevat stirring equipment						
Sampling equipment						
Hands (Cheesemaker)	1		4	10		9
Line direct after Cheesevat						
Line before draining vat						
Draining vat	4					
Draining vat stirring equipment	10					
Milling equipment	420					
Cutting equipment						
Hands (Workers; Cheddaring)	14	4	20	27	11	
Aprons			4			28
Molds (Lids)						
Molds	2		5			
Cheese cloth	8		22			
Hands (Pressing of cheese)	392	720			18	10
Cutting equipment at press	496					
Hands (Packaging)	310	640				
Plastic bags						
Draining vats (Air plates)	2	12	2	1		1
5 m Draining vats (Air plates)	2	12	4	2	1	1
10 m Draining vats (Air plates)	3		10		1	1
Press (Air plates)	2					
Packaging (Air plates)	2			3		

reported on the dominance of *D. hansenii* in 20 different cheese variants representing 48,3% of the yeast strains isolated. Similar findings were observed in a study on Brie and Camembert cheeses, purchased in Holland (Nooitgedacht and Hartog, 1988). Nakase et al. (1977) also confirmed that *Debaryomyces hansenii* species were predominant in imported European and American cheeses. The predominance of *Debaryomyces hansenii* species associated with various cheeses (de Boer and Kuik, 1987) is attributed to their ability to grow at low water activity levels, low temperatures, elevated salt concentrations and their lipolytic and proteolytic activity. The species are frequently recovered from cheese salt brines and other dairy products (Fleet and Mian, 1987; Gilmour and Rowe, 1981; Lenoir, 1984; Mrak and Bonar, 1939; Seiler and Busse, 1990; Szumski and Cone, 1962 Walker and Ayres, 1970). *D. hansenii* has also been isolated by other researchers from different cheeses (Banks and Board, 1987; Brodsky et al, 1982; Henson et.al, 1982; Hup and Stadhouders, 1972; Jarvis and Shapton, 1986; Koburger, 1971; Koburger and Farhat, 1975; Nooitgedacht and Hartog, 1988; Roostita and Fleet, 1996; Williams, 1986). Despite the frequent occurrence of the species in cheeses, Eliskases–Lechner and Ginzinger (1995) showed that *D. hansenii* causes no organoleptic defects during the ripening of cheeses.

Dekkera custersiana accounted for 19,05% of the total yeast isolates, isolated from the raw milk, equipment, air, hands, curd and cheese. Welthagen and Viljoen (1998) reported the presence of *D. custersiana* in Cheddar and Caciocavallo cheeses. *Dekkera anomala* and *Dekkera bruxellensis* were also isolated from the cheese and the equipment. Species of the genus *Dekkera* are characterized by peculiar metabolic properties producing acetic acid and very slow growth (Deak and Beuchat, 1987). They are responsible for (causing) spoilage of alcoholic beverages and soft drinks, often in anamorphic forms (*Brettanomyces)* (Deak and Beuchat, 1987) but are less frequently associated with dairy products.

Rhodotorula species were also present in high numbers (16,07% of the total isolates) represented by the species *R. mucilaginosa, R. glutinis* and *R. minuta*.

R. mucilaginosa species were isolated from the raw milk, equipment, air, hands, curd and cheese, representing 74,07% of the *Rhodotorula* isolates.

R. glutinis was isolated from the air and curd whereas *R. minuta* was present in the raw milk and final product. The presence of Rhodotorula species in high numbers corresponds with previous reports (Fleet, 1991; Viljoen and Greyling, 1995). Eliskases–Lechner and Ginzinger (1995) reported on the presence of *R. minuta* on cheese surfaces and in brines. Nooitgedacht and Hartog (1988) isolated *R. mucilaginosa* from Brie and Camembert cheeses whereas Fleet and Mian (1987) and Nakase et al. (1977) indicated on the dominance of *R. diffluens* strains in cheese samples.

Rhodotorula species are frequently associated with dairy product spoilage, causing pink spots on the surface of butter, cream and cheese (Skinner et. al, 1961; Walker and Ayres, 1970). The yeast species are typical cold tolerant basidiomycetes (Davenport, 1980; Schmidt–Lorenz and Gutschmidt, 1968) capable of growing at sub-zero temperatures and pH values as low as 2,4 (Pitt and Hocking, 1985). The *Rhodotorula* species are particularly regarded as harmful in milk and milk products, being able to peptonize casein and attack butterfat readily (Ingram, 1958; Fleet and Mian, 1987). Connell and Skinner (1953) described *Rhodotorula* species as common air contaminants.

Thichosporon beigelii isolates are represented by 14,88%, recovered from raw milk, equipment, rennet, hands, aprons, curd and cheese. Viljoen and Greyling (1995) reported that *T. beigelii* represented the second largest group of species associated with dairy products when studying the sources of contamination in a cheese factory. Seiler and Busse (1990) also obtained high numbers of *T. beigelii* strains from cheese brines. *T. beigelii* species play a substantial role in the spoilage of dairy products, being capable of metabolizing milk protein and fat (Szumski and Cone, 1962; Vorbeck and Cone, 1963) at low temperatures (Davenport, 1980). Engel (1986) and Gueho et al. (1992a, 1992b) also reported the presence of *Trichosporon* species in dairy products.

Candida species represented 12,5% of the total yeast isolates. *Candida rugosa* was isolated from the raw milk, equipment and cheese. This species is frequently recovered from yogurt and different types of cheeses (Banks and Board, 1987; Green and Ibe, 1987; Seham et al, 1982; Suriyarachchi and Fleet, 1981; Suarez and Inigo, 1982). Seiler and Busse (1990) reported the presence of *C. rugosa* isolated from cheese brines. *Candida rugosa* species are used as a starter culture for the maturation of Pultost cheese (Brendehaug), a special and traditional sour milk cheese made in Norway. *Candida versatilis* was present in the raw milk, equipment, air, hands, curd and cheese. Tilbury et al. (1974) isolated *C. versatilis* from cream desserts, Cheddar cheese and cottage cheese whereas Schimdt-Lorenz and Gutschmidt (1968) and Lenoir (1984) reported on the species presence in Camembert cheese. Roostita and Fleet (1996) also obtained various species of *Candida* in Camembert and Blue-viened cheeses. A yeasty flavour defect was detected in Cheddar cheese by Horwood (1987) who attributed it to low starter activity and high cheese moisture. Consequently, *Candida* species were able to produce high levels of ethanol, ethyl acetate and ethyl butyrate, commonly associated with a fruity flavour in cheese.

Other yeast species that were isolated in low numbers were, in order of frequency, *Torulaspora delbrueckii* (3,57%) and *Debaryomyces vanrijiae* (2,38%). *T. delbrueckii* was isolated from raw milk, rennet, hands and cheese, whereas *Debaryomyces vanrijiae* was isolated from the air, rennet and cheese. The presence of *T. delbrueckii* in Brie, Camembert cheese (Nooitgedacht and Hartog, 1988) cheese brines (Kaminarides and Laskos, 1992; Seiler and Busse, 1990), Kareisk cheese (Zein et al., 1983) and yoghurt (Mckay, 1992) is frequently reported.

The application of yeast species as part of the starter cultures for the manufacturing of mature Cheddar, is still the exception rather than the rule. However, yeast contamination can easily occur during the cheese-making process contributing substantially to the final product. Based on the results obtained in this study, we clearly demonstrated the frequent occurrence of yeasts associated with mature Cheddar making, originated as post-pasteurization

contaminants. It is therefore important to identify the yeasts that contribute to desirable qualities of dairy products. Those yeasts contributing positively have the potential to be incorporated in starter cultures for cheese manufacture. Furthermore, yeast contamination has to be controlled to prevent overgrowth of the normal starter cultures when the curd is salted and during temperature fluctuations. High levels of contaminating yeasts may be responsible for off-flavours, aroma and texture.

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CHAPTER 3

THE RESISTANCE OF DAIRY YEASTS AGAINST COMMERCIALLY AVAILABLE CLEANING COMPOUNDS AND SANITIZERS

1. ABSTRACT

Seven yeasts, isolated from a cheese factory (chapter 2), were screened for their resistance against normal sanitary practices in the industry. Ten commercial cleaning compounds and sanitizers, incorporated individually in UHT treated milk were tested for their inhibitory effect against the yeast isolates at varying temperatures.

Candida rugosa exhibited the high resistance to all the compounds. The "Peroxide Based Acid Sanitizer" proved to be the most effective inhibitor against most of the isolates causing the least growth after 45-60 min contact. None of the compounds used, however, was able to kill all the yeasts. The variation in temperature caused no significant differences in the compounds, inhibitory effect on the yeast isolates.

2. INTRODUCTION

Yeasts developing as natural contaminants play a substantial role in the manufacturing processes of cheese due to their ability to grow under environmental conditions unfavourable to many bacteria (Welthagen and Viljoen, 1998; Viljoen and Greyling, 1995; Fleet, 1990; Fleet and Mian, 1987; Seiler and Busse, 1990). Their progression is governed by the ability to grow at low temperatures, low water activity, high salt concentrations, fermentation/assimilation of lactose, production of lipolytic and proteolytic enzymes and utilisation of lactic and citric acids (Fleet, 1990; Fleet and Mian, 1987; Seiler and Busse, 1990).

High numbers of yeasts, exceeding counts of 10^4 cfu/g and even as high as 10^8 cfu/g are responsible for the spoilage of dairy products causing yeasty or fruity flavours, gassiness, slime formation and discolouration of products (Ingram, 1958; Walker and Ayres, 1970; Lenoir, 1984; Pitt and Hocking, 1985; Seiler and Busse, 1990; Rohm et al., 1992; Tudor and Board, 1993). Under poor hygienic conditions, an increase in the number of yeasts may result in early blowing and off-flavours during ripening of certain cheeses (El - Bassiony et al., 1980).

Typical dairy associated yeasts isolated from cheeses are dominated by *Kluyveromyces marxianus, Yarrowia lipolytica, Debaryomyces hansenii, Candida spp., Rhodotorula* and *Cryptococcus* species (Brocklehurst and Lund, 1985; El-Bassiony et al. 1980; Engel, 1986; Ingram, 1958; Lenoir 1984, Pitt and Hocking, 1985; Rohm et.al., 1992; Romano et al., 1989; Seiler and Busse, 1990; Tudor and Board, 1993; Viljoen and Greyling, 1995; Walker and Ayres, 1970). All of these yeast species are capable to survive under the selective environmental stresses associated with dairy products and may cause spoilage or contribute positively to the final product (Fleet, 1990).

Despite the high occurrences of yeasts, no studies attempted to elucidate the reasons for the high number of yeasts other than blaming it on post-pasteurisation contamination, development as secondary microflora or improper sanitation practices.

Proper disinfection of production facilities in the dairy industry is therefore imperative to minimise not only bacterial contamination but also yeast contamination to secure quality products. This requires that cleaning compounds and sanitizers must be active against the spoilage yeasts.

In this study the nature of the response of 7 dominant yeast species, frequently associated with dairy products (isolated from matured Cheddar) and the immediate environment, to commercial cleaning compounds and sanitizers was examined. In addition, we have compared their resistance against the cleaning compounds and sanitizers at different temperatures

3. MATERIALS AND METHODS

3.1 Microorganisms

Candida versatilis, C. rugosa, Debaryomyces hansenii, Dekkera custersiana, Rhodotorula mucilaginosa, Trichosporon beigelii and *Torulaspora delbrueckii* were strains isolated from matured Cheddar as described in Chapter 2. These cultures were maintained on yeast malt extract agar (YM) (Wickerham, 1951) and checked for purity by streak plating into this medium before use in experiments. The sources of origin are listed in Table 1.

3.2 Cultivation of yeast isolates

Sample preparation of the yeasts was conducted with UHT-treated milk purchased from local supermarkets. The composition of such milk is similar to

Table 1. Isolated yeasts at a cheese factory (chapter 2) and the sources of contamination

ORGANISMS	SOURCE
1. *Rhodotorula mucilaginosa*	Raw milk, equipment, air, hands, cheese curd and cheese
2. *Candida versatilis*	Raw milk, equipment, rennet, cheese curd and cheese
3. *Candida rugosa*	Raw milk, equipment and cheese
4. *Trichosporon beigelii*	Raw milk, equipment, rennet, hands, cheese curd, cheese and aprons
5. *Debaryomyces hansenii*	Raw milk, equipment, air, rennet, hands, cheese curd and cheese
6. *Dekkera custersiana*	Raw milk, hands, cheese, air, equipment and cheese curd
7. *Torulaspora delbrueckii*	Cheese, raw milk, hands and rennet

that of raw milk (Rosenthal, 1991). Milk (200ml) was aseptically dispensed into sterilised Erlenmeyer flasks (500 ml), inoculated with the relevant yeast cultures and incubated at 25°C on a rotary shaker at 160 rpm (throw = 50mm) for 48 hrs.

The yeast cells were harvested by centrifugation at 10 000 g for 5 min at 4°C. The final cell pellet was resuspended in sterile water to achieve a concentration of 1g/100ml (1%).

3.3 Cleaning compounds and sanitizers

The cleaning compounds and sanitizers comprised of nine commercial products normally used for cleaning/sanitation in the dairy industry. Cleaning compounds and sanitizers were mixed with distilled water according to the manufactures instructions and concentration. The cleaning compounds and sanitizers used are listed in Table 2.

3.4 Test procedures

The experimental yeast cultures (1%) were inoculated (1ml) into 10ml of the individual cleaning compounds and sanitizers solution. Contact times of 0, 10, 20, 45 and 60 min were used at 10 °C and 25°C. During incubation, samples (1,0 ml) of the culture were taken and analysed for viable yeast counts. Aliquots (1.0ml) were transferred into 9,0 ml of ringer solution and thoroughly mixed. Further dilutions were carried out as required for microbiological assays and plated by the spread plate technique onto duplicate plates of YM agar. YM plates were incubated for 5 days at 25°C and the surviving colonies counted.

Table 2. Description of the cleaning compounds and sanitizers.

PRODUCTS		DESCRIPTION/CLASS
W1	Chlorinated general cleaning agent	High foaming powder detergent
W2	Concentrated acid detergent	Concentrated acid detergent
W3	Germicidal hand soap	Synthetic germicidal hand soap
W4	Heavy duty caustic detergent powder	Foaming detergent / Alkaline
W5	Iodophor sanitizer	Fast acting sanitizer
W6	Pasteuriser detergent	Blend of alkalines, surfactants and several sequestering agents
W7	Peroxide based acid sanitizer	Advanced sanitizer
W8	Heavy duty chlorinated alkaline detergent	Non-foaming chlorinated alkaline
W9	Heavy duty caustic detergent powder	Foaming caustic detergent powder

4. RESULTS AND DISCUSSION

The microbial spoilage of dairy products is generally associated with the growth of bacteria (Cousin, 1982). Yeasts are, however, a frequent cause of spoilage of a wide range of dairy products. Despite the ability to cause spoilage, and references indicating post-pasteurisation contamination originating from the environment (Welthagen and Viljoen, 1998, 1999), and the yeasts greater resistance compared to bacteria (Ballou, 1976), little consideration has been given to the effect of cleaning compounds and sanitizers on these organisms. Although the heat-tolerance (Put and De Jong, 1982), activity of biocides (Jones et al. 1991), resistance against food preservatives, etc. (Romano and Suzzi, 1985) of vegetative cells and the ascospores are known, the authors mainly focussed on *Saccharomyces cerevisiae* as the challenge organism, and little is known of the non-*Saccharomyces* species present in dairy products. It is probable that, many yeasts may survive cleaning and disinfection procedures as ascospores (Jones et al. 1991). When vegetative cells (from mid-exponential phase) of *Candida versatilis, C. rugosa, Debaryomyces hansenii, Dekkera custersiana, Rhodotorula mucilaginosa, Trichosporon beigelii* and *Torulaspora delbrueckii* were exposed to different cleaning compounds and sanitizers currently applied in the dairy industry, their growth rates were inhibited and some of the yeast types was completely killed (Tables 3-9). No substantial differences in the inhibition of the growth of the yeasts were observed when the cleaning compounds and sanitizers were applied at 10°C or 25°C. *Candida rugosa* was the most resistant yeast species against the cleaning compounds and sanitizers, while *Debaryomyces hansenii* was the second most resistant yeast species against the cleaning compounds and sanitizers. This corresponds with results obtained by Bundgaard-Nielsen and Nielsen (1995) indicating that *D hansenii* is more resistant to disinfectants like chlorine dioxide, compared to related yeasts.

TABLES 3-9 : Cleaning compounds and sanitizers activity against yeasts isolated at a cheese factory (chapter 2)

Table 3 : *Rhodotorula mucilaginosa*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$156x10^5$				19 020	$156x10^5$				18 270
W2	$156x10^5$			23 200	8 620	$156x10^5$			20 120	7 930
W3	$156x10^5$			18 260	9 200	$156x10^5$				10 270
W4	$156x10^5$	128 000	3 140	720	460	$156x10^5$	119 000	2 980	690	390
W5	$156x10^5$		1 920	640	520	$156x10^5$		1 810	720	490
W6	$156x10^5$		2 810	730	280	$156x10^5$		3 120	690	270
W7	$156x10^5$	3 000	80	0	0	$156x10^5$	2 000	90	0	0
W8	$156x10^5$			9 370	4 920	$156x10^5$			10 210	5 370
W9	$156x10^5$				8 320	$156x10^5$				7 980

Table 4 : *Candida versatilis*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$384x10^5$				14 800	$384x10^5$				13 910
W2	$384x10^5$				12 100	$384x10^5$				14 200
W3	$384x10^5$				9 780	$384x10^5$				11 310
W4	$384x10^5$		14 080	1 590	580	$384x10^5$		15 080	1 620	620
W5	$384x10^5$	132 000	2 320	840	560	$384x10^5$	148 000	2 460	600	380
W6	$384x10^5$		9 600	980	490	$384x10^5$		10 200	1 070	380
W7	$384x10^5$	1 900	1 989	860	80	$384x10^5$	920	840	620	70
W8	$384x10^5$				5 310	$384x10^5$				6 120
W9	$384x10^5$		2 480	1 000	190	$384x10^5$		3 280	1 260	160

Table 5 : *Candida rugosa*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$216x10^5$				29 890	$216x10^5$				31 800
W2	$216x10^5$				19 630	$216x10^5$				17 190
W3	$216x10^5$				23 840	$216x10^5$				24 310
W4	$216x10^5$		14 280	1 260	580	$216x10^5$	598 000		980	490
W5	$216x10^5$	642 000		804	180	$216x10^5$	683 000	10 200	980	160
W6	$216x10^5$		7 260	620	480	$216x10^5$		8 320	710	510
W7	$216x10^5$	620 000		290	60	$216x10^5$	720 00		490	80
W8	$216x10^5$				7 210	$216x10^5$				9 170
W9	$216x10^5$	620 000		830	160	$216x10^5$	780 000		810	90

Table 6 : *Trichosporon beigelii*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$96x10^5$				18 620	$96x10^5$				17 620
W2	$96x10^5$				20 120	$96x10^5$				19 610
W3	$96x10^5$			14 520	8 930	$96x10^5$			12 820	9 260
W4	$96x10^5$	248 000	4 200	460	90	$96x10^5$	310 000	5 090	510	110
W5	$96x10^5$	3 200	640	0	0	$96x10^5$	280 000	560	0	90
W6	$96x10^5$	480 000		426	230	$96x10^5$	400 000	530	380	210
W7	$96x10^5$	340 000		500	140	$96x10^5$	310 000	690	480	80
W8	$96x10^5$				8 920	$96x10^5$			15 820	7 980
W9	$96x10^5$				7 920	$96x10^5$			12 820	6 950

Table 7 : *Debaryomyces hansenii*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$372x10^5$				20 160	$372x10^5$				18 600
W2	$372x10^5$			51 000	16 800	$372x10^5$			49 600	15 760
W3	$372x10^5$			16 540	7 280	$372x10^5$				9 620
W4	$372x10^5$		12 000	1 390	690	$372x10^5$		11 800	1 490	580
W5	$372x10^5$	782 000		1 080	520	$372x10^5$	862 000	8 700	980	490
W6	$372x10^5$		84 000	1 000	580	$372x10^5$		9 880	9 880	460
W7	$372x10^5$	980 000		620	320	$372x10^5$	1 021 000	8 000	580	170
W8	$372x10^5$				9 890	$372x10^5$				10 800
W9	$372x10^5$				8 200	$372x10^5$				9 670

Table 8 : *Dekkera custersiana*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$416x10^5$				12 000	$416x10^5$				11 800
W2	$416x10^5$	728 000	8 980	502 000	168 000	$416x10^5$	682 000	928 000	560 000	1 420
W3	$416x10^5$			10 540	5 860	$416x10^5$			10 520	4 820
W4	$416x10^5$	520 000	8 600	940	190	$416x10^5$	480 000	9 200	890	80
W5	$416x10^5$	460 000	980	0	0	$416x10^5$	392 000	860	0	0
W6	$416x10^5$	738 000	48 000	980	480	$416x10^5$	920 000	52 000	1 002	520
W7	$416x10^5$	680 000	1 680 000	1 010	280	$416x10^5$	810 000	1720 000		470
W8	$416x10^5$				8 930	$416x10^5$				10 500
W9	$416x10^5$			870 000	3 820	$416x10^5$			920 000	2 980

Table 9 : *Torulaspora delbrueckii*

	10°C					25°C				
Detergent	0 Min	10 Min	20 Min	45 Min	60 Min	0 Min	10 Min	20 Min	45 Min	60 Min
W1	$528x10^5$					$528x10^5$				19 600
W2	$528x10^5$	146 000	2 080	1 120	600	$528x10^5$	138 000	1 940	980	520
W3	$528x10^5$	890	250	50	20	$528x10^5$	1 000	290	30	10
W4	$528x10^5$	4 800	980	640	340	$528x10^5$	5 000	1 010	720	290
W5	$528x10^5$	620 000	920	640	320	$528x10^5$	71 000	880	580	300
W6	$528x10^5$	18 000	960	640	320	$528x10^5$	192 000	1 680	580	270
W7	$528x10^5$	460 000	1 040	20	10	$528x10^5$	380 000	1 020	10	0
W8	$528x10^5$				10 800	$528x10^5$				14 600
W9	$528x10^5$			6 700	8 640	$528x10^5$			5 600	8 250

The resistance of *D. hansenii* against the compounds may be a cause for concern, as the species proved to be dominant in various dairy products (Welthagen and Viljoen, 1998), being frequently recovered from salt brines (Mrak and Bonar, 1939; Walker and Ayres 1970; Seiler and Busse, 1990; Viljoen and Greyling, 1995), yoghurt (Suriyarachchi and Fleet, 1981), cheese (Szumski and Cone, 1962; Gilmour and Rowe, 1981; Lenoir, 1984) and raw milk (Fleet and Mian, 1987). In all of these studies, the species emerged as a post-pasteurisation contaminant, isolated from a wide range of sources including the brine, whey, curd, air, surface equipment, workers' hands and aprons. Consequently, they have to be controlled by cleaning compounds or sanitizers to prevent spoilage of the final product.

Rhodotorula mucilaginosa was killed within 45 min when exposed to the peroxide based sanitizer, whereas *Trichosporon beigelii* and *Dekkera custersiana* were killed when exposed to the iodophor sanitizer. None of the remaining yeasts was killed by any of the cleaning compounds or sanitizers. The resistance of the yeast species, attributed to their thicker cell walls (Jones et al. 1991), may lead to spoilage as they are all typical dairy associated yeasts (Comi et al. 1981; Eliskases - Lechner and Ginzinger, 1995; Kaminarides and Laskos, 1992; McKay, 1992; Skinner et. al, 1961; Seiler and Busse, 1990; Walker and Ayres, 1970; Zein et al., 1983) capable of peptonising casein and attack butterfat readily (Fleet and Mian, 1987; Green and Ibe, 1987; Ingram, 1958; Rohm et.al. 1992; Suriyarachchi and Fleet, 1981; Suarez and Inigo, 1982; Tilbury et al. 1974).

The "peroxide based acid sanitizer" proved to be the most effective inhibitor against all the yeasts, resulting in final counts ranging from zero to 3.2×10^2 cfu/ml after 60 min of contact. Poor killing effects of hydrogen peroxide (Bundgaard-Nielsen and Nielsen, 1995) and peracetic acid (Jones et al. 1991) were attributed to low concentrations or too short contact time (10 min).

Despite the general use of chlorinated cleaning compounds and concentrated detergents in the food industry, the compounds had little effect on the survival of the yeasts, resulting in the high viable yeast counts after 60 min of contact time. All the yeasts showed viable counts exceeding 10^4 cfu/ml. Bundgaard-Nielsen and Nielsen (1995) also reported poor killing effects of the alkaline disinfectants like potassium hydroxide and sodium hydroxide. High yeast counts were also observed with the use of heavy duty chlorinated alkaline and caustic detergents (Tables 3-9). The usage of iodophor as a sanitizer resulted in the total inhibition of *Trichosporon beigelli* and *Dekkera custersiana*, and generally exhibited good killing effects against most of the yeast species. The usage of this sanitizer in the South African dairy industry, however, was recently prohibited.

These results clearly demonstrate that, individual yeast species exhibit different responses to cleaning compounds and sanitizers and have the potential to survive on surfaces and during sanitation of processing equipment. Therefore, to obtain proper manufacturing hygiene it is important to determine the resistance of the dominant yeast types against all the compounds. Eventually, it may be necessary to apply more than one sanitizer to assure efficient cleaning.

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CHAPTER 4

KEY PROPERTIES OF YEASTS ISOLATED DURING THE MANUFACTURING AND RIPENING OF MATURED CHEDDAR CHEESE.

1. ABSTRACT

Seven yeast species (*Debaryomyces hansenii, Trichosporon beigelii, Torulaspora delbrueckii, Candida versatilis, Candida rugosa, Rhodotorula mucilaginosa* and *Dekkera custersiana)* were selected based on the frequency of their occurrence during processing of mature Cheddar cheese (Chapter 2). The growth of the yeast species in milk containing added sodium chloride (5%, 10% and 15%) and at different temperatures (7°C and 15°C) were studied. Depending on the conditions, all yeasts grew to a maximum population of $10^5 - 10^8$ cfu/ml. All the yeast isolates investigated were able to grow at low temperatures and high salt concentrations. According to the results obtained, no substantial differences were observed between the progression of the yeasts grown at 7°C and 15°C. The number of organisms, however, decreased when the NaCl contents increased. None of the strains was able to ferment lactose. Thirty-nine of the 151 yeast strains examined for hydrolysis of protein were positive, while 21 strains were positive for the hydrolysis of fat based on Tributyrin and 27 strains were positive on Rhodamine.

According to the results obtained, it is impossible to control the growth of undesired yeasts solely by adjustment of physcochemical parameters like NaCl, pH and temperature.

2. INTRODUCTION

Yeasts are frequently found within the microflora of a wide variety of cheeses (Roostita and Fleet, 1996a and 1996b; Fleet, 1990; Devoyod, 1990; Eliskases-Lechner and Ginzinger, 1995; Seiler and Busse, 1990; Viljoen and Greyling, 1995; Fleet and Mian, 1987; Lenoir, 1984). They are considered as natural contaminants of the cheese-making process and populations as high as 10^5 cfu/g were reported during the maturation stage of production. *Debaryomyces hansenii, Kluyveromyces marxianus, Yarrowia lipolytica* and various species of *Candida* were isolated from cheese during maturation and retailing, being present due to their ability to survive at low temperature and high salt concentrations (Choisy et al., 1987a,b; Fleet, 1990; Devoyod, 1990; Roostita and Fleet, 1996; Viljoen and Greyling, 1995; Lenoir, 1984; de Boer and Kuik, 1987; Nooitgedacht and Hartog, 1988; Besancon et al., 1992). Some yeast species contribute substantially to the development of flavour and texture of certain cheeses during the ripening process (Nunez et al., 1981; Schmidt and Daudin, 1983). Yeasts also contribute to the quality of cheese based on the production of lipolytic and proteolytic enzymes, fermentation of residual lactose and the utilisation of lactic acid (Choisy et al., 1987a, b; Fleet, 1990; Devoyod, 1990).

Microbial proteolysis is mainly responsible for spoilage in cheese, leading to the development of bitter flavours, followed by the production of strong ammoniacal odours and putrefaction (Lowrie and Lawrence, 1972; Lowrie, 1977). Enzymatic degradation of food lipids produces a range of free fatty acids that, depending on the type of acid and the concentration, confer characteristic off-flavours (Fleet, 1992). Jacobsen et al., (1989), Espinosa et al., (1990) and Petrovic et al., (1990) reported on the production of characteristic flavours and/or tastes in cheeses, due to lipolytic activities in the dairy and food industry. Lipase is also responsible for odour spoilage of food products (Nelson et al., 1991). Depending on the strain properties and the contamination level, the yeasts affect the ripening process positively (de-acidification, aroma substances) or negatively (smell and taste

defects). Yeasts contribute to the formation of components or precursors of aroma, like amino acids, fatty acids, esters, etc. based on proteolytic, lipolytic and esterifying processes (Lenoir, 1984).

Yeasts also contribute to the development of cheeses by stimulating the growth of the starter bacteria, due to the excretion of growth factors, like group B vitamins and amino acids (Lenoir, 1984). Siewert (1986) reported that yeasts produce substances with a stimulating effect on the growth of lactic acid bacteria and aerobic bacteria on the cheese surfaces. Furthermore, the yeasts ferment the residual lactose, influence flavour formation by producing volatile acids and carbonyl compounds, and prevent the formation of a "toad skin" on the cheese surface.

Commercial yeast cultures, used for the ripening of cheeses, contain different yeast species involving *Yarrowia lipolytica*, *Debaryomyces hansenii, Candida utilis, Candida valida, and Kluyveromyces lactis.* Various undesired contaminating yeasts, however, are frequently encountered during the making of cheese (Fleet, 1992). These yeasts are difficult to control during processing by adjustment of physcochemical parameters like pH, NaCl, water activity and temperature (Vivier et al., 1994). Therefore, in this study the influence of temperature and NaCl on the seven most dominant yeast species isolated in a cheese factory was examined. The relevant yeast species were also screened for lipolytic and proteolytic activities and the production of secondary metabolites. The data obtained from this study, form the basis for selecting the most suitable yeast to be incorporated as part of the starter cultures for the production of Cheddar cheese.

3. MATERIALS AND METHODS

3.1 Samples

Numerous yeasts were isolated from equipment contact surfaces (cheddaring vats, cheese vats and aprons), ingredients (rennet, calcium chloride, milk, salt and coloring agent) added during production, hands of workers, curd and final product (Chapter 2). All visually distinguished yeasts were cultured on Yeast extract malt extract (YM) agar (Wickerham, 1951) slants and stored at 4°C during the period of investigation.

3.2 Characterization of yeast isolates

Yeast isolates were identified, using the methods described by Kreger-van Rij (1984) and the computerized identification system of Barnett et al. (1990). The 7 dominant yeast isolates were selected based on the frequency of occurrences during processing (Chapter 2). The selected yeast strains and their sources are indicated in Table 1.

Table 1 Isolated yeast strains associated with mature Cheddar cheese making (chapter 2).

Yeast	Number of strains	Sources
Candida rugosa	5	Milk, Equipment and Cheese
Candida versatilis	16	Milk, Equipment, Air, Hands and Cheese
Debaryomyces hansenii	47	Milk, Equipment, Air, Rennet, Hands, Aprons, Curd and Cheese.
Dekkera custersiana	32	Milk, Equipment, Air, Hands, Curd and cheese.
Rhodotorula mucilaginosa	20	Milk, Equipment, Air, Hands and Curd.
Torulaspora delbrueckii	6	Milk, Rennet, Hands and cheese.
Trichosporon beigelii	25	Milk, Equipment, Rennet, Hands, Aprons, Curd and Cheese.

3.3 Preparation of suspensions of test microorganisms

Growth experiments were conducted in triplicate in UHT-treated milk purchased from local supermarkets. The composition of such milk is similar to that of raw milk (Rosenthal, 1991). Milk (200ml) was aseptically dispensed into sterilised 500ml Erlenmeyer flasks and inoculated with a loopful of the individual yeast cultures. Yeast cells were harvested by centrifugation at 11 000 r.p.m. after growth on an orbital shaker controlled at 25°C for 48 hrs. Sterile water was added, calculated according to the cell mass obtained.

3.4 Temperature

A 1% inoculum of the prepared yeast suspensions of each of the 7 yeast strains was transferred into 200 ml UHT milk, in 500ml Erlenmeyer flasks and incubated at 7°C and 15°C while shaking on an orbital shaker at 150 rpm. Every 8 hrs 1 ml samples were taken up to 112 hrs and diluted in 9,0 ml of ringer's solution (Dissolve 1 tablet in 500 ml of distilled water, sterilize in autoclave), (Merck) and thoroughly mixed. Further dilutions were prepared out as required for microbiological assays and plated by the spread plate technique onto yeast extract malt extract (YM) agar. YM plates were incubated for 5 days at 25°C. Surviving yeasts were detected by counting visible colonies on plates with between 30 – 300 colonies on the highest dilution.

3.5 Salt

Similar procedures were followed as described for temperature. The inoculated yeasts were grown in UHT milk containing different salt concentrations (5, 10 and 15%) and incubated at 25°C.

3.6 Lipase production on agar plates

Screening for lipase was done by streaking yeast strains on agar plates containing two different inducers, namely, (1) Olive oil and Rhodamine B, pH 7.0 (Kouker and Jeager, 1987) and (2) Tributyrin (glycerol tributyrate) (Lima, Teixeira and Mota, 1991). The plates were incubated for 3 – 6 days at 25°C.

3.7 Proteolytic activity on agar plates

Screening for proteolytic activity was done by streaking yeast strains on proteolytic agar (Ahearn et al., 1968). The plates were incubated for 3- 6 days at 25°C.

3.8 Physical and chemical analysis of the matured cheese and curd

The pH, salt, moisture content, water activity (a_w) and lactose content were determined at 0 hrs, 48 hrs, 12 days and 30 days, thereafter at 30 day intervals up to 6 months. The curd in the cheese molds represented the first sample and is indicated as 0 hrs. The pH of the homogenized cheese samples was measured at 24°C with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1982). The salt was determined as described by Marshall (1992). The water activity (a_w) was determined throughout ripening on a Novasina Thermoconstanter, Model TH 200. Lactose content was measured by means of a Waters HPLC system with a Biorad-aminex C42 Column and Refractive index detector. The moisture content was determined throughout ripening according to the method described by Marshall (1992).

4. RESULTS AND DISCUSSION

4.1 Physical and chemical analysis

The initial pH of matured Cheddar cheese was 5.37 (Bulk starters) and 5.48 (DVI starters) at the beginning of ripening (just before press) and decreased to 5.10 (Bulk starter) and 5.26 (DVI starter) after 12 days. The pH remained stable for the remaining period of maturation (Table 2). Welthagen and Viljoen (1998) reported that the pH of Cheddar was 5.42 at the beginning of ripening (just after processing), decreasing to a final value of 5.06 after 51 days of ripening.

No substantial changes were observed in the moisture during the study. The retaining of the values is attributed to the wrapping of the cheese in impermeable plastic film. The moisture content of the curd before press was 41.05 % (Bulk starter) and 41.45 % (DVI starter). After the cheese was wrapped the moisture varied between 37 % after 2 days to 36.45 % after 180 days for the bulk starter and 37.09 % after 2 days to 36.11 % after 180 days for the DVI starters (Table 2). The salt content varied between 1.95 % and 2.50 % when Bulk starters were used and between 1.59 % and 2.26 % when DVI starters were used (Table 2), salt content varies due to bad process controle.

Table 2 Average analytical data and chemical composition of matured Cheddar cheese made with DVI and Bulk starters over a period of 180 days

Days	PH	Salt	Moisture	Fat	A_w	Lactose
0	5.43	1.70 %	41.25 %	32.25 %	99.3 %	2.98 %
2	5.17	1.77 %	37.05 %	33.36 %	96.4 %	0.00 %
12	5.18	1.96 %	36.97 %	33.35 %	96.5 %	0.00 %
30	5.23	2.03 %	36.76 %	33.15 %	96.2 %	0.00 %
60	5.23	2.16 %	36.70 %	33.00 %	96.0 %	0.00 %
90	5.24	2.20 %	36.68 %	33.05 %	95.9 %	0.00 %
120	5.25	2.29 %	36.47 %	33.05 %	94.7 %	0.00 %
150	5.26	2.33 %	36.36 %	33.16 %	94.2 %	0.00 %
180	5.30	2.38 %	36.28 %	33.18 %	93.8 %	0.00 %

During the first 12 days of ripening, the % Fat content increased slightly due to surface evaporation (Table 2).

The changes in water activity of matured Cheddar cheese during the ripening period of 180 days are shown in Table 2. The water activity decreased from 99.3 at the end of cheddaring to 96.4 at day 2 and remained between 96.5 and 93.8 for the remainder of the ripening period (Table 2). According to Fleet (1992), the lowering in water activity during the ripening is due to surface evaporation of moisture. Welthagen and Viljoen (1998) reported an increase in water activity from day 1 to day 14, followed by a slight decrease after 26 days and remaining constant for the duration of the ripening period in Gouda cheese.

The lactose content initially present in matured Cheddar cheese after processing was 2.98, but was quickly utilized being depleted after 2 days of the ripening process (Table 2). The depletion of the lactose is attributed to the rapid growth of the starter cultures, utilizing the carbohydrate and contributed to organic acid production.

4.2 Influence of salt on yeast growth

NaCl is a major food additive and its use in the food processing industry is exceeded only by that of sugar (Reddy and Marth, 1995). The effect of salt on the growth and survival of the isolated yeasts is illustrated in Fig. 1 a-g. The growth rate decreased linearly when NaCl contents increased. *Rhodotorula mucilaginosa* (Fig. 1c), *Debaryomyces hansenii* (Fig. 1e), *Candida rugosa* (Fig. 1g), *Dekkera custersiana* (Fig. 1d) and *Candida versatilis* (Fig. 1f) were less sensitive to the 5 % salt concentration compared to *Trichosporon beigelii* (Fig. 1a) and *Torulaspora delbreuckii* (Fig. 1b). The salt concentration of mature Cheddar cheese in this study varied between 1.59 and 2.50 %. According to the

Fig. 1 a) The growth of *Trichosporon beigelii* at different salt concentrations

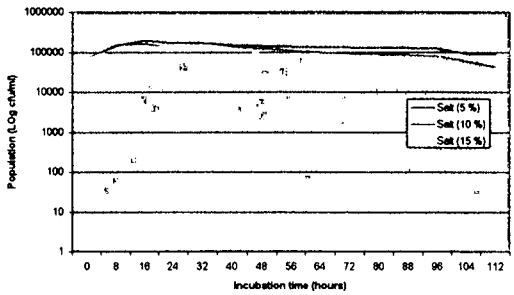

Fig. 1 b) The growth of *Torulaspora delbrueckii* at different salt cocentrations

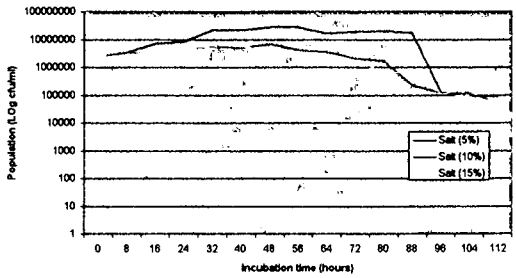

Fig. 1 c) The growth of *Rhodotorula mucilaginosa* at different salt concentrations

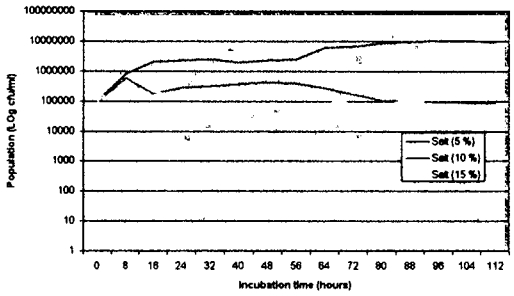

Fig. 1 d) The growth of *Dekkera custersiana* at different salt concentrations

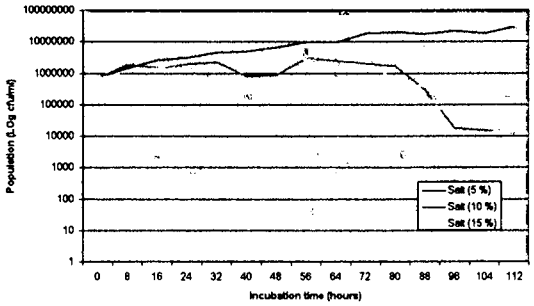

Fig. 1 e) The growth of *Debaryomyces hansenii* at different salt concentrations

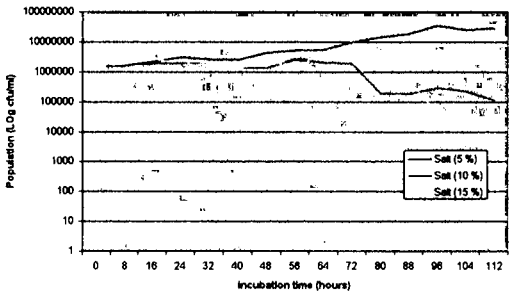

Fig. 1 f) The growth of *Candida versatilis* at different salt concentrations

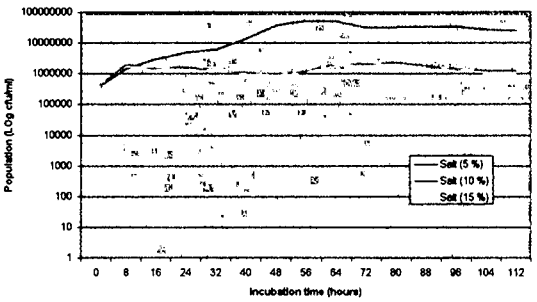

Fig. 1 g) The growth of *Candida rugosa* at different salt concentrations

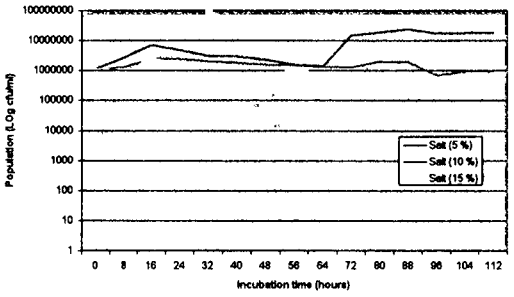

Fig. 1 The influence of salt on yeast growth

results obtained, 5 of the yeast species showed positive growth at salt concentrations as high as 5 %, (Fig. 1). *Trichosporon beigelii,* despite an initial increase in cell numbers within the first 16 hrs reaching counts as high as 1.9 x 10^5 cfu/ml (5 % salt), 1.6 x 10^5 cfu/ml (10 % salt) and 1.4 x 10^5 cfu/ml (15 % salt), exhibited a negative growth (organisms dying of) resulting in final cell numbers of 8.8 x 10^4 cfu/ml (5 % salt), 4.2 x 10^4 cfu/ml (10 % salt) and 3.2 x 10^4 cfu/ml after 112 hrs (Fig. 1a). *Torulaspora delbreuckii* cell numbers initially increased reaching maximum cell populations of 2.8 x 10^6 cfu/ml (5 % salt) after 56 hrs, 7.2 x 10^5 cfu/ml (10 %salt) after 48 hrs and 6.1 x 10^5 (15 %) after 32 hrs. Again, similar decreasing growth patterns were observed resulting in final counts as low as 5.2 x 10^3 cfu/ml after 112hrs (Fig. 1b). *R. mucilaginosa, D. hansenii, D. custersiana, C. versatilis* and *C. rugosa* cell numbers continued to increase when grown in milk with the addition of 5% salt. However, when the yeast isolates were grown in 10 and 15 % salt concentrations, a decrease in population counts were observed after 1 to 2 days (Fig. 1c-g). *D. hansenii, C versatilis* and *D. custersiana* showed substantial growth in milk with the addition of 5 % salt, reaching population numbers exceeding 10^7 cfu/ml after 112 hrs (Fig. 1d, 1e and 1f). According to the results obtained the growth of the yeast species, with the exception of *C. versatilis,* were inhibited by the high salt concentrations when grown for a period longer than 2 days (Fig. 1).

Roostita and Fleet (1996a) reported that the production of main primary and secondary products of lactose fermentation, ethanol, glycerol, lactic acid, acetic acid and propionic acid by yeasts (*Debaryomyces hansenii, Saccharomyces cerevisiae, Kluyveromyces marxianus, Candida lipolytica* and *Candida catenulata*) was not abnormally affected by the presence of 10 % NaCl. Seiler and Busse (1990) and Eliskases-Lechner and Ginzinger (1995) showed that *Candida versatilis, Candida rugosa, Debaryomyces hansenii, Trichosporon beigelii, Torulaspora delbrueckii* and *Rhodotorula minuta* are typical yeast species associated with cheese brine. *Debaryomyces hansenii* strains exhibited extreme salt tolerance (Seiler and Busse, 1990; Lenoir, 1984).

4.3 Influence of temperature on yeast growth

Temperature is the single most important environmental factor contributing to the ripening of Cheddar cheese. The ripening temperature for mature Cheddar cheese varied between 8°C and 11°C. Fig. 2 shows the variation in growth curves obtained when the yeasts were grown at 7°C and 15°C. Based on the results obtained, the yeasts were able to grow in milk at 7°C and 15°C. Their rates of growth and maximum cell densities decreased as the temperature in the milk was decreased (Fig. 2). All the yeast isolates reached maximum populations exceeding 10^9 cfu/ml when grown at 15°C, whereas only *T. delbrueckii, D. hansenii* and *C. rugosa* reached counts in excess of 10^9 cfu/ml when grown at 7°C. Roostita and Fleet (1996a) reported that the production of the main primary and secondary products was not affected abnormally when the yeasts were grown at low temperature (10°C). *Rhodotorula* species are typical cold-tolerant type yeasts (Davenport, 1980), capable of growing at sub-zero temperatures and pH values as low as 2.4 (Pitt and Hocking, 1985). *Debaryomyces hansenii* possesses the ability to grow well at low temperatures (Davenport, 1980). The ability of yeasts to grow well at low temperatures (5°C) explains the frequent occurrences in dairy products which, for the most part, are stored under refrigeration (Davenport, 1980).

4.4 Properties of yeast species that affect their growth in cheese

Growth in the presence of elevated salt concentrations, low temperatures and the ability to utilise lactic and citric acids are key determinants that cause the predominance of *Debaryomyces hansenii* in cheeses (Besancon et al., 1992). Some of the 47 strains of *D. hansenii* previously isolated (chapter2) exhibited extracellular

Fig. 2 a) The growth of *Torulaspora delbrueckii* at different temperatures

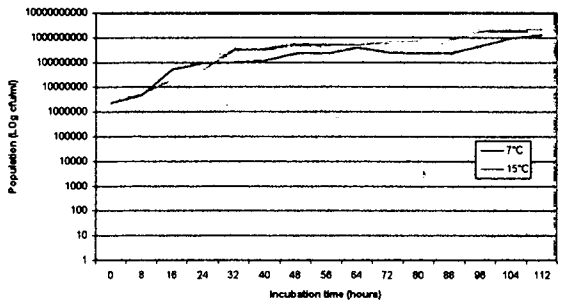

Fig. 2 b) The growth of *Rhodotorula mucilaginosa* at different temperatures

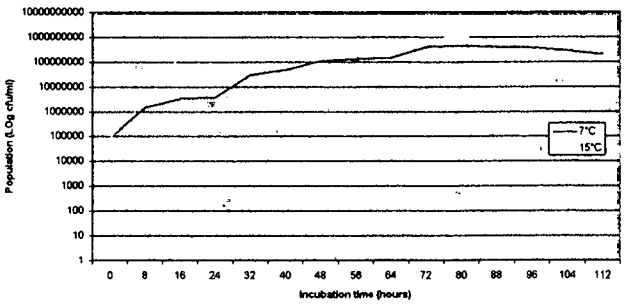

Fig. 2 c) The growth of *Dekkera custersiana* at different temperatures

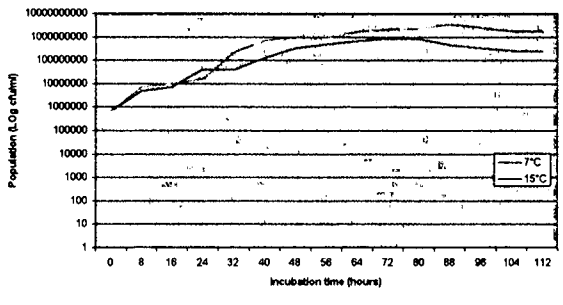

Fig. 2 d) The growth of *Debaryomyces hansenii* at different temperatures

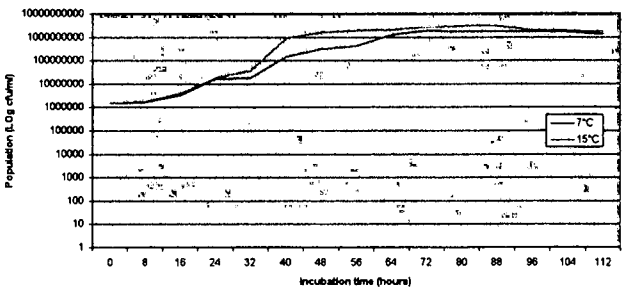

Fig. 2 e) The growth of *Candida versatilis* at different temperatures

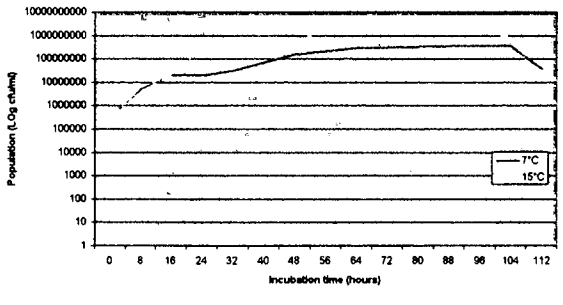

Fig. 2 f) The growth of *Candida rugosa* at different temperatures

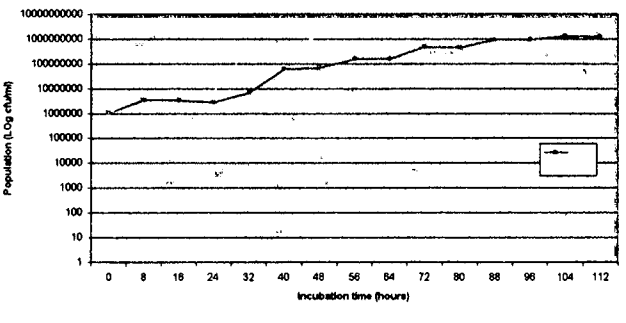

Fig. 2 f) The growth of *Trichosporon beigelii* at different temperatures

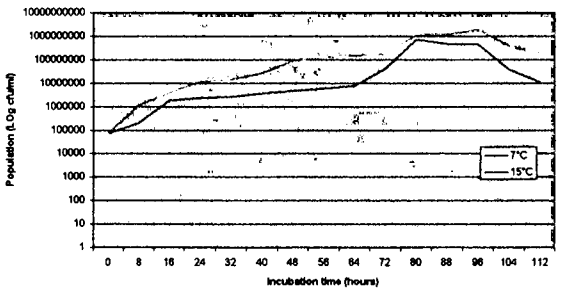

Fig. 2 The influence of temperature on yeast growth

proteolytic (2 strains) and lipolytic (Tributyrin, 8 strains and Rhodamine, 17 strains) activities (Table 2). Ahearn et al. (1968), Carini et al. (1975) and Roostita and Fleet (1996b) have noted the absence of such activities in these species. Besancon et al. (1992), however, found that some of the strains of *D. hansenii* proved to be positive for these reactions. Schmidt et al. (1979) indicated that strains of *Debaryomyces hansenii* have variable abilities regarding the production of proteolytic and lipolytic enzymes. Consequently, this species is capable of metabolising milk protein and fat. Welthagen and Viljoen (1998) reported that *Debaryomyces hansenii* exhibits extracellular lipolytic activity, but no proteolytic activity. High intracellular proteolytic activities are reported by Nunez et al. (1981) and Lenoir (1984). According to results obtained in this study there are certain strains of *Debaryomyces hansenii* that exhibits extracellilar proteolytic and lipolytic activities.

All the strains of *Dekkera custersiana* and *Candida rugosa* studied, showed strong lipolytic and proteolytic activities (Table 2). This is in agreement with the results reported by Wu et al. (1990) indicating positive lipolytic activity by *Candida rugosa* strains. Roostita and Fleet (1996a) reported that the proteolytic and lipolytic reactions of *Candida lipolytica* and *Candida catenulata* decrease when grown at low temperature (10°C) or NaCl (10%), but are not inhibited. *Trichosporon beigelii* showed positive lipolytic activity, but no proteolytic activity. Proteolytic and lipolytic activities were absent in the strains of *Candida versatilis* and *Torulaspora delbrueckii.* Only one of the 20 strains representing *Rhodotorula mucilaginosa,* tested positive for the hydrolysis of fat. According to Comi and Cantoni (1985), some strains of *Rhodotorula* possess lipolytic activity.

The proteolytic and lipolytic systems of yeasts comprise a mixture of several different proteases (Ogrydziak, 1993) and several different lipases (Ratledge and Tan, 1990), the individual activities of which could respond differently to changes in temperature and salt concentration. None of the strains studied, was able to

ferment lactose. According to Deak and Beuchat (1996) and Roostita and Fleet (1996a), the fermentation of lactose by lactose fermenting yeasts influences the aroma of the cheese by the formation of alcohol, inhibits acidification due to the utilisation of lactic acid, affecting the texture of the cheese, and cause the formation of CO_2.

Table 2

Properties of yeast species that effect their growth in cheese

Properties	*Debaryomyces Hansenii*	*Candida Versatilis*	*Trichosporon Beigelii*	*Rhodotorula Mucilaginosa*	*Candida Rugosa*	*Torulaspora Delbrueckii*	*Dekkera Custersiana*
Number of Strains tested	47	16	25	20	5	6	32
Fermentation Of lactose	0/47	0/16	0/25	0/20	0/5	0/6	0/32
Hydrolysis of Protein	2/47	0/16	0/25	0/20	5/5	0/6	32/32
Hydrolysis of Fat (Tributyrin)	8/47	0/16	11/25	1/20	1/5	0/6	0/32
Hydrolysis of Fat (Rhodamine)	17/47	0/16	5/25	0/20	5/5	0/6	32/32
Growth in 5% NaCl	47/47	16/16	25/25	20/20	5/5	6/6	32/32
Growth in 10% NaCl	47/47	16/16	25/25	20/20	5/5	6/6	32/32
Growth in 15% NaCl	47/47	16/16	25/25	20/20	5/5	6/6	32/32
Growth at 7°C	47/47	16/16	25/25	20/20	5/5	6/6	32/32
Growth at 15°C	47/47	16/16	25/25	20/20	5/5	6/6	32/32

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CHAPTER 5

THE INTERACTION BETWEEN YEASTS AND BACTERIA DURING THE MANUFACTURING OF MATURE CHEDDAR CHEESE

1. ABSTRACT

The development of yeasts and bacteria in matured Cheddar cheese by using Bulk- and DVI-starters, from 6 separate days, was monitored during a 180 days ripening period. After 12 days, the numbers of yeasts reached their maximum growth (1.31 x 10^3 cfu/g), followed by a rapid decline. No yeasts were present after 3 months of maturation. Total bacterial counts as high as 5.18 x 10^8 cfu/g were observed after 48 hrs. The salting of the curd resulted in a decrease in total bacterial loads and an increase in yeasts numbers. Coliforms as high as 8.6 x 10^2 cfu/g were present in the curd, but the numbers decreased to less than 10 cfu/g after 1 month. The pH of the cheese varied between 5.48 and 5.06 during the manufacturing of the curd and the ripening of the cheese. Moisture content remained between 41.45 % (curd) and 35.5 % (cheese after 6 months), whereas the salt concentration varied between 1.59 % and 2.50 % of the separate production days, during the ripening period. The difference in salt content between the different production days, was due to bad process control.

2. INTRODUCTION

Microbial interactions involving yeasts in cheese and fermented milk products have been reported by a number of authors (Lenoir, 1984; Devoyod, 1990; Kaminarides and Laskos, 1992; Robinson and Tamine, 1990). Yeasts interact either positively or negatively with other microorganisms in cheese; (1) by inhibiting or eliminating undesired microorganisms responsible for quality defects or possess potential pathogenic characters; (2) by inhibiting the starter culture; or (3) by supporting the function of the starter culture during the fermentation or maturation process. Yeasts furthermore stimulate the growth of other microorganisms, in particular moulds (*Penicillium, Geotrichum*) and bacteria (micrococci and corynebacteria) due to the excretion of growth factors (group B vitamins and amino acids) (Lenoir, 1984). Deiana et al. (1984) indicated the possibility that *Debaryomyces hansenii* inhibits or eliminates undesired microorganisms like *Clostridium butyricum* and *Clostridium tyrobutyricum* due to organic acid depletion. The killer factor of yeasts isolated from cheese brines, also affects bacterial and mould growth due to its antibacterial capabilities (Seiler and Busse, 1990).

The positive interaction between yeasts and the starter culture for surface ripening cheese is well documented (Besancon et al., 1992; Gripon, 1993; Hartley and Jezeski, 1954; Kaminarides and Anifantakis, 1989; Lenoir, 1984; Morris et al, 1951; Noomen, 1983). Yeasts also assist the development and growth of *Penicillium roqueforti* in blue cheese by gas production leading to curd-openness and the secretion of nutrients (Coghill, 1979). A vast range of yeasts could assist the starter cultures in cheeses by proteolytic and lipolytic activities (Besancon et al, 1992; Grieve et al, 1983; Gripon, 1993; Kalle et al, 1976), and the formation of amines (Adda et al, 1982). The yeasts thereby directly take part in the maturation, through the formation of aroma components.

This paper reports on the interaction between yeasts, coliforms and bacteria during the manufacturing of mature Cheddar cheese, by using DVI starters and Mass starters. The cell numbers and species of yeasts found during processing, the changes in physical and chemical composition of the cheese and the bacterial numbers are studied.

3. MATERIAL AND METHODS

3.1 Matured Cheddar cheese manufacture

Matured Cheddar cheese was manufactured at a commercial cheese factory in the Orange Free State in South Africa. The procedure for cheese-making was carried out as described by Kosikowski (1977).

3.2 Sampling methods and selection of isolates

Samples were taken during processing of the Cheddar cheese at selected sampling points, as indicated in Table 1. Liquid samples (10 ml) were diluted in 90 ml sterile quarter strength Ringers solution (Dissolve 1 tablet in 500 ml of distilled water, sterilize in autoclave), (Merck). For solid samples, 20g portions were placed in 180ml sterile Ringers solution, macerated in a Colworth 400 stomacher (London, UK) for 2 min and the liquid portions diluted. Further decimal dilutions were carried out as required for microbiological assays and plated in triplicate by the spread plate technique onto Plate count agar (Merck), Violet red bile dextrose agar (Merck) and Yeast extract Glucose Chloramphenicol Agar (YGC) (Merck, Darmstadt, Germany; pH 6.6). Plates for total counts were incubated for 48h at 25°C, Violet red bile dextrose agar plates were incubated for 24h at 32°C and YGC agar plates incubated for 120h at 25°C.

Table 1. Identification of yeasts isolated during the ripening of Cheddar Cheese

Isolates	Time								
	O hrs	48 hrs	12 days	1 month	2 months	3 months	4 months	5 months	6 months
Candida									
C. versatilis			+	+					
C. rugosa									
Debaryomyces									
D. hansenii	+	+	+	+	+				
D. vanrijiae		+							
Torulaspora									
T. delbreuckii		+							
Dekkera									
D. anomala		+							
D. custersiana	+	+							
D. bruxellensis		+	+						
Rhodotorula									
R. glutinis	+								
R. mucilaginosa	+								
R. minuta		+	+	+					
Trichosporon									
T. beigelii	+	+	+	+	+				

+ : Precence of yeasts in cheese.

Coliforms were detected by counting visible colonies on Violet red bile dextrose agar, whereas the total bacterial count was detected by counting colonies on total plate count agar. All yeast colonies were isolated from the highest dilution on plates containing YGC agar. The representative yeast isolates were sub-cultured on Yeast extract malt extract (YM) agar and incubated for 120h at 25°C for control of purity by colony morphology and microscopy. The pure cultures were stored at 4°C on YM agar during the period of investigation.

3.3 Sampling during ripening

Cheddar cheese from different batches and days, were kept under controlled conditions (8°C–11°C) and sampled as indicated in Table 1. Cheese samples were prepared for microbiological analyses by opening the cheese aseptically with a cheese trier. After sampling the cheese, the sampling holes in the cheese were closed with cheese plugs. Sampling of the cheese was performed as described in Chapter 2.

3.4 Physical and chemical analysis

The pH, salt, moisture content, water activity and lactose content were determined at 0 hrs, 48 hrs, 12 days and 30 days, thereafter at 30 day intervals up to 6 months. The curd in the cheese molds being the first sample, is indicated as 0 hrs. The pH of the homogenized cheese samples was measured at 24°C with a HI 9321 Microprocessor pH meter (HANNA Instruments) according to the method described by Kosikowski (1982). The salt was determined as described by Marshall (1992). The water activity (a_w) was determined throughout ripening on a Novasina Thermoconstanter, Model TH 200. Lactose content was measured by means of a Waters HPLC system with a Biorad-aminex C42

Column and Refractive index detector. The moisture content was determined according to the method described by Marshall (1992).

3.5 Characterization of yeast isolates

The representative yeast isolates were identified by using the methods described by Kreger-van Rij (1984) and the computerized identification system of Barnett et al. (1990). Results are discussed earlier (Chapter 2).

4. RESULTS AND DISCUSSION

Cheeses from 6 separate days production were examined over a six month period, by using bulkstarters during the first 3 days of production and Direct vat inoculation starters during the last 3 days production. Six cheeses were selected from each days production to represent the beginning, middle and end of each cheese vat manufacture. The cheeses were stored under normal conditions and examined at 48 hrs, 12 and 30 days, followed by 30 day intervals for pH, fat, moisture content, lactose content and water activity. Microbial analysis included counts of coliforms, total bacteria and yeasts.

4.1 Chemical and physical composition

Figure 1 shows comparative analytical data and chemical composition of Cheddar cheese during ripening over a period of 6 months. The pH of the

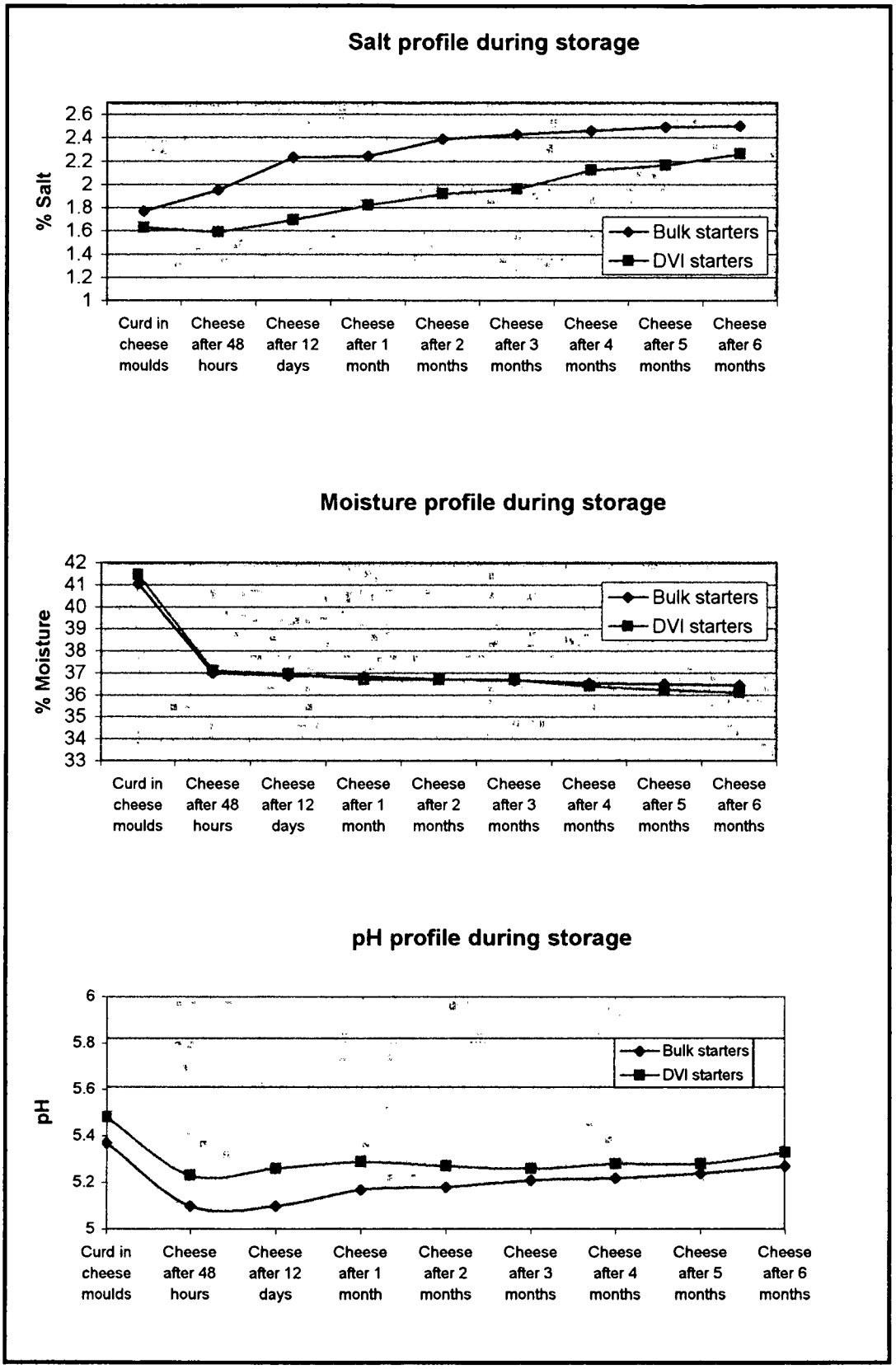

Fig. 1 Chemical and physical composition of matured Cheddar cheese

matured Cheddar cheese was 5.37 (Bulk starters) and 5.48 (DVI starters) at the beginning of ripening (just before press), decreasing to 5.10 (Bulk starters) and 5.26 (DVI starters) respectively after 12 days and increased to 5.27 after 180 days. The initial decrease in pH values corresponds with results obtained by Welthagen and Viljoen (1998) indicating a decrease in pH from 5.42 at the beginning of ripening to 5.06 after 51 days of ripening. The decrease in pH might be due to the bacterial utilization of lactose and other components during ripening at 8°C (Fox, 1987). Welthagen and Viljoen (1998) also reported a slight increase in pH values between 18 and 26 days attributed to the assimilation of lactic acid by the growth of yeasts and moulds (Fleet, 1990). Therefore, as the moulds or yeasts neutralized the acidity of the cheese, the pH increased (Lenoir, 1984; Schlesser et al., 1992). The subsequent gradual rise in the pH is most likely caused by the progressive breakdown of proteins (Prentice and Brown, 1983).

No substantial changes in the moisture and salt levels were observed during this study. This is not surprising since the cheeses were wrapped in impermeable plastic film. The moisture content of the curd before press was 41.05 % (Bulk starter) and 41.45 % (DVI starter). After the cheese had been wrapped, the moisture content varied between 37.00 % after 2 days to 36.45 % after 180 days for the bulk starters. The moisture content of the cheese prepared with the DVI starters varied between 37.09 % after 2 days to 36.11 % after 180 days. Prentice and Brown (1983) reported that the moisture content remains between 35.5 % and 36.2 % over one year during the ripening of Cheddar cheese. The salt content of the cheeses varied between 1.95 % and 2.50 % when Bulk starters were used and between 1.59 % and 2.26 % when DVI starters were used, varies in salt content was because of bad process control.

The water activity decreased from 99.3 at the end of cheddaring to 96.4 after 2 days and remained between 96.5 and 93.8 for the remainder of the ripening

period. According to Fleet (1992) the lowering in water activity during the ripening is due to surface evaporation of the water. Welthagen and Viljoen (1998) reported an increase in water activity from day 1 to day 14, followed by a slight decrease and thereafter the aw remained constant for the rest of the ripening period in Gouda cheese. The lactose content initially present in matured Cheddar cheese after processing was 2.98 % but was depleted after 2 days of the ripening process.

4.2 Microbial enumeration during the ripening of the cheese (Fig. 2)

a) **Bulk starters:** Total bacterial counts of 3.0 x 10^5 cfu/ml, yeasts (6.3 x 10^3 cfu/ml) and coliforms (2.24 x 10^3 cfu/ml) were found in the raw milk. After pasteurization, total bacterial counts decreased to 5.2 x 10^3 cfu/ml whereas no yeasts or coliforms were observed. This proves that all yeasts and coliforms present after pasteurization during processing and maturation originated as post-pasteurization contaminants derived from the environment. The major sources of contamination were the hands of the cheddaring workers and the air. After milling of the curd, the total bacterial population present varied between 5.2 – 10.9 x 10^7 cfu/ml. The numbers decreased to 3.6 – 6.4 x 10^4 cfu/ml within 24 hrs after salting, followed by an increase to 1.9 x 10^8 cfu/ml after 12 days. Maximum yeast counts in the cheese for a specific cheese vat were observed after 12 days represented by 1.31 x10^3 cfu/g. No yeasts, however, were detected after 4 months.

   The yeast counts of the three cheeses representing the beginning, middle and end of a specific cheesevat, differed substantially between 2.1 x 10^1 – 2.86 x 10^3 cfu/g. There is no proper explanation fr the fact that yeasts can be present in high numbers in one cheese, and absent in another, manufactured

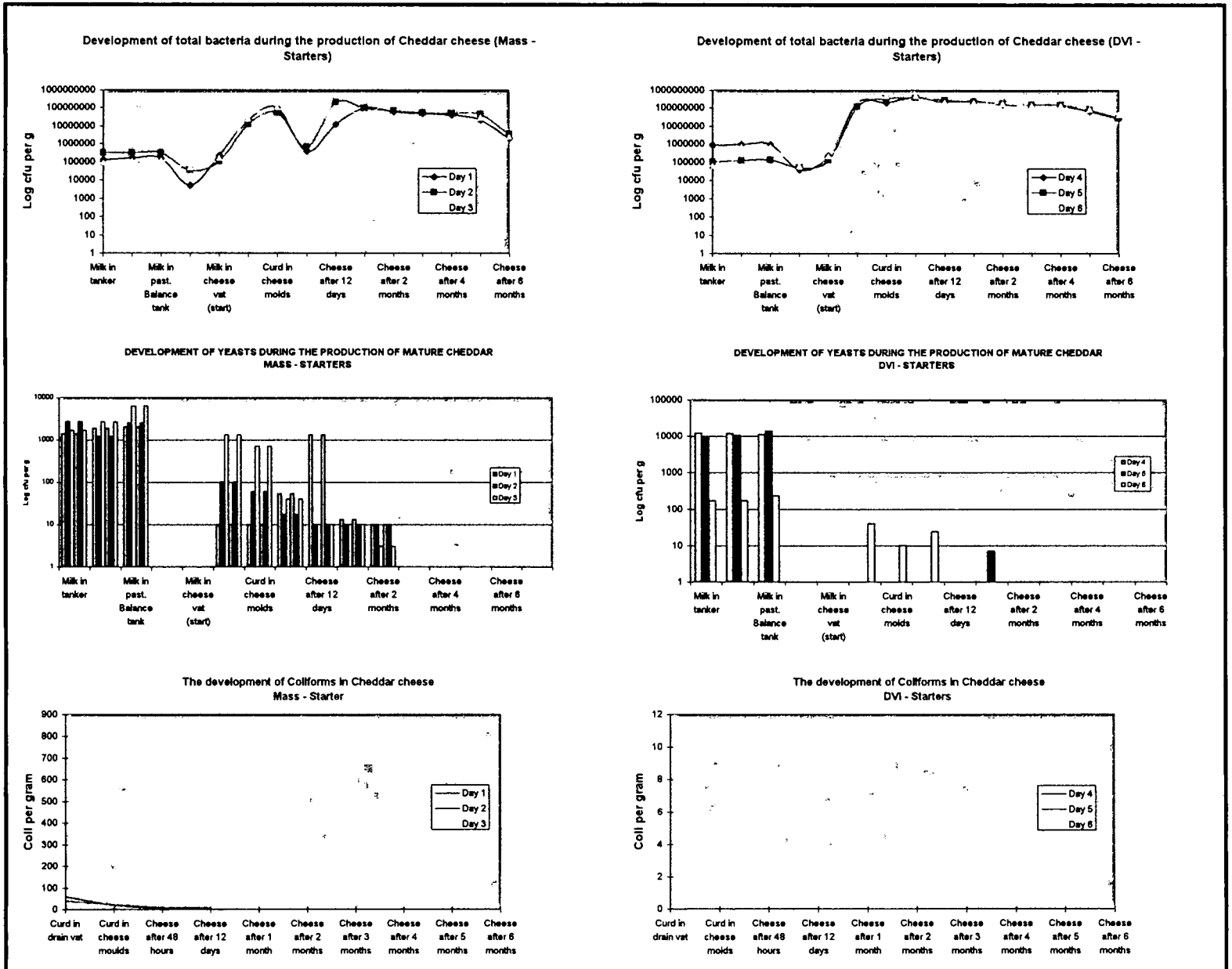

Fig. 2 THE DEVELOPMENT OF MICRO-ORGANISMS DURING THE PRODUTION OF MATURE CHEDDAR CHEESE, BY USING DVI- AND MASS - STARTERS

under the same conditions. Yeast counts as high as 10^7 and 10^8 cfu/g cheese have been reported (Koburger, 1971; Hup and Stadhouers, 1972; Koburger and Farhat, 1975; Henson et al, 1982; Brodsky et al, 1982; Williams, 1986; Jarvis and Shapton, 1986; Banks and Bourd, 1987). Yeasts, however, can be present at high numbers without affecting the quality of the product (Prentice and Brown, 1983). The number of yeasts increased during the salting process of the curd, whereas the total bacteria count decreased.

Coliforms as high as 8.6×10^2 cfu/g were found in the curd and in the drain vat. Numbers, however, decreased to 2.05×10^2 cfu/g after 48 hrs, to 2.7×10^1 cfu/g after 12 days, and less than 10 cfu/g after 1 month. The normal standard for coliforms in Cheddar cheese is less than 100/g (Prentice and Brown, 1983).

b) **DVI starters:** Total bacterial counts of 8.6×10^5 cfu/ml, yeasts (1.3×10^4 cfu/ml) and coliforms (8.8×10^2 cfu/ml) were obtained in the raw milk. After pasteurization, total bacterial counts decreased to 3.8×10^4 cfu/ml whereas no yeasts or coliforms were detected. After milling of the curd, the total bacterial population present was $1.96 - 5.18 \times 10^8$ cfu/ml and the number increased to $3.6 - 4.8 \times 10^8$ cfu/ml within 48 hrs and remained constant during the earlier part of maturation. The salting of the curd had no effect on the progression of the total bacteria as observed during the implementation of bulk starters (Fig 2) the lower salt content in the cheese during the use of DVI starters, might be responsible for this. The growth rate of the total bacteria was much higher with DVI starters compared with the Bulk starters (Fig 2). Yeast populations present in the curd and product were much lower.

The lower number of yeasts present may be attributed to a reduction in environmental contamination. The air contamination (3 colonies/15 min) as

well as the contamination of the equipment, hands etc. were much lower, because of higher focus on hygiene during the use of DVI starters.

The substantial decrease in the total bacterial counts (Fig 1) during the salting process while using mass starter cultures is a typical indication of the interaction between yeasts and bacteria. Despite the normal mutualistic interaction between these organisms that exists in restricted dairy environments, a competitive interaction was induced attributed to the change in environmental conditions by the salt. Yeasts are more resistant to high salt concentrations and therefore, have to be controlled during the production process to prevent overgrowth resulting in spoilage or failure of the starter cultures to initiate cheese development. The cultures, therefore, have a competitive advantage over the contaminating yeasts and coliforms. Coliforms, however, are inhibited by the increase in the amount of organic acids resulting in a decrease in pH (Prentice and Brown, 1983). Prentice and Brown (1983) reported that 96 % of Cheddar cheese samples examined, contained coliforms up to day 224.

4.3 Yeast species isolated during ripening

Twelve different yeast species were represented during the maturation of the cheese (Table 1). High numbers of yeasts were obtained during the processing of the curd (10^3 cfu g^{-1}) and the cheese (10^2 cfu g^{-1}) after 2 months. The number of yeasts corresponds with results obtained by Welthagen and Viljoen (1998) indicating yeasts counts of 10^2 cfu g^{-1} in Cheddar cheese after 51 days of ripening. Yeast species isolated from the cheese comprised of *Trichosporon beigelii* (16), *Debaryomyces hansenii* (9), *Dekkera custersiana* (7), *Candida versatilis* (5), *Rhodotorula minuta* (3), *Rhodotorula mucilaginosa* (3), *Candida rugosa* (3), *Dekkera bruxellensis* (2),

Dekkera anomala (2), *Torulaspora debrueckii* (1), *Rhodotorula glutinis* (1) and strains of *Debaryomyces vanrijiae* (1). The predominant species during the making of matured Cheddar cheese were *Trichosporon beigelii* and *Debaryomyces hansenii*, which frequently exceeded counts of 10^3 cfu g^{-1}, and which varied between species during the processing and ripening stages.

According to Viljoen and Greyling (1995), *Debaryomyces hansenii* and *Candida albidus* were the predominant species frequently associated with Cheddar cheese-making, while Welthagen and Viljoen (1998) reported that *Debaryomyces hansenii, Candida albidus* and *Yarrowia lipolytica* were the predominant species associated with Cheddar cheese-making. In this survey, however, *Thichosporon beigelii* strains represented the largest group of yeasts recovered from the cheese during the ripening. Viljoen and Greyling (1995) reported that *Trichosporon beigelii* represented the second largest group of species during a study to identify the sources of yeast contamination in a cheese factory. The number of yeasts and types, however, may vary between different cheese factories.

The results obtained indicate that contaminating yeasts made a significant contribution to the overall microbial ecology of mature Cheddar cheese, despite being produced from pasteurized milk. The wide variety of yeast species isolated during this study indicates a strong contaminating tendency of matured Cheddar cheese during the manufacturing and ripening processes. Conversely, only a few species proved to be dominant at all times, which may be attributed to the selective environmental influences or competition between species for survival.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Yeasts play a very important role in the ripening and manufacturing of a wide variety of cheeses due to their physiological characteristics. These include the ability to multiply at low temperatures, low moisture content, high salt concentrations, and their resistance towards physico-chemical stresses (Fleet and Mian, 1987; Seiler and Busse, 1990; Warth, 1991; Deak and Beuchat, 1996). Limited studies have been conducted on the composition of yeast flora of matured Cheddar cheese, and their positive and/or detrimental role during the manufacturing and ripening process. The function of yeasts in Cheddar cheese making, remains only partly explored, due to the lack of pre-ordained studies and research in this area which result in partial, occasional and incomplete information which fail to reflect the significant role of yeasts.

1. The incidence of yeasts associated with matured Cheddar cheese

The application of yeast species as part of starter cultures for the manufacturing of matured Cheddar cheese is still the exception rather than the rule. However, yeast contamination occurs during the cheese making process contributing substantially to the final product. Based on the results obtained in this study, we clearly demonstrated the frequent occurrence of yeasts associated with mature Cheddar making. Yeasts are generally heat sensitive and therefore can be assumed to be post-pasteurization contaminants. The hands of workers, the air and equipment were the major sources of contamination after pasteurization. It is therefore important to identify the yeasts that contribute to desirable qualities of dairy products, cheeses in particular with a view to the active use of yeasts as

starter cultures in dairy products. Those yeasts have the potential to be used as starter cultures for cheese maturation. Yeast contaminants have to be controlled, to prevent over-growth of the normal starter cultures, especially when the curd is salted or during a change in temperature. High levels of yeasts can be responsible for off-flavours. Yeasts exhibited strong growth in cheeses during storage. A total of 168 yeast strains were isolated from matured Cheddar cheese and in the vicinity of the processing line. Species of the genera *Debaryomyces, Dekkera* and *Rhodotorula* were the most frequently isolated yeasts. Other genera encountered were *Trichosporon, Candida* and *Torulaspora.* Strains of *Debaryomyces* species proved to be predominant, accounting for 30,36% of the total isolates, which is in agreement with results reported in the literature (de Boer and Kuik, 1987; Welthagen and Viljoen, 1998).

The predominance of *Debaryomyces, Rhodotorula*, *Candida* and *Thrichosporon* species in the present study, could be related to their ability to produce extracellular proteases or lipases or both and the utilization of organic acids.

2. The resistance of dairy yeasts against commercially available cleaning compounds and sanitizers

Yeasts spoilage is recognized as a problem primarily in fermented milk and cheeses (Brocklehurst and Lund, 1985; Engel, 1988; Fleet, 1990; Rohm et. al., 1990 a, b; Rohm et. al., 1992; Tudor and Board, 1993). Therefore, it is very important to control the progression of yeasts by using good cleaning compounds and sanitizers.

The dominant yeasts associated with matured Cheddar cheese were screened for their resistance against nine commercial cleaning compounds and sanitizers currently implemented in the dairy industry. *Candida rugosa* proved to be the most resistant to all the compounds, while *Dekkera custersiana* was the most vulnerable against the cleaning compounds. The "Peroxide Based Acid Sanitizer"

is selected as the most effective inhibitor against most of the isolates causing almost no growth after 45-60 min. None of the compounds used, however, were able to kill all the yeasts, within 60 minutes. Different isolates of the yeast species responded differently to the cleaning compounds and sanitizers. To maintain proper manufacturing hygiene, it is important to determine the resistance of the spoilage yeasts during processing and in the product. Based on the results obtained in this study, it proved necessary to implement a special cleaning program including at least two different cleaning compounds or sanitizers containing different active components to control yeast contamination. The contact time and concentration of the product implemented, must be selected according to the specific equipment washed and according to the suppliers specifications.

3. Key properties of yeasts isolated during the manufacturing and ripening of matured Cheddar cheese

All the yeasts investigated have the ability to grow at low temperatures (7°C) and high salt concentrations (15%). According to the results obtained, there was no substantial difference between the progression of the yeasts grown at 7°C and 15°C. The counts, however, decreased linearly when NaCl contents increased. Therefore, it is impossible to control the growth of undesired yeast by adjustment of physiochemical parameters like NaCl, pH and temperature only. None of the strains were able to ferment lactose. Thirty-nine of the 151 yeast strains examined for hydrolysis of protein were positive, while 21 strains were positive for the hydrolysis of fat (Tributyrin) and 27 strains positive for the hydrolysis of fat (Rhodamine).

4. The interaction between yeasts and bacteria during the manufacturing of mature Cheddar Cheeses

In this study we monitored all the changes in chemical composition, the pH, a_w, % fat, % lactose, % salt and % moisture content at intervals from day 1 to 180

days during the ripening of matured Cheddar cheese. The number of yeasts, total bacteria and coliforms were also monitored during the ripening of the cheese. Yeasts were present at large numbers without affecting the quality of the cheese. The yeast numbers increased during the salting process of the curd, while the total bacteria count decreased probably due to the intolerance against higher salt concentrations (Fleet, 1992). The results obtained indicate that slower growth of the starter bacteria (slower acid production) in the drainvat and after the salting process influenced the growth of yeast contaminants and coliforms positively. Coliforms and yeasts are generally heat sensitive and therefore can be considered as post-pasteurization contaminants. In this study we proved that yeasts have to be controlled during the production process, to prevent over-growth of the starter bacteria resulting in spoilage. No visible yeasts were detected after four months and no coliforms (< 10/g) after 1 month of ripening.

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CHAPTER 7

SUMMARY

Yeasts play an important role in dairy products causing spoilage or contribute positively to the ripening of some cheeses due to their lipolytic and proteolytic activities and the ability to grow at low temperatures, pH and water activity, and high salt concentrations. Despite various factors affecting the survival and growth of yeasts in cheese, the food commodity is considered as a potential habitat of yeasts. Cheese renders an ideal medium for the survival and progression of yeasts due to the availability of the necessary nutrients. A historical review of the incidence of yeasts, their interaction with bacteria and their properties, during the manufacturing and ripening of matured Cheddar cheese is given in Chapter 1. The history of Cheddar cheese making, the fermentation of milk during cheese making, different starter cultures and methods of application and occurrence of yeasts are highlighted.

In Chapter 2 a survey was undertaken with the objective of identifying the predominant yeast contaminants associated with matured Cheddar cheese production. A total of 168 yeasts strains, representing 12 different species, isolated from matured Cheddar cheese and the immediate environment were isolated. Once the identity of the yeast flora associated with the cheese had been established, the survey was extended to determine the sources of yeast infection during the processing of the matured Cheddar cheese. The results obtained, showed that yeasts contributed substantially to the deterioration as well as the ripening of the matured cheese, especially when stored under environmental conditions exhibiting, low temperature, water activity and pH values and high salt concentrations.

In Chapter 3 seven of the most dominant yeasts frequently associated with dairy products are screened for their resistance against nine commercial cleaning compounds and sanitizers commonly used in the dairy industry. None of the compounds used, however, were able to sufficiently kill the yeasts, within 60 minutes.

In Chapter 4 the influence of temperature and NaCl on the dominant yeasts isolated in a cheese factory, during the manufacturing and ripening of matured Cheddar cheese was examined. Relevant yeast species were screened for lipolytic and proteolytic activities, and their ability to ferment lactose. The chemical and physical characterition including pH, fat content, water activity, salt and moisture were determined and its relevance to the growth and survival of yeasts studied.

The interaction between yeasts, coliforms and total bacteria during the manufacturing and ripening of matured Cheddar cheese, by using DVI- and Mass- starters was studied in Chapter 5. Results obtained indicate that yeast contaminants have to be controlled during the salting process to prevent overgrowth of the starter cultures. During the salting stage, the curd is prone to yeast contamination and may result in excessive loads due to the competitive growth advantage of the species and the simultaneous bacterial inhibition. This may contribute to product spoilage or stimulate the growth of the starters adding to the formation of aroma components.