SURVIVAL AND GROWTH OF *BACILLUS CEREUS* DURING GOUDA CHEESE MANUFACTURING

BY

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I declare that the dissertation herewith submitted for the degree of MInst Agrar Food Processing at the University of Pretoria, has not been submitted by me for a degree at any other university or institution of higher education.

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OPSOMMING

Titel: Oorlewing en groei van *Bacillus cereus* tydens Gouda-kaas vervaardiging

deur

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Die hoof doelstelling van die studie was om die oorlewing en groei van *Bacillus cereus* te bepaal gedurende die vervaardiging van Gouda-tipe kaas. Die kaas is in 'n toetsaanleg vervaardig met melk wat vooraf kunsmatig gekontamineer is met 10^2 *B. cereus* selle per ml melk. *B. cereus* getalle is bepaal deur uitplating op *B. cereus* selektiewe medium en melksuurbakteriegetalle is bepaal op lakaat-agar en MRS-agar (Man-Rogosa-Sharpe). Monsters vir mikrobiologiese analise is geneem van die melk voor stremming, van die wrongel gedurende die snyproses, by die stadium waar die helfte van die wei verwyder is, by die finale weiverwydering, toe die wrongel in die vorms geplaas is, van die kaas na samepersing en na insouting, na 1 week, na 2 weke, na 4 weke en na 6 weke. Chemiese analise is na 6 weke op die kaas gedoen.

B. cereus spore is geaktiveer deur pasteurisasie by 63°C vir 30 min. Hulle het ontkiem en die vegetatiewe selle het gegroei gedurende die vervaardigingsproses en het maksimum waardes van ongeveer 10^4 kve per gram wrongel bereik by die vorming-stadium (ongeveer 3,5 h na stremming). Na samepersing (ongeveer 16 h na strem) het die lewensvatbare selle verminder na minder as 1×10^1 kve/g. Na insouting (40 h na stremming) kon geen *B. cereus* opgespoor word nie. Op hierdie stadium het die kondisies in die kaas, veral die verlaagde voginhoud en a_w, laer Eh, hoë soutkonsentrasie, uitgeputte laktosevoorraad gekombineer met hoë suurheid, die groei van *B. cereus* geïnhibeer. *B. cereus* het geen effek gehad op die groei van melksuurbakterieë gedurende die kaasvervaardingsproses nie. Melksuurbakterieë het vermeerder van 10^7 tot 10^9 kve per gram wrongel gedurende die kaasvervaardigisngproses en het vir 6 weke redelik konstant gebly op 10^9 kve/g.

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ABSTRACT

Title: Survival and growth of *Bacillus cereus* during Gouda cheese manufacturing

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The primary objective of this study was to determine the survival and growth of *B. cereus* during manufacturing of Gouda type cheese. The cheese was prepared in the pilot plant from pasteurised milk artificially contaminated with *B. cereus* spores to give a final concentration of 10^2 *B. cereus* spores per ml of cheese milk. *B. cereus* was enumerated by surface plating on *B.cereus* selective media and lactic acid bacteria were enumerated on lactic agar and MRS agar (Man-Rogosa-Sharpe). Samples were taken for microbiological analysis of the milk before renneting, curd at cutting, at half whey removal, at final whey removal, at hooping of the curd, the cheese after pressing, after brining, after 1 week, after 2 weeks, after 4 weeks and after 6 weeks. Chemical analysis were done on cheese after 6 weeks.

Spores of *B. cereus* were activated by pasteurisation at 63°C for 30 min. The spores germinated into vegetative cells which grew and reached a maximum of approximately 10^4 cfu/g at the hooping stage (about 3,5 h after renneting). After pressing (approximately 16 h after renneting) the viable cells were reduced to less than 1×10^1 cfu per gram. After brining (40 h after renneting) *B. cereus* was not detected. At this stage the conditions of the cheese , particularly lower moisture content and a_w, lower Eh, high salt content, depleted lactose content combined with high acidity inhibited the growth of *B. cereus*. *B. cereus* did not affect the growth of lactic acid bacteria during cheese manufacturing. Lactic acid bacteria grew from 10^7 to 10^9 cfu per gram of curd during cheese manufacturing and stayed fairly constant at about 10^9 for 6 weeks.

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CHAPTER 1

INTRODUCTION

Gouda is a semi-hard cheese variety made from cow's milk and originated from Holland (Kosikowski, 1978). Milk used for cheese-making should be of a good bacteriological quality to avoid undesirable fermentation and enzymic reactions and without any substances that inhibit or interfere with the growth of the starter bacteria (Chapman & Sharpe, 1981). Milk is an excellent medium for microbial growth. Milk as produced in the alveoli of a healthy cow is free from microorganisms, but the farm environment makes it impossible to exclude bacteria during milking (Andersson, Ronner & Granum, 1995). The bacterial load of raw milk ranges from 10^3 per ml when hygiene is good, to 10^7 per ml when hygiene is poor (Chapman & Sharpe, 1981). The bacteria consist of psychrotrophs, small numbers of lactic acid bacteria, spore forming Gram-positive rods, coryneform bacteria, micrococci and coliforms (Chapman & Sharpe, 1981). Psychrotrophs will continue to multiply during transport and storage of milk. Pasteurisation (72°C for 15 s) of the milk prior to Gouda cheese manufacture will reduce the microbial load, but the thermoduric microorganisms with psychrotrophic properties survive (Andersson *et al*, 1995) and of importance to this research are the species *Bacillus cereus*.

Occurrence of *B. cereus* in milk has been reported since 1916, and this bacterium is a common contaminant of raw milk produced on some dairy farms (Ahmed, Moustafa & March, 1983). It can also be found in large numbers in dairy products (Ahmed *et al*, 1983). In a survey by Wong, Chen & Chen (1988) on dairy products, 52% of ice creams, 35% of soft ice creams, 29% of milk powders, 17% of fermented milks, and 2% of pasteurised milks and fruit flavoured milks were found to be contaminated with *B. cereus*. The concern about it's presence in dairy products is that it may cause certain defects in the products or produce toxins and thus cause food poisoning.

At counts above 2×10^5 /ml *B. cereus* causes off flavours such as unclean, fruity, bitter, putrid, rancid and yeasty (Meer, Baker, Bodyfelt & Griffiths, 1991). When growth continues, the product shows the defect sweet curdling in homogenised low-pasteurised

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milk and bitty cream in low-temperature pasteurised milk. It has been estimated that more than 25% of the shelf-life problems encountered with pasteurised milk are due to the proliferation of *Bacillus* species (Griffiths, 1992). Although low numbers of *B. cereus* in foods pose no direct health hazard, if these foods are mishandled, growth of this organism might result in a direct health hazard. Large numbers of *B. cereus* produce enterotoxin in the food before it is consumed and cause illness (Macrae, Robinson & Sadler, 1993).

There are two types of food poisonings caused by *B.cereus*, namely the emetic and the diarrheal type (Wong *et al*,1988; In't Veld, Soentaro & Notermans, 1993; Granum, 1994). A large number of viable cells of *B. cereus* is required to cause illness; numbers in excess of 10^5 - 10^6 per gram have been encountered in food suspected of causing illness (Ahmed *et al*, 1983). Foodborne illness caused by *B. cereus* is considered as a food intoxication rather than a food infection, so a significant level of growth by the organism would be required to synthesize the necessary level of extracellular toxin (Ahmed *et al,* 1983).

The objective of this study was to determine the survival and growth of *B.cereus* during manufacture of Gouda type cheese.

1.1 Statement of the problem

Under modern processing conditions, milk and milk products can be successfully kept almost free from contamination after pasteurisation. Although this has significantly improved the keeping quality of packed products in general, micro-organisms surviving pasteurisation now determine the keeping quality of the products. An example of such a thermoduric micro-organism is *B. cereus* which has been found to be more difficult to control in the dairy industry. Apart from it's presence in almost all milk received at dairy factories, it's spores are very hydrophobic and will attach to the surfaces of the pipelines in dairy plants, where they can multiply quite rapidly and even sporulate (Andersson *et al*, 1995). Since the spores survive pasteurisation, *B. cereus* is often setting the limits of keeping quality of milk and milk products. Apart from its role in the spoilage of heat-treated milk and several other dairy products, the production of toxins by some strains pose a risk to the safety of dairy products.

1.2 Objectives of the project

A considerable amount of research has been carried out on the growth and survival of *B. cereus* in milk and during yoghurt manufacture (Wong & Chen, 1988; Driessen, 1992) but little if any research has been done on growth of *B. cereus* during Gouda cheese manufacturing.

The primary objective of the project was

To determine the growth and survival of *B.cereus* during manufacture of Gouda type cheese.

The secondary objectives were

To determine whether *B. cereus* would affect the growth of lactic acid bacteria during Gouda cheese manufacturing.

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CHAPTER 2

LITERATURE REVIEW

2.1 *BACILLUS CEREUS*

2.1.1 Occurrence

B. cereus is impossible to eliminate in the food industry and has been found to be associated with various forms of food spoilage and food poisoning (Kramer & Gilbert, 1989). It can be introduced into milk supplies from water, udder and teat surfaces, or from soil and milk stone deposits, gaskets and processing equipment (Meer *et al*, 1991; Kalogoridou-Vassiladou & Tsiantas, 1992). Soil has been shown to contain 10^5 - 10^6 spores per gram (Andersson *et al*, 1995). *B. cereus* may also be isolated from rice, dairy products, meat, spices and eggs (Kramer & Gilbert, 1989). The bacterium survives different environmental stresses which makes it very difficult for the food industry to exclude *B. cereus* from their products.

Milk was known to be contaminated with *B. cereus* before the organism's pathogenicity was established (Johnson, 1984). It causes a defect known as "bitty" cream or sweet curdling of milk (Overcast & Atmaram, 1974; Johnson, 1984), the source of the milk contamination having been traced to mastitic cows and to cans of milk that were allowed to stand after emptying (Johnson, 1984). Spores of *B. cereus* may occasionally survive UHT treatment and may be one of the main spoilage organisms if the milk is not recontaminated after pasteurisation (Stadhouders & Beumer, 1993). *B. cereus* has also been reported to cause defects in yoghurt (Driessen, 1992). According to Driessen (1992) all spores of *B. cereus* found in milk products originated from raw milk, contamination with spores was higher in winter than in late summer. Davies & Wilkinson (1973) have pointed out that "new" products such as puddings, presumably the shelf stable variety, may present problems with *B. cereus*. Outbreaks of foodborne illnes involving vanilla slices and macaroni and cheese dishes were thought to have been caused by contamination from the milk powder used in their preparation (Jonhson, 1984).

Bacillus spp. comprised 90% of rice paddy soil bacteria (Asanuma, Takana & Yatazawa, 1979). The high association of the emetic syndrome with rice dishes is thus not surprising since *B. cereus* is usually present in rice. The organism has also been isolated from other Oriental dishes and ingredients, such as egg rolls and vegetables (Sly & Ross, 1982) and therefore rice should not be viewed as the only potential contributor to diarrheal toxins in Oriental foods.

Dried products like legumes have also been shown to be contaminated with *B. cereus*. The organism has been isolated from cooked Mexican-style beans (Johnson, 1984). Blakey & Priest (1980), pointed out the potential hazard of soaking dried legumes before cooking. This practice is necessary for a satisfactory product; however, growth of indigenous *B. cereus* does occur and numbers can reach hazardous levels. Numerous spices have been shown to contain *B. cereus* and it has been reported that heavy use of spices in Hungarian cooking may have contributed to the high reported incidence of *B. cereus* illness outbreaks in that country (Johnson, 1984). Garlic extracts, however, have been shown to inhibit growth of *B. cereus* (Johnson, 1984).

2.1.2 General characteristics

Certainly a major concern to food microbiologists is the destruction of microorganisms. As *B. cereus* forms spores most of the concern revolves about destruction of the spores rather than the vegetative cells.

2.1.2.1 Morphological and biochemical characteristics

B. cereus is classified in the family Bacillaceae. Cells are large rods (0,5 - 2,5 x 1,2 - 10,0 μm) with rounded or squared ends and are often arranged in pairs or chains (Stanier, Adelberg & Ingraham, 1979). *B. cereus* cells are Gram-positive and motile by peritrichous flagella (Frankland & Frankland, 1974). The spores are very hydrophobic and they have a suitable spore morphology which makes them easily attach to pipes and surfaces (Andersson *et al*, 1995). The spores are covered with long appendages (Figure 1) which promotes adhesion and this compounds the problem of contamination from equipment and utensils.

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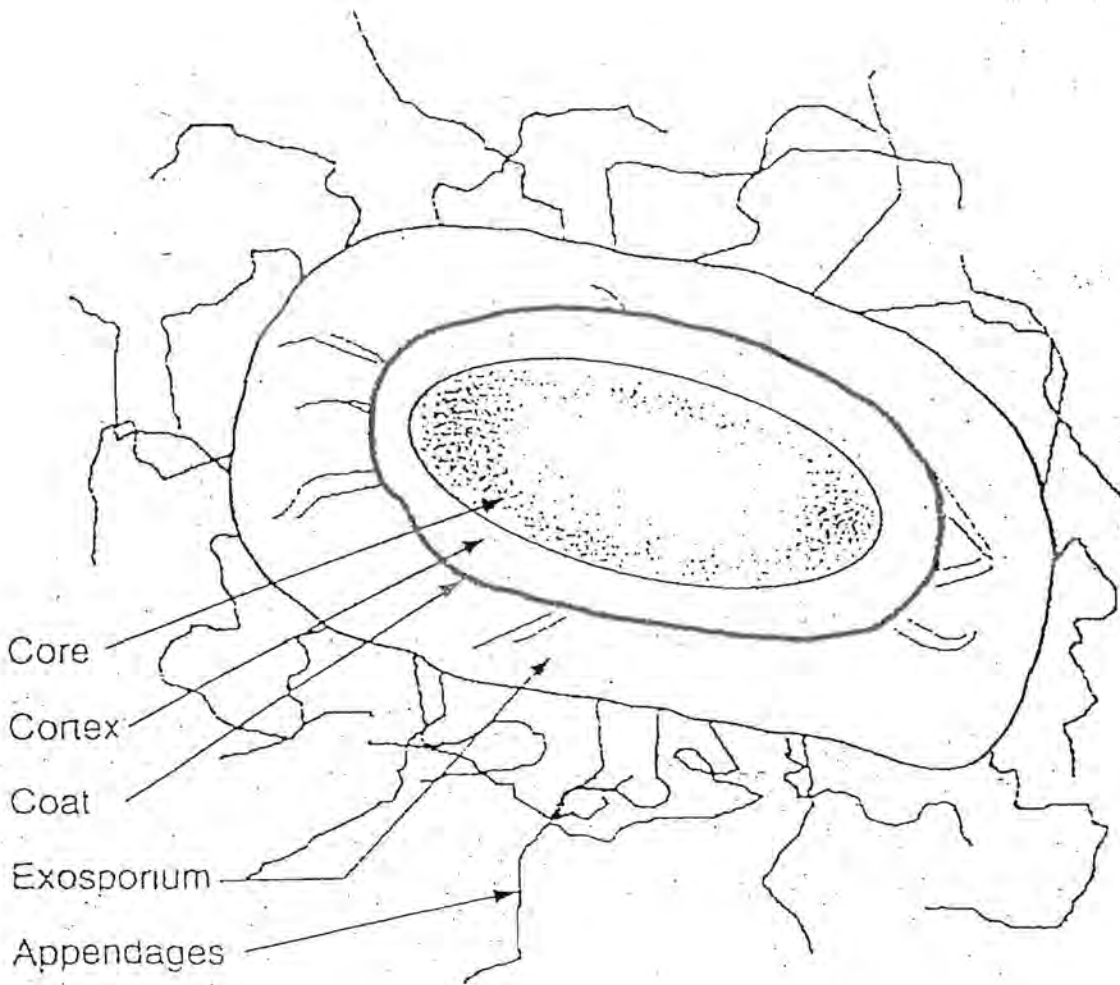
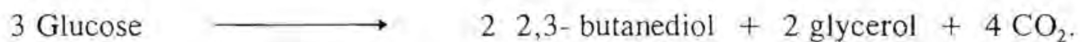


Figure 1 The different layers of *B. cereus* spores (Andersson *et al*, 1995)

Growth has been demonstrated over the pH range 4.9 to 9.3 (Jay, 1992). *Bacillus cereus* can grow at minimum temperatures of 4-5°C and maximum temperatures of 48-50°C. It grows well on *B. cereus* selective agar containing egg yolk emulsion and forms rough, dry colonies with a ring of dense precipitate (Mossel, Koopman & Jongerius, 1967). *B. cereus* is catalase positive, ferments glucose with the production of 2,3-butanediol, glycerol and carbon dioxide (Frankland & Frankland, 1974; Stanier *et al*, 1979). Fermentation can be represented as:



$$3 \text{ Glucose} \longrightarrow 2 \text{ 2,3-butanediol} + 2 \text{ glycerol} + 4 CO_2.$$

B. cereus reduces nitrate to nitrite and is lecithinase positive. It does not ferment mannitol, xylose or arabinose. It has been shown to be inhibited by high levels of spoilage microorganisms, nisin, 0.02% sorbic acid and 0.40% potassium sorbate (Steele & Stiles, 1981). Antibiotics effective against *B.cereus* are aureomycin, dihydrostreptomycin, terramycin, bacitracin, oxytetracycline, chloramphenicol, and gentamycin (Johnson, 1984). Slight inhibition was observed with neomycin, cloxacillin, ampicillin and penicillin but there was no inhibition of *B. cereus* by polymyxin (Johnson, 1984)

2.1.2.2 Isolation and identification of *B.cereus*

Isolation of *B. cereus* from food involves plating on differential and selective media. The most commonly used media formulation include: mannitol-egg yolk-polymyxin (MYP) agar, KG agar, blood agar, polymyxin-pyruvate-egg yolk mannitol bromothymol blue agar (PEMBA) (Meer *et al*, 1991). Several of these formulas use polymyxin as an inhibitory agent for competitive organisms and are designed to use the mannitol negative, lecithin hydrolyzing nature of *B. cereus* for differentiation. Typical *B. cereus* colonies on PEMBA plates are peacock blue with a surrounding zone of precipitation, while colonies on the plates that contain bromocresol purple are mauve in color with a zone of precipitation (Szabo, Todd & Rayman, 1984).

2.1.2.3 Heat resistance of *B. cereus* spores

Various theories have been proposed for the physiological mechanism for heat resistance, including several mechanisms based on partial dehydration, hydrostatic pressure applied to the protoplast by the cortex and an expansive cortex (Johnson, Nelson & Busta, 1982; Marquis, Sim & Shin, 1994). Shehata & Collins (1972) measured the heat resistance in sterilised milk of psychrotrophic spores which had been isolated from pasteurised milk (Table 1).

Table 1 Thermal resistance data for spores of selected psychrotrophic bacilli (Meer *et al*, 1991)

Organism	D-Value at				z-value
	85°C	90°C	92.5°C	95°C	
			(min)		°C
B. cereus	18.5	5.8	3.4	1.8	9.4
B. pumilus	16.5	5.1	2.7	1.4	9.7
B. laterosporus	20.5	6.5	3.9	2.1	10.1
Bacillus DPL	18.9	6.6	4.2	2.4	11.0

D-values at 90°C varied between 5.1 and 6.6 min. Meer *et al* (1991) reported that D-values for *B. cereus* strains isolated from a number of dairy products were in the range of 2 to 5.4 min at 90°C. The heat resistance of *Bacillus* spores may be affected by the composition of the suspending media (Shehata & Collins, 1972). In particular lipids have been shown to confer a protective effect on spores (Meer *et al*, 1991).

2.1.2.4 Germination and growth of *B. cereus*

Most of *B. cereus* and related species isolated from dairy products were able to grow below 10°C but strains from the dairy environment and from paperboard mills had higher minimum growth temperatures. According to Meer *et al* (1991), the conceptional minimum growth temperature for *B. cereus* averaged 15°C. There was a correlation between minimum growth temperature and fatty acid composition of *B. cereus*.

Milk contains bacterial spores that exhibit a range of genetically predetermined germination rates (Meer *et al*, 1991). These can be differentiated into fast, intermediate and slow germinating types. It has been shown that a factor is present in milk that promotes germination of *B. cereus* (Meer *et al*, 1991). The germination factor was thought to be an organic compound with a molecular weight below 200 kDa and which was active at concentrations of 10 μg/ml or less. There is evidence that the germinant was associated with small peptides and may in fact be hippuric acid.

According to Chung & Cannon (1971), strains of psychrotrophic *Bacillus* species examined had a lag phase of 8 to 14 d and a generation time of 22 to 26 h at 7°C. Typical growth parameters for psychrotrophic bacilli isolated from dairy products are shown in Table 2.

Table 2 Growth parameters of *Bacillus* spp. grown in milk (Meer *et al*, 1991)

Species	Temperature of growth					
	2°C		6°C		10°C	
	Lag[a]	Gen[b]	Lag	Gen	Lag	Gen
B. cereus[c]	NG[d]	NG	78	17	24	4
B. circulans[e]	60	28	33	11	31	7
B. mycoides[f]	NG	NG	92	22	33	4

[a] Lag time in h

[b] Generation time in h.

[c] Mean for four strains

[d] No growth after 21 d incubation

[e] Mean of four strains

[f] Mean for two strains

2.1.2.5 Effect of heating on spore germination

Heating influences germination in two ways. Firstly, by a direct effect upon the spore and secondly by affecting the medium within which the spore may germinate. The first of these concerns the activation temperature. It has long been known that the germination of most spores is activated by a period of sub-lethal heat treatment (Setlow, 1994). Davies & Wilkinson (1973) reported that little activation of the spores occurred at the normal pasteurisation temperature (71,7°C for 15 s) used for commercial pasteurisation. However an elevation of this temperature significantly increased the level of germination. The spores survived pasteurisation temperatures of 80°C for 10 min (Stone & Rowlands 1952). Figure 2 shows that for pasteurisation temperatures above 55°C the extent of germination of spores activated at 75°C for 30 min increased markedly to a maximum at 65-75°C, a range within which the temperature for commercial HTST pasteurisation unfortunately falls (Davies & Wilkinson, 1973).

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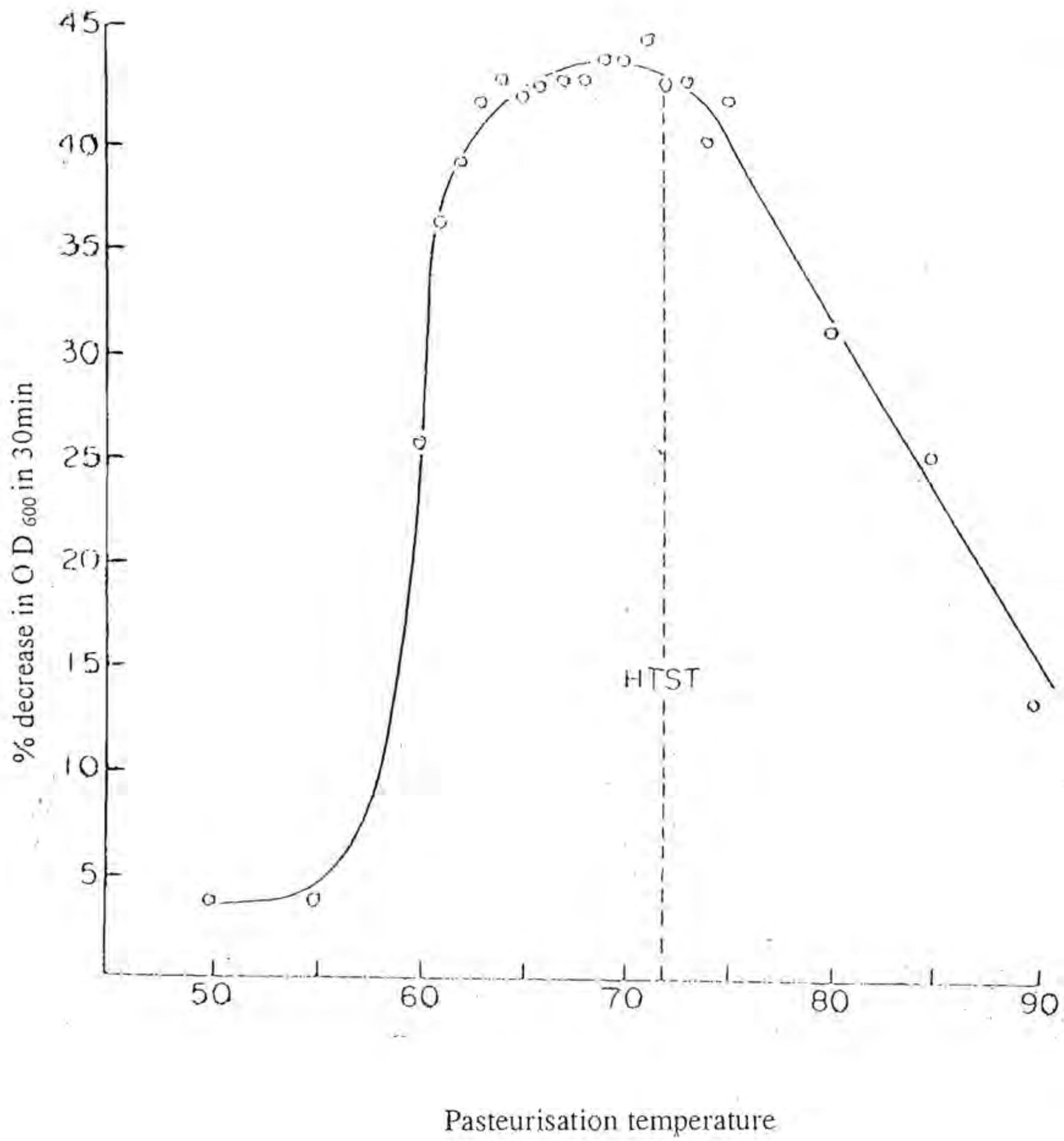

Pasteurisation temperature

Figure 2 Germination of spores of *B. cereus* T16 previously activated in water at 75°C for 30 min and then pasteurised in milk for 15 s over a range of temperatures (Davies & Wilkinson, 1973)

2.1.2.6 Effect of source on germination of *B.cereus* spores

Meer *et al* (1991) reported that the rate at which bacterial spores germinated depended on their specific source. For instance spores isolated from soil and faeces tended to be fast germinators, while those isolated from raw milk and soiled milking equipment appeared to be slower germinators. Davies (1977) suggested that large numbers of *B. cereus* spores in pasteurised milk can be derived as dairy processing plant contaminants, rather than the common assumption that spores are always introduced at the dairy farm. Davies (1977) also noted that *B. cereus* spores isolated from raw milk were slower germinators than isolates from pasteurised milk. This could be indicative that *B. cereus* isolates from pasteurised milk were most likely derived from the dairy plant (i.e. post process contaminants).

2.1.3 Toxin production

B. cereus produces seven types of toxins which can be divided into four groups of toxins namely enterotoxins, haemolysins, phospholipase C and emetic toxin (Table 3). Six of these toxins are produced and secreted by cells during vegetative growth. The seventh toxin is the emetic toxin which is most probably produced from components in foods during growth of *B cereus*. According to In't Veld *et al,* (1993) and Granum (1994), *B. cereus* is known to cause two distinct food-borne illness syndromes, one is caused by diarrhoeal toxins, while the other is characterised by the emetic toxins.

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Table 3 The toxins of *Bacillus cereus* (Granum, 1994)

Toxin	**Molecular weight (mature molecule)**	**Signal peptide size**	**Characteristics**
Enterotoxin	≈40 kDa	Not detected	Membrane damage, heat labile, susceptible to proteolysis
Haemolysins Cereolysin	≈56 kDa	Not detected	Thiol activated, heat labile, little susceptible to proteoloysis
Haemolysin II	≈ 30 kDa	Not detected	Heat labile, susceptible to proteolysis
Sphingomyelinase	34 kDa	27 amino acids	Stable matallo-enzyme (Mg^{2+}), heamolysin
Phospholipase C Phosphatidylinositol hydrolase (PIH)	34 kDa	31 amino acids	Non-metallo enzyme, sequence homology to other pro- and eukaryotic enzymes
Phosphatidylcholine hydrolase (PCH)	27 kDa	38 amino acids	Stable metallo-enzyme (Zn^{2+}, Ca^{2+})
Emetic toxin	5-7 kDa	Not detected	Heat stable to 121°C, non-metabolic product, lipid (?)

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2.1.3.1 Haemolysin

Three different haemolysins are known from *B. cereus* and have been characterised quite well. The cereolysin is a thiol activated protein that cross-react with streptolysin-O and has a calculated molecular weight of about 55 kDa (518 amino acids) (Granum, 1994). It is heat labile but relatively little susceptible to proteolysis (Granum, 1994). It is responsible for the main haemolysis of *B. cereus*, is lethal when injected in mice and is inhibited by cholesterol and serum. The secondary haemolysin has a molecular weight of about 30 kDa, is heat labile and is easily degraded by proteolytic enzymes (Granum, 1994). It is not inhibited by cholesterol.

Sphingomyelinase is well characterised and is most probably responsible for heat stable haemolysis (Tomita, Taguchi & Ikezawa, 1991). It is a metallo-enzyme (Mg^{2+}) and works best as a haemolysin through the so-called hot-cold incubation (37-4°C) (Granum, 1994).

2.1.3.2 Phospholipase C

Bacillus cereus produces three different types of phospholipase C (Granum, 1994). They have all been cloned and are well characterised. Phosphatidylinositol hydrolyse (PIH) is a protein that specifically hydrolyse phosphatidylinositol (PI) and PI-glycan containing membrane anchors, which are important structural components of one class of membrane proteins. The enzyme is synthesized as a 329 amino acids protein with a signal peptide of 31 amino acids resulting in a mature enzyme of about 34 kDa (Granum, 1994).

Phospatidylcholine hydrolase (PCH) hydrolyses phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Granum, 1994). PCH is synthesized as a 283 amino acid protein leaving 245 amino acids in the mature protein.

Sphingomyelinase (SM) is larger with 333 and 306 amino acids in the synthesized and mature protein respectively. It needs Mg^{2+} and is inhibited by Zn^{2+} and Ca^{2+}.

2.1.3.3 Diarrheal toxin

The diarrheal toxin is heat labile and sensitive to trypsin and ponase (Jay, 1992). It is produced over a pH range of 6,0 to 8,5. Growth and toxin production have been demonstrated at 4°C (Jay, 1992). The syndrome is mild and it develops within 8 to 16 h and lasts for 6 to 12 h (Jay, 1992; Granum, 1994). Symptoms consist of nausea (but vomiting is rare), cramplike abdominal pains, tenesmus and watery stool. Fever is generally absent (Jay, 1992). The symptoms of this toxin are similar to those caused by *Clostridium perfrigens*. The foods involved in the diarrheal illness have been quite varied, ranging from vegetables and salads to meat dishes and casseroles. The enterotoxin probably consists of three proteins (Mr 43000, 39000 and 38000 respectively). The enterotoxin is produced during the log phase of the growth cycle and it is relatively unstable to heat (destroyed at 55°C for 20 min)

2.1.3.4 Emetic toxin

The emetic toxin differs from the diarrheal toxin by being heat and pH stable. The toxin is sensitive to trypsin and pepsin (Jay, 1992; Granum, 1994). The emetic toxin is produced over the range 15 to 50°C with an optimum between 35 to 40° C (Jay, 1992). The enterotoxin is unstable at pH lower than pH 4 and is produced in the presence or absence of oxygen. The pH range is pH 2-11 (Goepfert, Spira, Glatz & Kim, 1972; Granum, 1994). Intoxication is rapid and occurs within 1-5 h, with nausea and vomiting, occasionally accompanied by diarrhea which lasts for less than 24 h (Granum, 1994). This form of *B. cereus* food poisoning is more severe and acute than the diarrheal form. The incubation period and symptoms mimic those of *Staphylococcus aureus* food poisoning. Illness may be due to toxin ingestion or toxin produced by ingested live cells (Granum, 1994). Emetic syndrome is often associated with fried or boiled rice dishes, pasteurised cream, spaghetti, mashed potatoes and vegetable sprouts (Jay, 1992). The number of organisms necessary to cause illness are 2 x10^9/g (Jay, 1992). The emetic toxin is a small protein (Mr < 10 000) and is stable to heating at 126 °C for 90 min.

2.1.3.5 Comparison of emetic and diarrheal type strains

According to Johnson (1984), there was little difference between growth rates of *B. cereus* strains isolated from diarrheal or emetic outbreaks, or strains naturally occurring in rice. Germination was more extensive for diarrheal strains than emetic strains in trypticase soy broth and in rice (Johnson, 1984). According to Johnson (1984), antibiotic sensitivity and fatty acid

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composition of emetic and diarrheal *B. cereus* were similar. Certain diarrheal strains could ferment mannose or salicin while no emetic strains had these abilities. According to Johnson (1984), the symptoms produced by the strains were related to the food involved, rather than the strain. Johnson (1984) reported that a distinction between diarrheal and emetic types could be made with an API system and numerical methods of identification. *B. cereus* strains isolated from food involved in food borne illness outbreaks lacked the ability to hydrolyse starch. According to Johnson (1984), there was no difference in biochemical properties, antibiotic sensitivity, cerecin production, or phage sensitivity between emetic and diarrheal types.

2.1.4 Toxin detection methods

Several investigators of *B. cereus* food poisoning have used various methods to detect both the diarrheal and emetic toxins. According to Meer *et al* (1991), a toxin detection kit based on RPLA is available to assay for *B. cereus*. The method was used to investigate three outbreaks of food intoxications caused by psychrotrophic *B. cereus*. The foods implicated in each outbreak were homemade vegetable pie, codfish and pasteurised milk. These outbreaks caused diarrhea and abdominal cramps. The *B. cereus* isolated from the vegetable pie and codfish were positive for enterotoxin production as determined by the RPLA test kit. The outbreak involving pasteurised milk caused nausea and vomiting, but the bacilli isolated were negative for enterotoxin production as determined by the RPLA test. *B. cereus* was recovered from all three foods at levels of 0.2 to 0.4 x 10^6 cfu/g. Additionally, this study reported that about 25% of psychrotrophic strains of *B. cereus* isolated from commercial pasteurised milk samples were enterotoxin positive (Meer *et al*, 1991)

The RPLA test kit was also used by Griffiths (1990) to determine the toxigenic activity of psychrotrophic *B. cereus* isolated from raw or pasteurised milks. Of these *B. cereus* strains, 85% demonstrated toxin production when grown in brain heart infusion (BHI) broth at 25°C. Four strains exhibiting high levels of toxin under these growth conditions were tested for their ability to synthesize toxin in sterile reconstituted skim milk at 6, 10, 15 and 21°C. Toxin production increased with increasing temperature, but the toxin was produced during growth in milk at all temperatures under stagnant conditions.

2.1.5 Public health problems

Kramer & Gilbert (1989) summarised a number of foodborne outbreaks caused by *B. cereus* (Table 4).

Table 4 Examples of food poisoning outbreaks where *B. cereus* was the probable etiological agent (Kramer & Gilbert, 1989)

Country	Type of food	*B. cereus* isolated /g or /ml
Germany	Meat-balls	Not recorded
Sweden outbreaks	Meats and meat products	Not recorded
Norway	"Yellow pudding desserts"	1.3×10^7
Italy	Chicken soup	6.0×10^7
Norway	Vanilla sauce	$2.5 \times 10^7 - 1.1 \times 10^8$
Netherlands	Mashed potatoes, meats, rice dishes, puddings, soups	$5 \times 10^5 - 2 \times 10^8$
Hungary	Vegetable soup	Not recorded
Hungary outbreaks	Sausage, vegetable dishes, cream pastries, soups	$3.6 \times 10^4 - 9.5 \times 10^8$
USA	Meat- loaf	10^7
UK	Rice dishes	$10^6 - 10^7$

The discrepancy between cases of food poisoning and reported cases involving dairy products could be due to the mild and short illness periods experienced by individuals (not outbreaks) who might attribute the sickness to other foods consumed with the cheese. Rangasamy *et al* (1994) found *B. cereus* in various Victorian milk and dairy products (Table 5). Ahmed *et al* (1983) reported the presence of *B. cereus* in 9% of raw milk, 35% pasteurised milk, 14% Cheddar cheese and 48% of ice cream in the United States of America. Apart from public health problems, *Bacillus cereus* is associated with sweet curdling of milk and bitty cream (Stone & Rowlands, 1952). Ingestion of food containing 10^4 - 10^7 cells or spores per gram may cause food poisoning (Granum, 1994). Therefore surveillance of *B. cereus* in cheese

preparations will remain significant as there is no process control that can completely avoid its presence. Preservatives like $NaNO_3$ are utilised in some countries, however its use is forbidden in other countries. In some countries such as the United States of America lysozyme has been tried as an inhibitor for *B.cereus* (Johnson *et al*, 1990).

Table 5 Occurrence of *B. cereus* in milk and dairy products manufactured in Victoria (Rangasamy *et al*, 1994)

Dairy product	No of samples	No of samples with *B. cereus*		Range of counts (cfu/ml or g)
		KG agar	PEMBA agar	
Raw milk	24	6	3	<10
Pasteurised milk	12	4	2	<10-28
Yoghurt	15	3	4	60-430
Cheddar cheese	10	4	2	30-100
UHT milk	10	0	0	-
Milk powder	10	3	1	30-960
Ice cream	10	4	4	30-160
Total	91	24	16	-

2.1.6 Prevention of growth of *B. cereus*

The widespread distribution of *B. cereus*, the ability of spores to survive long-term storage in dried products, and the thermal resistance of spores help to explain the wide variety of foods that have been implicated in *B. cereus* food borne illness outbreaks. The fact that the organism naturally exists in numerous foods prevents the establishment of rigorous specifications that would preclude the presence of *B. cereus* in ingredients (Johnson, 1984; Macrae *et al*, 1993). Therefore, one should assume that *B. cereus* is always present and should take measures to prevent growth during food handling.

Bryan (1972) has reviewed factors that contribute to food borne illness outbreaks. Inadequate cooling was the most important factor leading to disease outbreaks. Inadequate cooking, preparation of food far in advance, and infected personnel were also important factors. Control

of *B. cereus* food poisoning should rely on the prevention of spore germination and prevention of multiplication of vegetative cells in prepared food (Macrae *et al*, 1993). Therefore foods should be cooled rapidly to 4°C or maintained above 63°C, and they should be reheated thoroughly to a temperature above 74°C before serving (Johnson, 1984; Macrae *et al*, 1993). These recommendations are applicable to any food product in which growth of *B. cereus* is possible.

In milk many methods have been investigated as means of controlling the growth of *B. cereus*. However, due to strain variations, no method can be considered ideal (Griffiths, 1992). The most appropriate action still remains good husbandry practices to minimise entrance of this ubiquitous organism into the milk supply and to keep milk as cold as possible during milk production and storage.

2.1.7 Legislation

According to the South African regulation relating to Herbs and Spices (R1468) of the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972) any foodstuff containing *B. cereus* in 20 g of sample is deemed harmful or injurious to human health. Notermans, Dufrenne, Teuns, Beumer, Giffel & Weem (1997) noted that for most European countries numbers of $10^4 - 10^5$ per ml or gram of a product at the expiry date are considered to be the critical limits of acceptance of a particular food. For epidemiological surveys any counts greater than 10^5 per gram would indicate *B. cereus* as the etiological agent of outbreaks of food poisoning. For cheese products mere presence of *B. cereus* is sufficient cause of concern and levels above 10^5 per gram indicate bad manufacturing practices (Johnson *et al*, 1990)

2.2 STARTER CULTURES

The preservation of food by fermentation is one of the oldest methods of food preservation known to mankind (Scott, 1986). The lactic acid fermentation is widely used during the manufacture of fermented dairy products. Such a fermentation process is the result of the presence of microorganisms known as starter cultures. The most important group of starter bacteria is the lactic acid bacteria which include the genera *Streptococcus, Leuconostoc* and *Lactobacillus* (Tamime, 1981). In the dairy industry, interest is mainly focused on the genera *Streptococcus, Lactobacillus* and *Leuconostoc*. The genus *Streptococcus* is widely

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used in the cheese industry and examples from this genus are *S. lactis (Lactococcus lactis)*, *S. lactis* subsp. *diacetylactis (Lactococcus lactis* subsp. *lactis* biovar *diacetylactis)*, *S. cremoris (Lactococcus lactis* subsp. *cremoris)* and *S. thermophilus*. These organisms are homofermentative, producing only lactic acid from glucose, and with the exception of *S. thermophilus*, are classified as mesophilic bacteria (Tamime, 1981). Of the genus *Leuconostoc*, only *L. cremoris* and *L. dextranicum* are associated with dairy starter cultures. They are heterofermentative organisms capable of producing lactic acid, diacetyl, carbon dioxide and aroma compounds such as ethanol and acetic acid from glucose (Tamime, 1981). The genus *Lactobacillus* is divided into three main groups; *Thermobacterium, Streptobacterium* and *Betabacterium*. The former two sub-groups are homofermentative and the species used in the dairy industry are L. *bulgaricus*, *L. lactis, L. acidophilus, L. helveticus, L. casei and L. plantarum* (Tamime, 1981). However, various strains of microorganisms can be used as starter cultures in the dairy industry.

2.2.1 Classification of dairy starter cultures

Starter cultures may consist of either single strains, multiple strains of the same species, or a mixture of different species or genera; thus giving the dairy industry the opportunity to manufacture different types of fermented dairy products. Table 6 illustrates the application of those microorganisms which are required for the production of cheese.

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Table 6 Lactic acid bacteria employed as starter cultures for cheese-making (Tamime, 1981)

Bacteria	Examples of usage
Mesophilic starters[a]	
L. lactis subsp. *cremoris* and *L. lactis* subsp. *lactis*	Hard-pressed cheese, e.g. Cheddar, Gouda; Mould-ripened, e.g. Stilton
L. lactis subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp.	Soft, ripened, e.g. Camembert, Feta, many other types
L. lactis subsp. *cremoris* *L. lactis* subsp. *lactis* biovar *diacetylactis* *Leuconostoc cremoris*	Soft, unripened, e.g. Coulommier, Cottage cheese, Quarg, Cream cheese
Thermophilic starters[b]	
S. thermophilus with *L. helveticus*, *L. lactis* or *L. bulgaricus*	Swiss-type, e.g. Emmenthal; Italian, very hard, e.g. Parmesan; Semi-soft, smear types e.g. Limburger
Mixed starters	
L. lactis, *S. thermophilus* or *S. faecalis* and *L. bulgaricus*	Italian pasta filata type, e.g. Mozzarella, Provolone

[a] Optimum growth temperature 20-30°C

[b] Optimum growth temperature 37-45°C

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Mesophilic lactic starter cultures (optimum temperature 20 - 30°C) are widely used. In the cheese industry they are divided into single, multiple or mixed strains. Single strain starters consist only of one type of organism, but in practise they are rarely used. Mixed strain starter cultures consist of known numbers of single strains, so that the starter can be used for an extended period of time during the cheese-making season (Kosikowski, 1978; Tamime, 1981). A mixed strain starter is a combination of *L. lactis, L. lactis* subsp. *cremoris* and the gas and aroma producing mesophilic lactic acid bacteria, *L. lactis* subsp. *lactis* biovar *diacetylactis, Leuconostoc cremoris* and/or *L. dextranicum* (Kosikowski, 1978; Tamime, 1981). The lactic acid bacteria ferment lactose to form mainly lactic acid. The mixed mesophilic cultures are also called the flavour producers because of their ability to produce diacetyl, the flavour compound from citrate fermentation (Cogan, 1995). Depending on their citrate-utilising component (Cit^+) mixed mesophilic cultures are assigned to the following types: D type with Cit^+ L*actococcus* species as the only flavour producer, L type with Cit^+ *Leuconostoc* as the only flavour producer or DL types with both flavour producers present. O or N type cultures are those lacking flavour producers (Cogan, Peitersen & Sellars, 1991). The classification of dairy starter cultures is summarised in (Figure 3). Successful production of cheese relies completely on choosing the right organism (Tamime, 1981).

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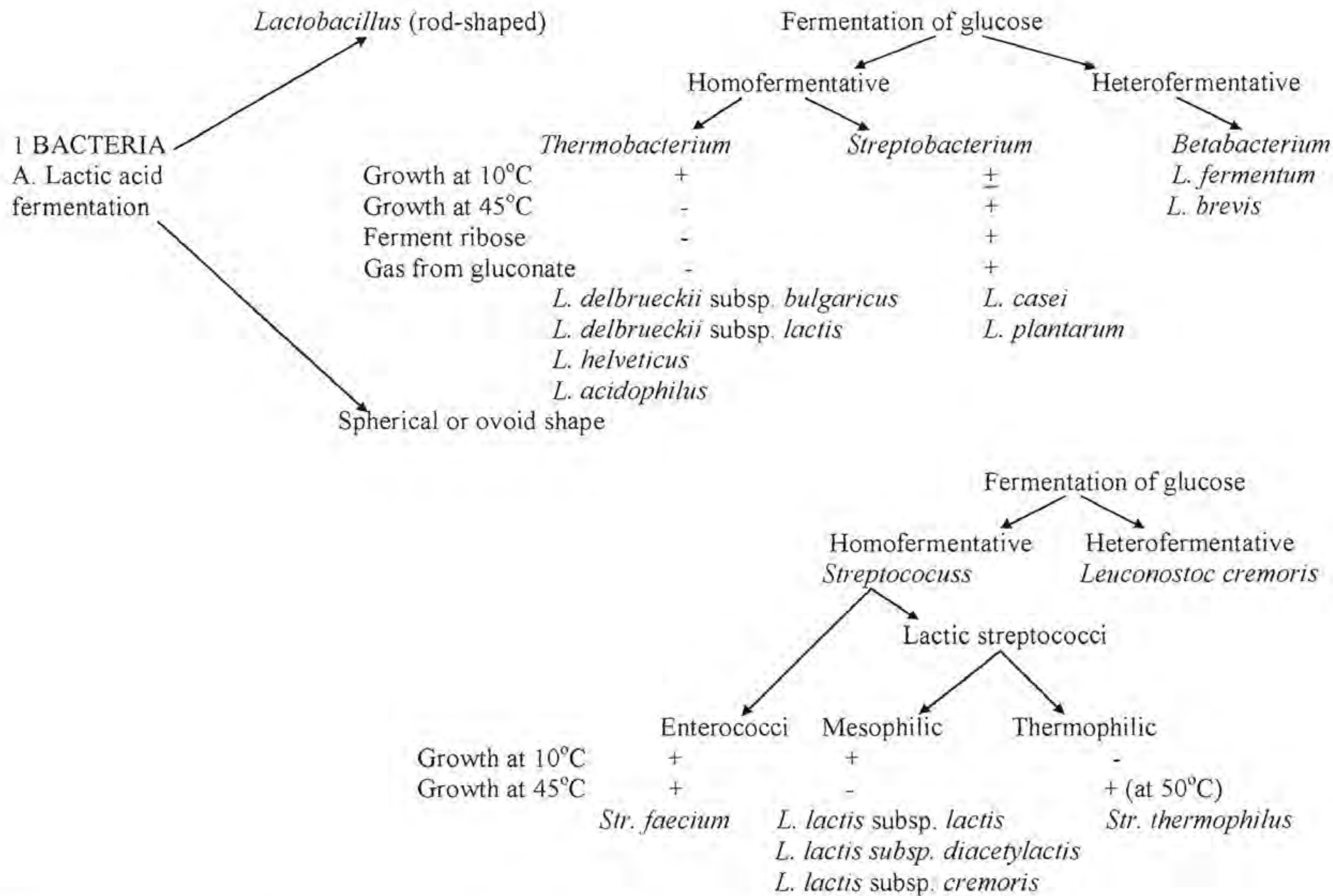

Figure 3 Classification and differentiation of dairy starter cultures (Tamime, 1981)

2.2.2 Fermentation of lactose by lactic acid bacteria (LAB)

Fermentation of lactose to lactate by lactic acid bacteria is the major metabolic reaction occurring in cheese manufacture. Addition of starter culture to the milk results in an initial population of ~ 1 x 10^7 cells per ml and they increase to ~ 1 x 10^9 cells per gram of cheese (Cogan *et al*, 1991). Such large populations ensure rapid metabolism of residual lactose and result in lactate concentration of 100-170 mmol per kg of cheese. The rate of lactose fermentation depends, however, on the manner of salting the cheese. In brine-salted cheese (e.g. Dutch and Swiss types), diffusion of salt into the cheese is relatively slow and lactose metabolism occurs over an extended period. This contrasts with dry-salted cheeses (e.g. Cheddar) in which the salt is mixed intimately with the curd and, especially at high concentrations, retards metabolism of residual lactose by the starter culture (Cogan, 1995).

The lactic acid bacteria share the property of producing lactic acid from hexoses. They are divided into two groups based on end products of glucose metabolism. Those that produce lactic acid as the major or sole product of glucose fermentation are the homofermentative types (Figure 4 - 1A). Those lactics that produce equal molar amounts of lactate, carbon dioxide and ethanol from hexoses are designated heterofermentatives (Figure 4 - 1B). All members of the genera *Pediococcus*, *Sreptococcus*, *Lactococcus*, *Vagococcus* and some of the lactobacilli are homofermentative, while *Leuconostoc* spp., as well as some lactobacilli are heterofermentative.

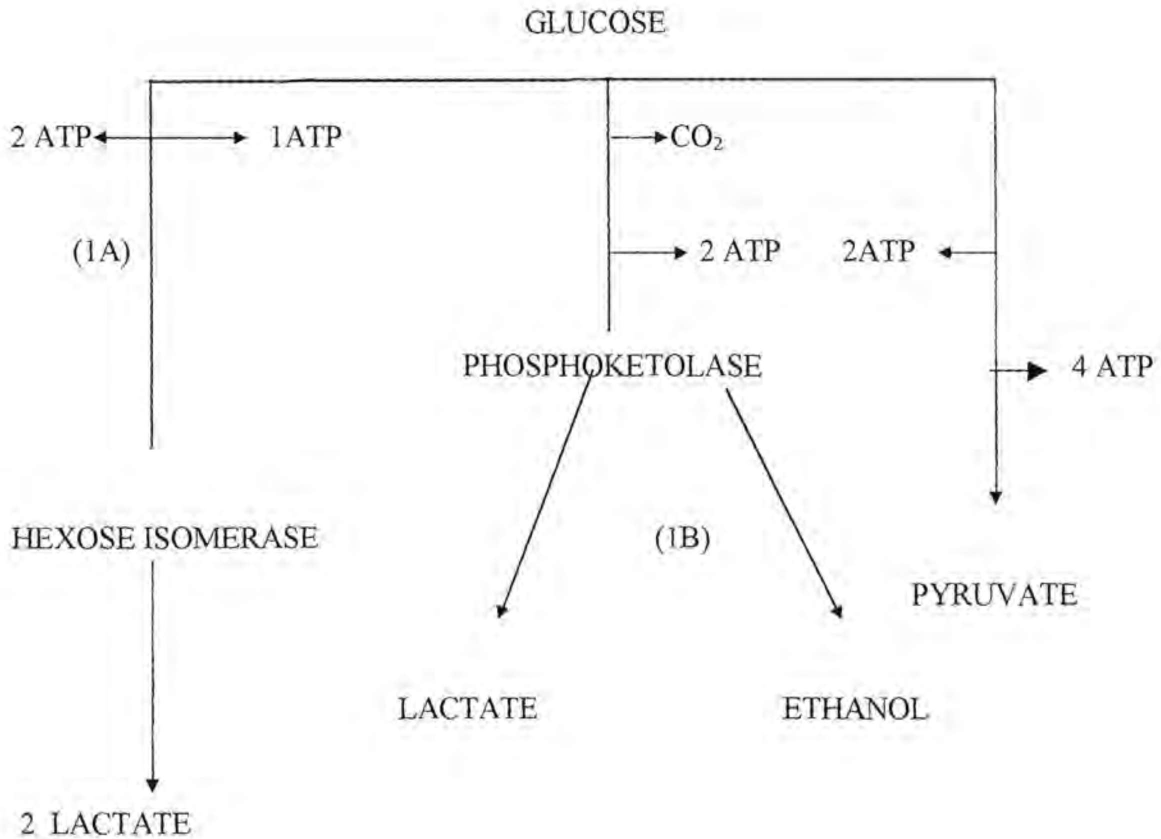

Figure 4. Generalised pathways for the production of some fermentation products from glucose by : (1A) Homofermentative lactics; (1B): heterofermentative lactics (Jay, 1992).

2.2.3 Factors affecting growth of starter cultures

For many years the problem of bacteriophage has been the most serious one confronting the cheesemaker because of the economic losses it entails (Cogan *et al*, 1991). Phage multiplication decreases the ability of the culture to produce acid, which in turn decreases the expulsion of whey, i.e. synerisis of the curd and in the extreme cases complete inhibition of acid production or "dead vats" occur (Cogan *et al*, 1991). Extreme care must be exercised during the transfer of cultures to maintain them free from bacteriophage contamination. Removing the calcium ions from the culture media, or binding them by addition of phosphates, oxalates or citrates is an effective way to control this contamination (Rosenthal, 1991). A number of other factors can be responsible for the inhibition of starter activity in milk (Table 7).

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Table 7 Factors responsible for inhibition of starter cultures (Rosenthal, 1991)

Natural inhibitors in milk	The content varies with the individual animal and can be destroyed by heat
Mastitis milk	Phagocytosis of the starter organism by the leucocytes
Free fatty acids in rancid milk	Correct handling of raw milk. Rejection of rancid milk
Antibiotic residues in milk	Rejection of antibiotic milk
Bactericide residues	Negligent operation may leave sanitizer residues in milk. Quaternary ammonium compounds have the greatest inhibiting effect, even at concentration of 3 mg/l, chlorine compounds are inhibiting above 5 mg/l, while iodophors show no effect up to 50 mg/l.
Bacteriophage contamination	Prevention of phage contamination

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2.2.4 Effect of lactic acid bacteria on survival and growth of *B. cereus*

Lactic acid bacteria are useful for the enrichment of flavour, palatability, therapeutic value and their ability to suppress many undesirable microorganisms and pathogens (Roberts & Skinner, 1983). From historical times production of lactic and acetic acid and the consequent reduction in pH has been utilised to preserve or prolong the shelf life of many foods including cheese. Research has also unearthed some other inhibitory substances produced such as hydrogen peroxide, alcohol, acetaldehyde, diacetyl and bacteriocins like nisin. However, *B. cereus* produces nisinase which can counter the effect of nisin if sufficient quantities are produced (Goepfert *et al,* 1992). Beuchat, Clavero & Jaquette (1997) demonstrated that 1 μg nisin per ml of beef gravy was lethal to *B. cereus*, the effect being greater on vegetative cells than spores. *B. cereus* is a poor competitor and it grows well only when other microorganisms have been reduced by heat or pasteurisation. The antagonism may be derived from substrate depletion and the reduction of oxidation-reduction potential (Eh). In the absence of competing flora *B. cereus* can grow to numbers that can cause food poisoning (Goepfert *et al,* 1992).

Lodi & Malaspina (1992) studied the interaction of *B. cereus* and the lactic acid bacteria in fresh cheeses (Figure 5). Lactic acid bacteria affected *B. cereus* differently in three types of fresh cheeses which were inoculated with varying quantities of vegetative cells. In fresh cheese type A, *B. cereus* decreased rapidly and disappeared within a week; on the contrary when inoculated into fresh cheeses type B and type C, *B. cereus* decreased only slightly. Strains of lactic acid bacteria which yield substances or an environment inhibiting *B. cereus* when used to produce dairy products continue to be the subject of research.

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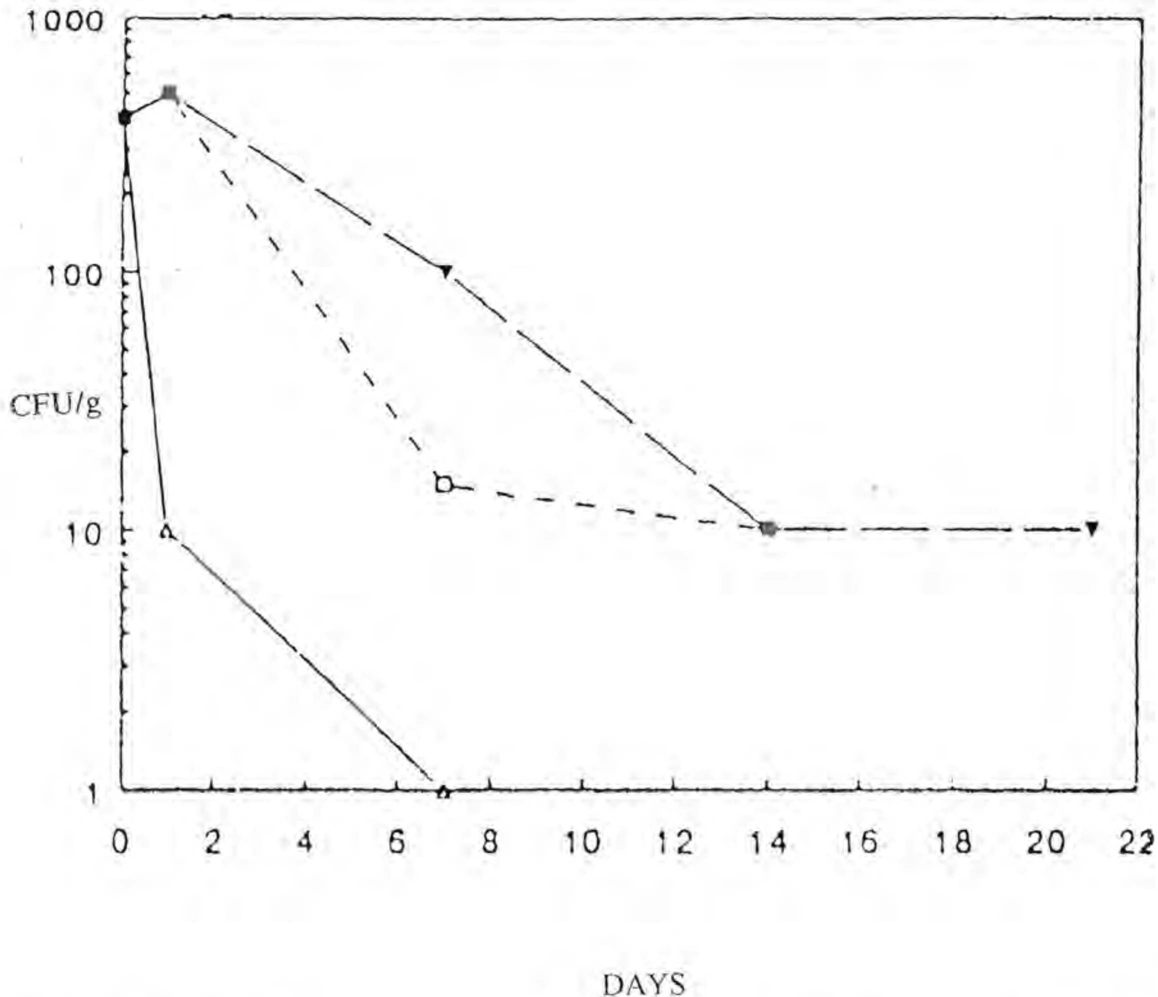

DAYS

Figure 5 Growth of *Bacillus cereus* artificially inoculated in three types of cheeses (–Δ– type A, –□– type B, –▼– type C) (Lodi & Malaspina, 1992).

2.2.5 Antimicrobial substances produced by lactic acid bacteria

Lactic acid bacteria are capable of producing substances other than organic acids (lactate and acetate) that are antagonistic toward other microorganisms (Daeschel, 1989). These substances are produced in much smaller amounts and include hydrogen peroxide, diacetyl, bacteriocins and secondary reaction products such as hypothiocyanate generated by the action of lacto-peroxidase on hydrogen peroxide and thiocynate.

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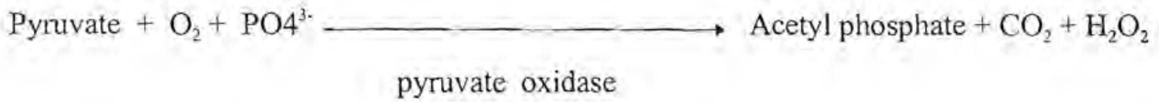
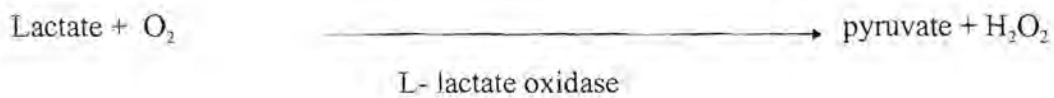
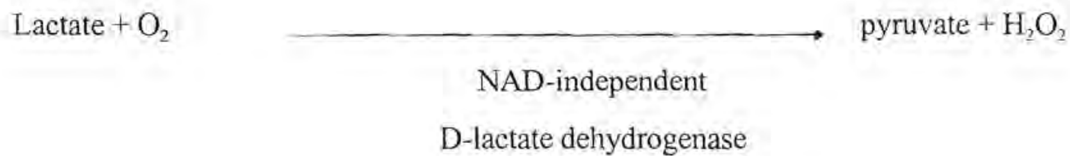
2.2.5.1 Hydrogen peroxide

The lactobacilli have the ability to generate hydrogen peroxide in the presence of oxygen through the action of flavoprotein oxidases or NADH oxidases (Figure 6).







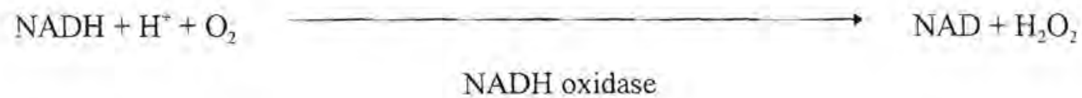


Figure 6 Mechanisms of hydrogen peroxide generation by lactic acid bacteria (Daeschel, 1989).

According to Kandler & Weiss (1986), accumulation of hydrogen peroxide in growth media can occur because lactobacilli do not possess the catalase enzyme. In raw milk hydrogen peroxide generated by lactic acid bacteria can react with endogenous thiocynate (catalysed by lactoperoxidase) to form a "Lactoperoxidase antibacterial system" which can be useful in extending the shelf life of unrefrigerated raw milk (Bjork, 1993).

2.2.5.2 Diacetyl

Diacetyl is best known for the buttery aroma that it imparts to cultured dairy products. This substance is produced by the genera *Lactococcus, Leuconostoc, Lactobacillus* and *Pediococcus* (Daeschel, 1989). Apart from being a flavour compound, Jay (1982) showed that 300 µg/ml diacetyl was inhibitory to non-lactic acid bacteria that were Gram-positive. Lactic acid bacteria were not inhibited at a concentration of 350 µg/ml (Jay, 1982). Although diacetyl is generally recognised as safe (GRAS), its utility as food preservative is limited because of the relatively large amounts needed to provide preservation.

2.2.5.3 Bacteriocins

Bacteriocins are a type of antimicrobial substances, protein in nature, which produce inter- and intra-species effects (Daeschel, 1989). A number of bacteriocins have been produced by the genera *Lactobacillus, Pediococcus* and *Lactococcus*. Bacteriocins and bacteriocin-like substances produced by strains of lactobacilli and lactococci are mainly active against Gram-positive organisms (Spahr & Url, 1994). Some of these substances are Bavaricin A produced by *Lactobacillus bavaricus* M140 and Plantaricin UGI produced by *Lactobacillus plantarum* UGI (Enan, El-Essawy, Uyttendale & Debevere, 1996). Nisin is produced by some strains of *Lactococcus lactis*. Its antimicrobial effect is directed against the bacterial cytoplasmic membrane. It is particularly effective against spore forming organisms e.g *Bacillus* and *Clostridium* because of their vulnerable cytoplasmic membrane (Spahr & Url, 1994).

The minimum inhibitory concentration of nisin against these bacteria varies from 0,25 to 500 IU/g when measured on an agar diffusion test. The most important application in dairy products is in processed cheese to inhibit growth of anaerobic sporeformers. Concentrations of 100-200 IU per gram of cheese are used for this purpose (Spahr & Url, 1994).

2.3 GOUDA CHEESE MANUFACTURING

2.3.1 Overview of Gouda cheese manufacture

Gouda, like other cheese varieties, is made from cow's milk, using rennet and starter cultures. The variety of cheese produced is determined by the type of milk used, the type of starter (lactic acid bacteria) responsible for the development of acid, characteristic features and flavour. The starter culture used for Gouda cheese is a mixture of streptococci, leuconostoc and lactobacilli (Kosikowski, 1978; Tamime, 1981). Typical Gouda cheese has a distinct yellow colour, waxy body, with a mild nutty flavour and a few holes evenly distributed throughout the cheese (Kosikowski, 1978).

2.3.2 Basic steps in the manufacture of Gouda cheese

The basic procedure for cheese-making is shown in Figure 7.

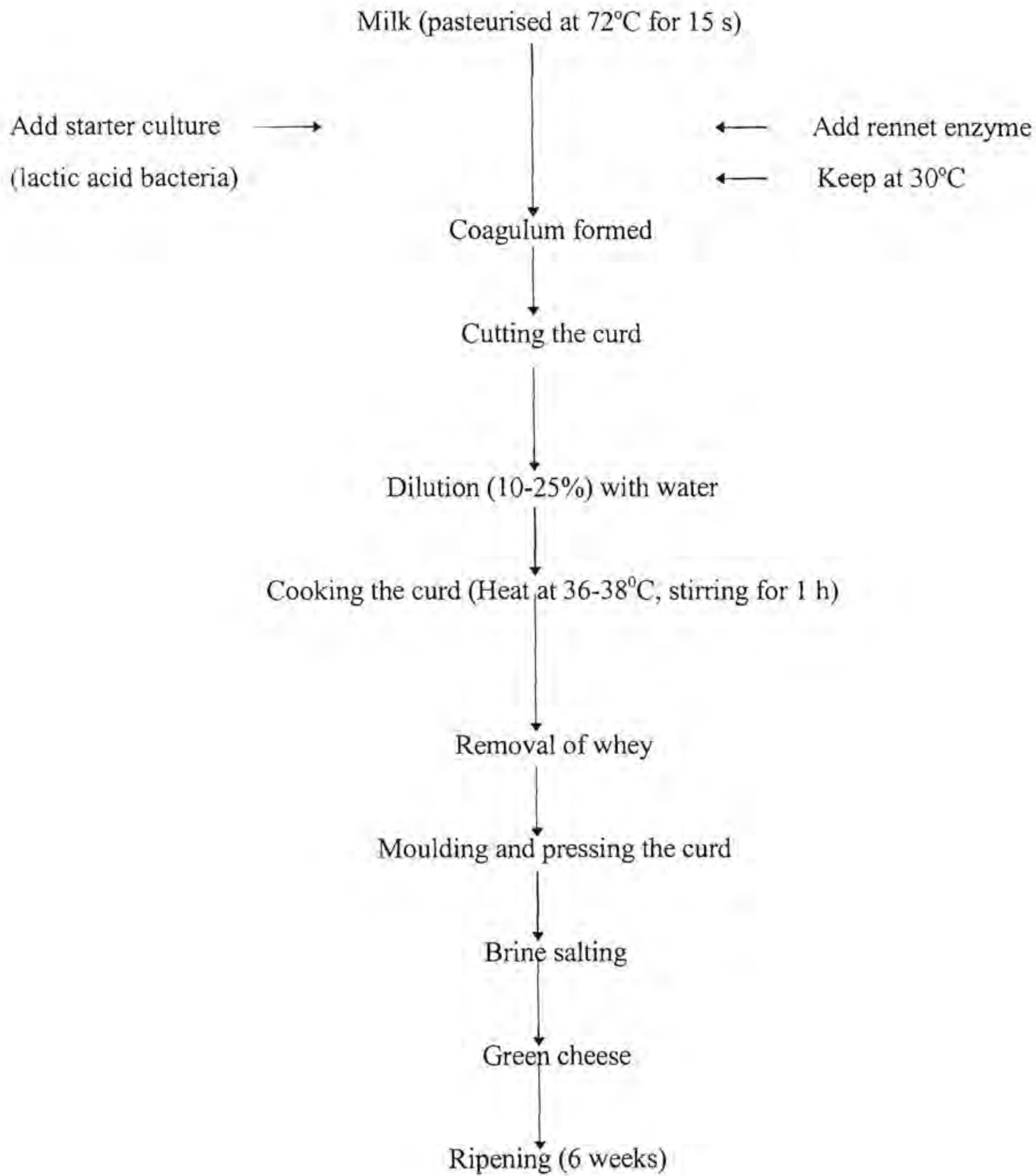


Figure 7 Flow diagram for cheese manufacture

2.3.2.1 Coagulation (Curd formation)

Pasteurised milk is inoculated with a starter culture and rennet added to form a curd. Renneting of milk takes place in three phases:

a) An enzymic, destabilising phase where the protective colloidal nature of k-casein is destroyed and para-k-casein is formed within the casein micelles (Tamime, 1981). This phase can occur at refrigeration temperatures.

b) A non-enzymic, coagulation phase which can proceed only at higher temperatures.

c) A mainly proteolytic phase which takes place around pH 5.2 - 5.8 and includes the breakdown of milk proteins to peptides. The last phase is essential for cheese-ripening. Peptides formed are utilised and further degraded by the starter cultures (Kosikowski, 1978; Tamime, 1981).

2.3.2.2 Cooking (scalding) and whey removal

The curd is cut into small particles. These are lightly scalded by removing about 10-25% of whey and replacing it with an equal amount of water at 50 - 60°C. Addition of water raises the vat temperature and also dilutes the lactose content of the curd. The growth of lactic acid bacteria is thus decreased resulting in Gouda cheese with higher pH. High cooking temperatures help in lowering the moisture content and proteins are changed resulting in a more elastic, spongy type of curd.

The starter bacteria are trapped in the coagulum at rennetting and most are retained in the curd particles after cutting. The trapped lactic acid bacteria continue to ferment lactose within the curd particles after cutting. The acid produced will also accelerate expulsion of moisture. The extent of acid production by lactic acid bacteria has a marked effect on the chemical and physical composition of the curd and control of acid development is important (Kanawjia, Toyoda, Kitamura & Ahiko, 1991; Johnson, Steele, Broadbent & Weimer, 1998).

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2.3.2.3 Salting

After the whey has been drained off, curds are filled into moulds and pressed lightly. The cheese is then salted in a brine bath. After brining the cheese is dried to form a coat and ripened for about six weeks. Lactic acid bacteria vary in their tolerance to salt. Strains of *L. lactis* subsp. *cremoris* are inhibited by 2% salt but *L. lactis* subsp. *lactis* can withstand up to 4% salt. Suppression by salt of the growth of undesirable proteolytic, lipolytic and other spoilage bacteria, such as butyric acid bacteria is of importance in Gouda cheese where low levels of lactic acid are produced during curdmaking (Tamime, 1981; Scott, 1986).

Brining of the cheese serves not only as a means to get salt into the cheese, but also to cool the cheese and delay gas formation (Johnson *et al*, 1998). In most cheese varieties, salt concentration attain levels of 1.6 - 2.5% in the total cheese which would not affect most of the pathogenic bacteria in cheese, but it is realised that salt is dissolved in the aqueous phase of the cheese only, the actual site of bacterial growth. In the aqueous phase salt reach levels of 2.2 - 6.5% and these will at least slow down the growth rate of most bacteria and may even have a lethal effect on the more sensitive ones (Spahr & Url, 1994).

2.3.2.4 Ripening

The cheese taken out of the press has a mild acid taste and aroma but does not possess the characteristic cheese flavour (Kanawjia *et al*, 1991). During ripening, characteristic changes take place regarding the body, texture and flavour of the cheese (Scott, 1986). The chemical changes responsible for cheese-ripening are:

a) Fermentation of lactose: Lactose is fermented to lactic acid, small amounts of acetic acid and propionic acid, CO_2 and diacetyl.

b) Proteolysis and lipolysis: These changes are brought about by enzymes from lactic itself and microorganisms growing within or on the surface of the cheese.

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The starter bacteria die out during ripening, as do most organisms present in the curd. Survival of *B.cereus* is also compromised by the production of lactic acid within the curd, the salt, the low a_w and Eh. Only the lactobacilli, which may be present in fresh curd in small numbers, multiply and these may reach levels of $10^6 - 10^8$ g^{-1} in cheese in 3 - 6 weeks (Chapman & Sharpe 1981; Scott, 1986). Gouda cheese with a relatively low moisture content, can be matured for a longer time and this results in more protein breakdown and rather more flavour in the cheese (Chapman & Sharpe, 1981). High levels of contaminants in milk may result in spoilage even at satisfactory concentration of moisture, salt and acidity.

2.4 Nutritional aspects of cheese

Milk is often described as nature's most nearly perfect food. Cheese, however, because of the selective concentration of components, differ from milk in this respect. The dairy industry turns milk, a short life commodity, into cheese with a shelf life of from 4 – 5 d up to 5 – 10 m (Scott, 1981).

In addition, microbial fermentation adds a new dimension of nutrition to cheese. Lactic acid, lactates, free fatty acids, amino acids, carbonyls and a variety of smaller molecular compounds are formed, and these influence the nutritive property of the fermented foods (Kosikowski, 1978). Furthermore, natural antibiotics which do not normally appear in dairy foods of unfermented nature are produced by the added bacterial cultures (Kosikowski, 1978)

Table 8 Nutrients of cheese per 100 g of cheese (adapted from Scott, 1986)

Food	Protein N x 6.26 (g)	Fat (g)	Calcium (mg)	Iron (mg)	Thiamine (mg)	Retinol (vitamin A) (μg)	Riboflavin (mg)	Ascorbic acid (Vitamin C)	Nicotinic acid (mg)	Energy (kcal)
Cheddar	26.0	33.5	800	0.5	0.04	310	0.5	0	0.5	406
Soft cheese	22.8	25.5	150	0.4	0.06	155	0.4	0	0.4	285
Cottage cheese	13.6	4.0	60	0.1	0.09	32	0.3	0	0.3	96

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CHAPTER 3

MATERIALS AND METHODS

3.1 Manufacturing of Gouda cheese

3.1.1 The milk

Milk was obtained from the University of Pretoria's experimental farm. A vegetable colouring (annatto) and rennet (Rennilase Type T, manufactured by Novo Industries, Denmark) were obtained from the University of Pretoria's existing stock. The materials were kept in a cold room at 4°C.

3.1.2 The starter culture

3.1.2.1 Mother cultures

The starter culture used, was a commercial culture CHN_{22} (a mixed culture of *Lactococcus lactis* and *L. lactis* subsp. *cremoris*). The stock cultures were supplied in feeze-dried form by CHR Hansen's Laboratories (Denmark) and distributed locally by Darleon (Johannesburg). Reconstituted skim milk was dispensed in 10 ml portions in McCartney bottles and the caps were screwed on. The bottles of reconstituted milk were then sterilised at 121°C for 15 min and cooled to room temperature. The freeze-dried culture was activated by aseptically transferring a small amount into 10 ml sterilised milk. This was then incubated at 22°C. After incubation the starter culture (mother culture) was cooled to less than 5°C and kept in a refrigerator. The mother culture was maintained by daily subculturing in sterilised milk, using a 1% inoculum (0.1 ml in 10 ml sterile milk), incubation at 22°C for 12-16 h and storing the culture at < 5°C in a refrigerator.

3.1.2.2 Bulk cultures

The bulk starter culture was prepared by inoculating 10 ml of the mother culture into 1000 ml of sterilised reconstituted milk (1% inoculation rate), incubating the inoculated milk at 22°C for 12-16 h and then cooling the bulk culture to < 5°C and keeping it at this temperature until it was used.

3.1.3 Manufacturing of the cheese

Gouda type cheese was manufactured in the pilot plant of the Department of Food Science (University of Pretoria) using the method described by Kosikowski (1978). Milk was pasteurized in a batch pasteurizer at 63°C for 30 min and cooled down to 30°C. The milk was then inoculated with 0.75% of the starter culture and then rennet added at the rate of 3 ml per 100 *l* of milk. After approximately 30 min at 30°C, the coagulated milk was cut using knives with blades 6 mm apart. Ten minutes later part of the whey was removed and replaced with warm water (32 - 42°C) to 10% of the milk volume. After another ten minutes, the curd was slowly heated from 30°C to 36°C over a period of 30 to 45 min. When the curd had attained a temperature of 36°C, whey was drained to 50% of the original volume of milk. The curd was held at 36°C for 1 - $1^1/_2$ h. The whey was then drained and the curd was put into warm moulds and pressed lightly for about 3 - 4 h. After pressing, the cheeses were redressed and left in the mould overnight without applying pressure. The green cheese (~ 10 kg each) was cut into six pieces (~1,6 kg each) and placed in a brine solution (20% $^w/_w$ salt, pH 5 at 12 - 15°C) for 24 h. The green cheese was removed from the brine and allowed to dry for two days in the cheese curing room. After drying, the green cheese was vacuum packed and ripened in a curing room at 12 - 15°C (RH 80 - 85%) for 6 weeks. Examples of the cheese manufacturing records are given in Appendix 1.

3.2 Preparation of *B.cereus* spore suspensions

3.2.1 Growth and sporulation of the cultures

B. cereus spores were produced on KG agar slants (Refai, 1979). Growth from a previous grown culture was suspended in 2 - 5 ml sterile distilled water by gently removing the growth from the agar medium with the aid of a sterilised (flamed) glass rod. This suspension was used as inoculum. The prepared agar slants (100 ml) were inoculated with a 0,5 ml of inoculum, spreading the inoculum as evenly as possible over the slant. The inoculated slants were incubated for 3 - 7 d at 30°C. After incubation the cultures were left on the bench at room temperature until ~80% sporulation had occurred. The rate of

sporulation was determined by preparing a smear of the culture on a glass slide, staining with crystal violet stain and examining under the microscope (Carl Zeiss Co., West Germany). Spores appeared as unstained oval structures.

3.2.2 Harvesting of the spores

Spores were washed off the sporulation medium with sterile distilled water using a sterile (flamed) glass rod to dislodge growth from the agar slants. Spores were gently removed from the medium and pooled into a sterile Ehrlenmeyer flask. The pooled suspension was then transferred to a sterile centrifuge tube which was covered with an aluminium foil cap. The tubes were balanced and centrifuged for 10 min at 3000 r/min. The supernatant was poured off and the spores were washed by adding fresh sterile distilled water. The spores were then resuspended with the aid of a sterile glass rod and centrifuged again. The spores were washed and centrifuged five times. After washing the spores, they were resuspended in sterile distilled water and sterile glass beads were added to separate clumps by hand shaking. The concentration of the spores was then adjusted with sterile distilled water to $\sim 10^7$ per ml (40-80% Transmission and wavelength of 420 nm) using a spectrophotometer (Spectronic 20D, Milton Roy, USA.). The concentration was checked by the Breed Microscopic count method (Harrigan & McCance, 1976). The spore suspension was then stored at 4-7°C in sterile McCartney bottles.

3.3 **Survival and growth of *B. cereus* during Gouda cheese manufacturing**

3.3.1 Experimental design

In order to determine the survival and growth of *B. cereus* during Gouda cheese manufacturing, two batches of cheese were prepared.

Batch 1: Experimental cheese with *B. cereus* spores added

A batch of 100 *l* of whole fresh milk was inoculated with 10 ml of a spore suspension (approximately 10^7 spores of *B. cereus* per ml) to give a spore concentration of $10^2 - 10^3$ per ml in the milk. The milk was thoroughly mixed and pasteurised at 63°C for 30 min. After pasteurisation the milk was used to manufacture Gouda cheese.

Batch 2: Control cheese without *B.cereus* spores added

A second batch of 100 *l* of whole fresh milk, to which no *B. cereus* spores were added, was pasteurized at 63°C for 30 min. After pasteurization the milk was used to manufacture Gouda type cheese.

3.3.2. Sampling

Samples were taken at different stages during the Gouda cheese manufacturing and analysed for the parameters as shown in Table 9.

Table 9 Sampling plan during Gouda cheese manufacturing showing process steps and stages at which samples were drawn for microbiological and chemical analyses

Time in weeks (wk)	Process Step	*B. cereus*	LA	MRS	pH	TA
0	Before rennetting	+	ns	ns	+	+
0.004	Curd at cutting	+	+	+	+	+
0.005	At $^{1}/_{2}$ whey removal	+	+	+	+	+
0.013	At final whey removal	+	+	+	+	+
0.023	At hooping (moulding)	+	+	+	+	ns
0.095	After pressing	+	+	+	+	ns
0.238	After brining	+	+	+	+	ns
1	After 1 week	+	+	+	+	ns
2	After 2 week	+	+	+	+	ns
4	After 4 weeks	+	+	+	+	ns
6	After 6 weeks	+	+	+	+	ns

+ sample taken

ns sample not taken

LA Lactic agar

MRS Man-Rogosa-Sharpe agar

TA Titratable acidity

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3.4 Microbiological analyses

3.4.1 Preparation of dilutions

Tenfold serial dilutions of the samples were made by aseptically transferring 25 g of sample into 225 ml of sterile saline peptone water to give a 10^{-1} dilution (IDF, 1992). Samples were then mixed for 20 s using a Stomacher 400 laboratory blender (Seward Laboratory UAC, Britain). Further tenfold dilutions of up to 10^{8} were made by transferring 1 ml of successive serial dilutions into universal bottles containing 9 ml of sterile saline peptone water.

3.4.2 Plate counts by the spread-plate technique

Plates for the spread-plate technique were prepared by pouring 12 to 15 ml of the relevant sterile culture media into sterile petri-dishes. The plates were dried at 35°C in an incubator. The dried plates were then inoculated with 0.1 ml of the homogenate or dilutions thereof and appropriately labeled. Each inoculum was spread over the surface of the dried plates using a sterile bent glass rod. After incubation the colonies were counted using a Scientific Colony Counter (Stuart Scientific Co., UK) and the results were expressed as cfu/g or cfu/ml depending on whether the sample was a solid or liquid.

3.4.3 *B. cereus* counts

B. cereus selective medium (Oxoid CM617) was used for the enumeration of *B. cereus*. The media were prepared according to the manufacturer's instructions and poured into sterile petri-dishes for spread-plate technique (3.4.2). The inoculated plates were incubated at 30°C for 24 h, the number of colonies were counted and expressed as cfu/g or cfu/ml of sample. The plates were also examined for typical *B. cereus* colonies which were rough in texture, turquoise to peacock blue in colour and surrounded by a greyish zone of egg yolk precipitate. Typical *B. cereus* colonies were confirmed by Gram staining (Harrigan & McCance, 1976). The stained smear preparations were examined under oil immersion microscope (Carl Zeiss, West Germany) for spore production.

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3.4.4 Lactic acid bacteria

Lactic agar (Merck) and MRS agar (Oxoid CM361) were used for the enumeration of lactic acid bacteria. The media were prepared according to the manufacturer's instructions and poured into petri-dishes for the spread-plate technique. The inoculated plates were incubated at 37°C for 48 h. Presumptive lactic acid bacteria were confirmed using the Gram staining test (Harrigan & McCance, 1976). The stained smear preparations were examined under an oil immersion microscope (Carl Zeiss, West Germany). The colonies that tested Gram positive were recorded as lactic acid bacteria

3.5 Chemical analyses

3.5.1 pH

The pH was measured using a combined glass electrode connected to a standard pH-meter DL 25 (Metler - Toledo AG, Switzerland).

3.5.2 Titratable acidity

The per cent acid present in the whey was determined by titration by measuring 9 ml of the whey sample in duplicate into 250 ml flasks. To this, 9 ml distilled water and 0.5 ml of phenolphthalein indicator were added and thoroughly mixed. The mixture was then titrated against 0.1 N sodium hydroxide to a pink colour that marked the end point. The per cent lactic acid present was calculated using the formula:

$$\% \text{ lactic acid of lactic acid} = \frac{N \times V \times ME \text{ of lactic acid} \times 100}{100}$$

Where: N is the normality of the sodium hydroxide solution

V is the volume of sodium hydroxide used to reach the end point

ME is the milli-equivalent of lactic acid

$$= \frac{\text{molecular weight of lactic acid}}{100} = 0.09008$$

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3.5.3 Fat content of milk

The Gerber fat test as described by Kosikowski (1978) was used.

3.5.4 Fat content of cheese

The method as described by Kosikowski (1978) was used.

3.5.5 Salt content of cheese

The method as described by IDF (1972) was used .

3.5.6 Moisture content

The method as described by Kosikowski (1978) was used.

3.6 Statistical Analyses

Statistical analyses were done using SPSS Version 8.0 for Windows (SPSS Inc., Chicago, USA). Numbers of bacterial counts were converted to log $_{10}$ cfu/ml or g. Means and standard deviations were calculated. The least significant difference between the means was determined.

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CHAPTER 4

RESULTS

4.1 Changes in *B. cereus* and lactic acid bacteria numbers during the cheese manufacturing

Table 10 and Figure 8 show changes in *B. cereus* and lactic acid bacteria numbers in cheese made from milk inoculated with *B. cereus* spores (experimental cheese) and cheese without *B. cereus* spores (control cheese). *B. cereus* spores survived and germinated into vegetative cells which grew from 10^2 cfu/g and reached a maximum of 10^4 cfu/g at hooping about 3.5 h after renneting (0.023wk). Numbers of *B. cereus* increased by 2.2 log cycles, whereafter they rapidly decreased to less than 1 x 10^1 cfu/g approximately 16 h after renneting.

B. cereus did not affect lactic acid bacteria numbers. Lactic acid bacteria numbers increased by 1.7 log cycles from 10^7 to slightly more than 10^9 cfu/ g and stayed fairly constant throughout the manufacturing and ripening process. *B. cereus* was not found in the control cheese and there was no significant difference ($p > 0.05$) in the numbers of lactic acid bacteria between the experimental and the control cheese (results not shown on the graph since they would overlap).

Table 10. Changes in *B. cereus* and lactic acid bacteria numbers during the manufacturing of Gouda cheese

Time weeks (wk)	Experimental cheese ($\log_{10}$ cfu/g) (MRS)[3]	(LA)[3]	BC[3]	Control cheese ($\log_{10}$ cfu/g) (MRS)[3]	(LA)[3]	BC[3]
0	[4]ns	ns	2.241^{a} (0.089)	ns	ns	0.000
0.004	[1]7.294^{a} [2](0.133)	7.331^{a} (0.080)	2.361^{a} (0.093)	7.419^{a} (0.041)	7.443^{a} (0.079)	0.000 0.000
0.005	7.628^{b} (0.047)	7.599^{a} (0.116)	3.466^{b} (0.046)	7.000^{b} (0.115)	7.703^{b} (0.072)	0.000 0.000
0.013	8.062^{c} (0.106)	8.089^{b} (0.161)	3.531^{b} (0.026)	7.834^{b} (0.073)	7.859^{b} (0.066)	0.000
0.023	8.171^{b} (0.081)	8.113^{b} (0.034)	4.414^{c} (0.129)	8.146^{c} (0.050)	8.149^{c} (0.060)	0.000 0.000
0.095	8.796^{d} (0.129)	8.799^{c} (0.146)	0.000	8.681^{d} (0.126)	8.710^{d} (0.127)	0.000
0.238	9.033^{de} (0.051)	9.037^{c} (0.072)	0.000	9.037^{e} (0.022)	9.037^{e} (0.011)	0.000
1	9.058^{e} (0.051)	9.049^{c} (0.058)	0.000	9.062^{e} (0.026)	9.063^{e} (0.025)	0.000
2	9.034^{de} (0.049)	9.025^{c} (0.059)	0.000	9.052^{e} (0.027)	9.049^{e} (0.020)	0.000
4	9.028^{de} (0.005)	9.031^{c} (0.024)	0.000	9.036^{e} (0.020)	9.033^{e} (0.028)	0.000
6	9.006^{de} (0.013)	9.001^{c} (0.018)	0.000	8.987^{e} (0.020)	9.001^{e} (0.021)	0.000

1. Means with different superscripts in the same column are significantly different from each other ($p < 0.05$).
2. Numbers in parentheses are standard deviations
3. Lactic acid bacteria on MRS and lactic agar (LA) and *B.cereus*, (BC) on Oxoid CM 617 agar
4. ns, not sampled

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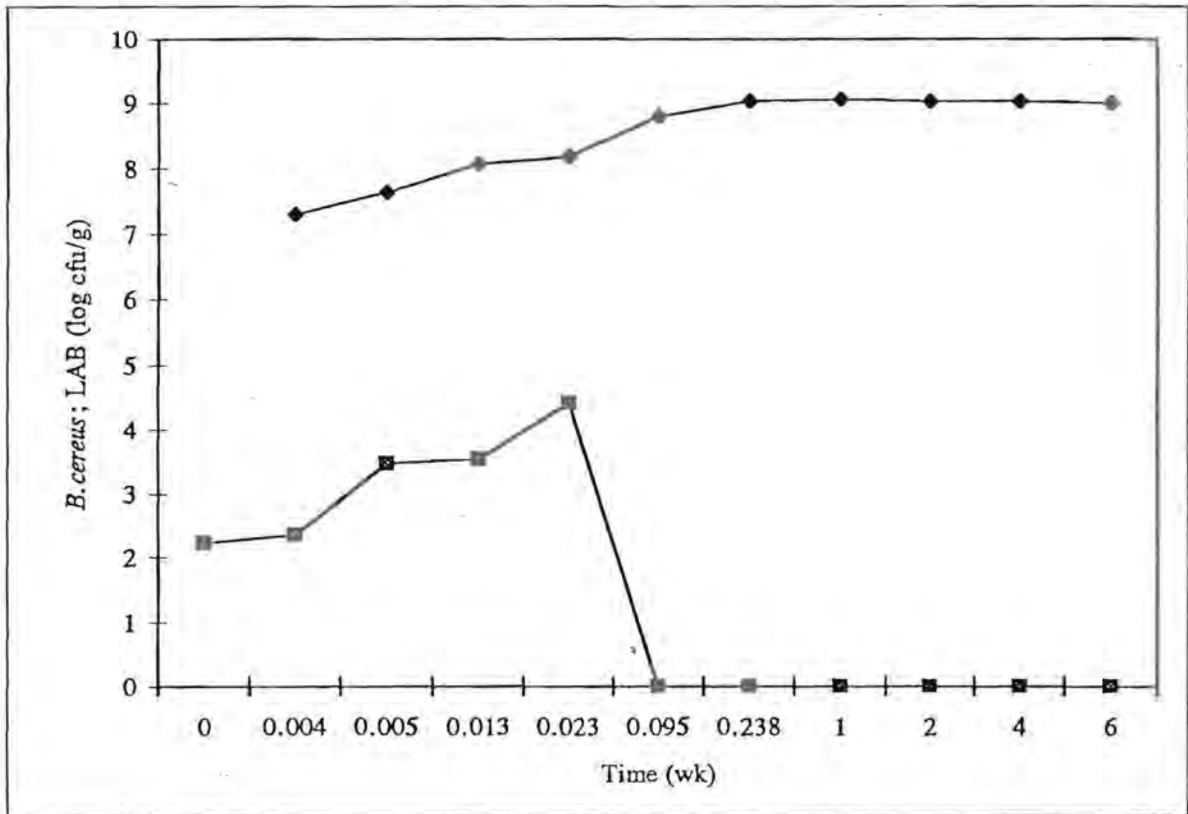

Figure 8. Changes in *B. cereus* (—■—) and lactic acid bacteria numbers (—◆—) during Gouda type cheese manufacturing from milk inoculated with *B. cereus* spores.

4.2 Changes in pH, *B. cereus and* lactic acid bacteria numbers during cheese manufacturing

Table 11 and Figure 9 show the changes in pH, and numbers of lactic acid bacteria and *B. cereus* during manufacturing of Gouda type cheese from milk inoculated with *B. cereus* spores. A general decrease in pH was observed from pH 6.6 to pH 5.16. *B. cereus* grew from approximately 10^2 cfu/g to a maximum of about 10^4 when pH was pH 6.17 whereafter it decreased sharply to < 10 cfu/g. The lactic acid bacteria increased steadily from approximately 10^7 to slightly over 10^9 cfu/g and maintained this average count throughout the cheese manufacturing and ripening process.

Table 11. Changes in pH, numbers of lactic acid bacteria (LAB) and *B. cereus* during manufacturing of Gouda type cheese from milk inoculated with *B. cereus* spores.

Time in weeks (wk)	pH	LAB (MRS) (log_{10} cfu/g)	*B. cereus* (log_{10}cfu/g)
0	6.6 (0.00)	[3]ns	[1]2.241[a] [2](0.089)
0.004	6.43 (0.06)	7.293[a] (0.133)	2.361[a] (0.093)
0.005	6.43 (0.06)	7.628[b] (0.047)	3.466[b] (0.046)
0.013	6.27 (0.06)	8.062[c] (0.106)	3.531[b] (0.026)
0.023	6.17 (0.06)	8.171[d] (0.081)	4.414[c] (0.129)
0.095	6.13 (0.06)	8.796[d]	0.000
0.238	5.17 (0.01)	9.033[de]	0.000
1	5.16 (0.01)	9.058[e]	0.000
2	5.16 (0.01)	9.034[de]	0.000
4	5.16 (0.01)	9.028[de]	0.000
6	5.16 (0.01)	9.006[de]	0.000

1. Means with different superscripts in the same column are significantly different from each other ($p < 0.05$)
2. Numbers in parentheses are standard deviations
3. ns, not sampled

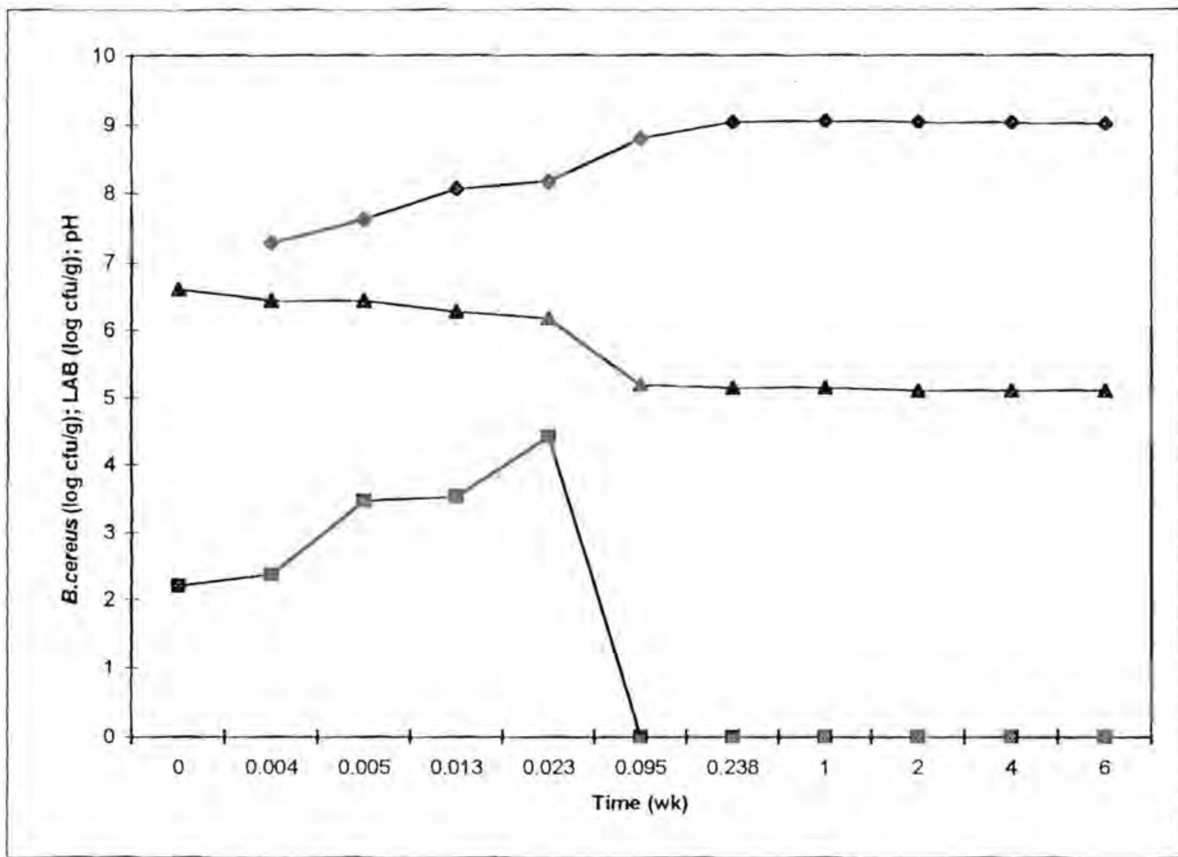

Figure 9. Changes in pH (-▲-); numbers of lactic acid bacteria (-◆-) and *B. cereus* (-■-) during manufacture of Gouda type cheese from milk inoculated with *B. cereus* spores.

Table 12 and Figure 10 show changes in pH and numbers of lactic acid bacteria in cheese without *B. cereus* spores (control cheese). The pH decreased from pH 6.60 to pH 5.15. Lactic acid bacteria grew steadily from 10^7 to slightly over 10^9 (1.7 log cycles) and maintained the maximum count throughout ripening. *B. cereus* was not found in the control cheese (<10/g).

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Table 12. Changes in pH and numbers of lactic acid bacteria during the manufacture of control Gouda type cheese (no *B. cereus* spores added)

Time in weeks (wk)	pH	LAB on MRS ($\log_{10}$ cfu/g)
0	6.60 (0.00)	[3]ns
0.004	6.43 (0.06)	[1]7.419[a] [2](0.041)
0.005	6.40 (0.00)	7.000[b] (0.115)
0.013	6.30 (0.00)	7.834[b] (0.073)
0.023	6.15 (0.07)	8.146[c] (0.050)
0.095	6.11 (0.01)	8.681[d] (0.126)
0.238	5.19 (0.01)	9.037[e] (0.022)
1	5.17 (0.01)	9.062[e] (0.026)
2	5.16 (0.01)	9.052[e] (0.027)
4	5.15 (0.01)	9.036[e] (0.020)
6	5.15 (0.01)	8.987[e] (0.020)

1. LAB, lactic acid bacteria on MRS
2. Means with different superscripts in the same column are significantly different from each other ($p < 0.05$)
3. Numbers in parentheses are standard deviations
4. ns, not sampled

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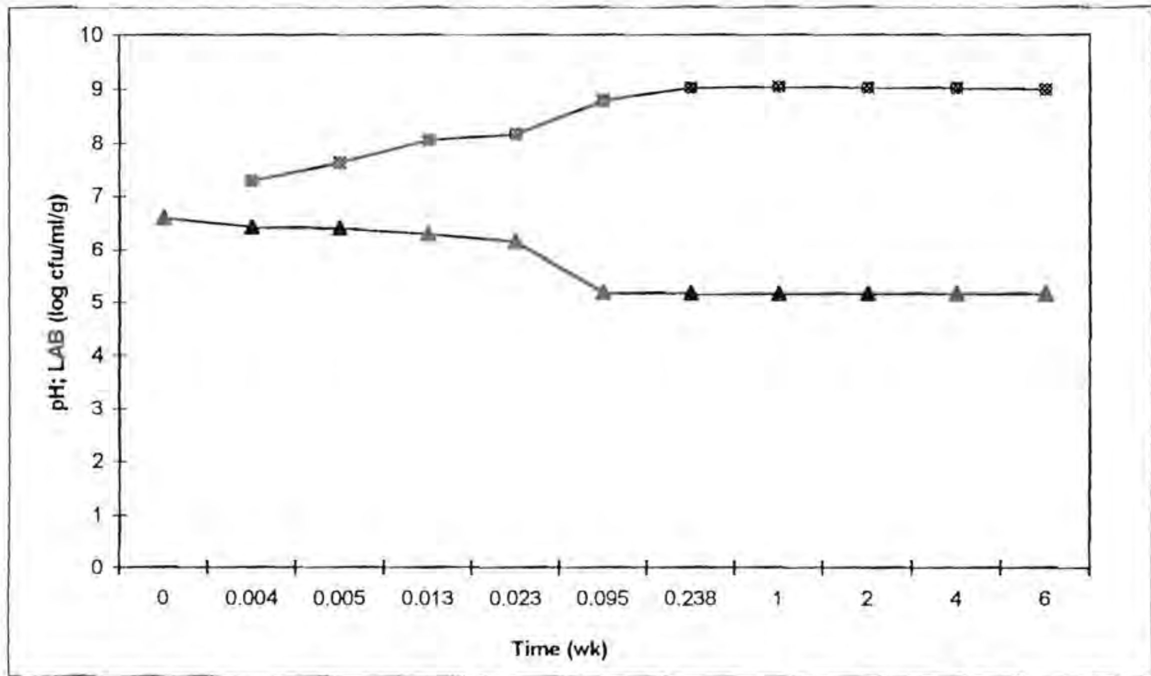

Figure 10. Changes in pH (-▲-) and numbers of lactic acid bacteria (-◆-) during manufacturing of control Gouda type cheese (milk not inoculated with *B. cereus*)

4.3 Chemical analyses of the cheese at 6 weeks

Chemical analysis were done at 6 weeks mainly to check the quality of the cheese. Table 13 shows the pH, % salt content, moisture content and % fat content of the experimental and the control cheese at 6 weeks.

Table 13. Means of pH, % salt content, moisture content and % fat content of the experimental and control cheese at 6 weeks

Analysis	Experimental cheese	Control cheese
pH	5,16	5,15
% Fat content	31,2	30,0
Moisture content	40,2	39,8
% Salt content	2,0	1,88
% salt in moisture	4,74	4,51
% fat in dry matter	52,22	49,83

Colony morphology of *B. cereus*

Typical *B. cereus* colonies with a dry rough surface and a blue-purple base surrounded by a ring of dense precipitate were isolated from the curd and whey during the early stages of the cheese manufacturing

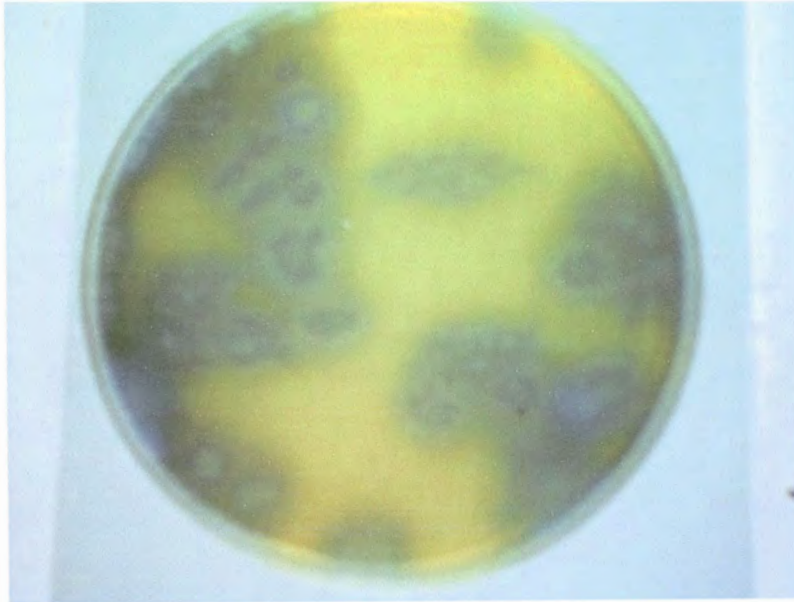

Figure 11 Colony morphology of *B. cereus* colonies grown on *B. cereus* selective agar

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CHAPTER 5

DISCUSSION

Vegetative cells of *B. cereus* are easily inactivated by heat, but the spores are activated by the heat treatment of pasteurisation, which triggers their germination and outgrowth (Andersson *et al*, 1995; Meer *et al*, 1991; Driessen, 1992). Meer *et al* (1991) found that more than 95 % of the spores in milk were activated at pasteurisation temperature and germinated in the milk. According to Driessen (1992) the extent to which the spores grow out in milk is dependent on the species or even the variant of the species and on the condition of storage of milk. In a study by Wong *et al* (1988) vegetative cells of *B.cereus* were killed or inactivated within 40 min in fermented milk while about 31% of spores survived the 7 d test period. In cheese milk inoculated with 10^2 *B. cereus* spores per ml of milk in the present study, *B. cereus* multiplied during cheese-making to a maximum of 10^4 cfu per g at hooping, i.e. after about 3h 50 min. After pressing (approximately 16 h after renneting) *B. cereus* numbers were reduced to less than 1 x 10^1 cfu/g of cheese curd. The level of vegetative cells of *B. cereus* did not exceed 10^5 per ml or per gram, a number which has been reported to cause illness (Johnson, 1984). The results of the present study are in contrast to those of Langeveld & Cuperus (1992) who found that only a minority of *B. cereus* spores present in milk before pasteurisation both germinated and grew quickly enough to be important for deterioration of the product.

The initial growth of *B. cereus* was expected because after pasteurisation the milk was free from other competitor micro-organisms and the *B. cereus* spores were activated by the heat treatment (Andersson *et al*, 1995) and conditions were still quite favourable for their growth. *B. cereus* grew nearly at the same rate as the lactic acid bacteria during the early stages of lactic acid fermentation until the LAB reached numbers of 10^8/g and the pH dropped to below 6.17. Thereafter the numbers of *B. cereus* decreased rapidly. Work by Wong & Chen (1988) on germination and growth of *B. cereus* in non-fat milk with added lactic acid bacteria also showed that *B. cereus* was not affected by lactic acid bacteria at the beginning of the fermentation process, but was affected strongly with continued fermentation. Killing or

inactivation of vegetative *B. cereus* cells at pH below 6.17 may not be attributed to reduction in pH and lactic acid production only but to a number of factors which act synergistically such as substrate competition, changes in oxidation-reduction potential and production of antimicobial agents (Wong *et al* 1988). According to Wong *et al* (1988) antimicrobial agents such as hydrogen peroxide, formate, acetate or lactate enhanced the inhibitory activity of lactic acid bacteria.

When considerable lactose had been fermented to lactic acid and the pH reduced to pH 5.2, growth of *B. cereus* was inhibited strongly. The reduction of *B. cereus* at pH lower than 6.1 is in accordance with the results found by Driessen (1992) during manufacture of yoghurt. As the acidity increased vegetative cells of *B. cereus* failed to survive, the counts went down from 10^4 to less than 1×10^1 cfu/g of cheese curd. At this stage the condition of the cheese curd, particularly low moisture content (a_w), lack of oxygen (low Eh), high salt concentration and depleted lactose content combined with high acid content may have inhibited the growth of *B. cereus* (Chapman & Sharpe, 1981). However, it has been previously demonstrated that inhibition of pathogens by lactic acid bacteria is not only dependent on acid production and decreased pH (Frank, Marth & Olson, 1978).

At hooping (moulding) the numbers of lactic acid bacteria surged well ahead of the numbers of *B. cereus*. After pressing *B. cereus* was not isolated, however lactic acid bacteria remained flourishing throughout the manufacturing and ripening process reaching numbers as high as 10^9 cfu/g. According to Kosikowski (1978) bacterial numbers of 10^8 to 10^9 cfu/g are enough to enhance cheese ripening. Both experimental and control cheese thus contained enough lactic acid bacterial cells (10^9 cfu/g) to accomplish ripening of the cheese. Analysis of variance showed no significant difference ($p > 0.05$) in the isolation rate of lactic acid bacteria on the two media, lactic agar and MRS agar.

In the present study the increase in the titratable acidity was not significantly correlated with changes in pH. According to Scott (1986) pH readings measure hydrogen ion activity in the milk, the coagulum, the curds and finally the solid cheese, whereas the titratable acidity measurements are made on liquids (milk and whey). Because of the differences between the

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systems being measured, there was no correlation between pH readings and titratable acidity, so the cheese manufacturer cannot switch from one form of measurement to another.

The moisture content of the cheese was satisfactory. According to Kosikwoski (1978) a maximum of 42% moisture is allowed in washed curd cheese. The high moisture content enhance ripening. The salt content and the fat content of the cheese were acceptable in a way that they are comparable to those mentioned in the literature which range from 1.5 – 2.2 % salt and 30% fat (Kosikwoski, 1978). Salt tend to play a role in cheese-making mainly to restore the calcium balance of the cheese milk, also by controlling the moisture content of the cheese, through aiding in whey expulsion. Salt also tend to increase the elasticity of the curd by forming a colloidal calcium phosphate and it also inhibits growth of microorganisms (Kosikowski, 1978).

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

B. cereus spores germinated and the vegetative cells multiplied during Gouda cheese manufacturing. The level of vegetative cells which was reached during cheese manufacturing was below the level of 10^5 cfu/g which was reported to cause illness or significant spoilage of milk and milk products. Growth of *B. cereus* during Gouda cheese manufacturing will be inhibited by pH, high lactic acid levels or by other factors such as hydrogen peroxide production, nutrient reduction, decreased lactose content or a decrease in the redox potential (Wong & Chen, 1988).

Lactic acid bacteria present in the curd continued to multiply in the maturing cheese and reached levels of 10^9 per gram in cheese of 6 weeks old. Growth of *B. cereus* was not affected by growth of the lactic acid bacteria during the early stages of the cheese-making process, but their continued growth resulted in a decrease in the numbers of *B. cereus* later in the cheese-making process until they could no longer be isolated from the cheese.

Lactic acid bacteria inhibit the growth of pathogens and spoilage organisms in milk and milk products and contribute remarkably to texture and flavour development of the cheese. The cheese manufacturer requires simple, rapid, sensitive and low cost methods for assessing the presence of pathogenic and spoilage organisms or their toxins and by-products in Gouda type cheese.

No viable cells of *B. cereus* were found in the cheese at the end of the manufacturing process in particular because of decreased lactose content, low a_w, low Eh, high lactic acid content and reduced pH. Therefore, it can be concluded that *B. cereus* was able to grow during early stages of cheese manufacturing but was not able to survive during the final stages of the manufacturing process and ripening of the cheese.

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With a view to future research in this field, it is recommended that the effects of major fermentative organic acids on growth and germination of vegetative cells and endospores of *B. cereus* be studied.

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APPENDIX 1 CHEESE MANUFACTURING RECORDS

CHEESE MANUFACTURING RECORD

CONTROL CHEESE A

Type of Cheese: GOUDA   Date: 2/7/98

Milk: % Fat: 3.7   Acidity (%) 0,15

Starter: Type CHN 22

Item/Action		Quantity	% Fat	Temp (°C)	Acidity %	pH	Time
Milk		100 ℓ	3.7		0,15	6,6	
Starter	0.75%	750 ml					
Cheese milk		100 ℓ		31			2.00
Colouring	1,25 ml/100 ℓ	10 ml					2.00
$CaCl_2$	50 ml/100 ℓ	50 ml					2.00
Rennet	3 ml/100 ℓ	30 ml		31			2.00
Cut				31	0,12	6,4	2.40
First removal of whey				31			2.55
Water added				31	0,10	6,4	3.05
Start heating				31			3.15
Acid development: 1							
2							
3							
End heating				36			3.55
Remove half of whey				36	0,12	6,3	4.10
Final whey removal				36	0,13	6,2	5.50
Cheddaring: start_____							
end _____							
Milling							
Salting							
Pressing				36			6.00

Yield: Number of cheeses: 3   Mass: 11,5 kg

Analyses: % Fat: 30   Moisture: 41,5   % Salt: 1,85   pH: 5,06

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CHEESE MANUFACTURING RECORD

EXPERIMENTAL CHEESE A

Type of Cheese: GOUDA Date: 2/7/98

Milk: % Fat: 3.7 Acidity (%) 0,15

Starter:Type CHN_{22}

Item/Action	Quantity	% Fat	Temp (°C)	Acidity %	pH	Time
Milk	100 ℓ	3.7		0,15	6,6	
Starter 0,75%	750 ml					
Cheese milk	100 ℓ		31			
Colouring 1,25 ml/100 ℓ	10 ml					2.45
$CaCl_2$ 50 ml/100 ℓ	50 ml					2.45
Rennet 3 ml/100 ℓ	30 ml		31			2.45
Cut			31	0,12	6,4	3.20
First removal of whey			31			3.35
Water added			31	0,11	6,4	3.45
Start heating			31			4.00
Acid development: 1						
2						
3						
End heating			36			4.40
Remove half of whey			36	0,12	6,3	5.25
Final whey removal			37	0,13	6,1	5.55
Cheddaring: start_____						
end _____						
Milling						
Salting						
Pressing			37			6.10

Yield: Number of cheeses:3 Mass: 10,9 kg Analyses: % Fat: 30,2

Moisture: 39,8 % Salt: 1,8 pH: 5,09

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CHEESE MANUFACTURING RECORD

CONTROL CHEESE B

Type of Cheese: GOUDA Date: 29/8/98

Milk: % Fat: 3.6 Acidity (%) 0,15

Starter: Type CHN 22

Item/Action	Quantity	% Fat	Temp (°C)	Acidity %	Acidity pH	Time
Milk	100 ℓ	3.6		0,15	6,6	
Starter 0,75%	750 ml					
Cheese milk	100 ℓ		31	0,15	6,6	12.30
Colouring 1,25 ml/100 ℓ	10 ml		31			12.30
$CaCl_2$ 50 ml/100 ℓ	50 ml		31			12.30
Rennet 3 ml/100 ℓ	30 ml		31			12.30
Cut			30,5	0,12	6,5	1.30
First removal of whey			30,6			2.15
Water added			30	0,10	6,5	2.30
Start heating			30			2.25
Acid development: 1						
2						
3						
End heating						3.45
Remove half of whey			37,2	0,11	6,3	3.55
Final whey removal			36	0,13	6,11	4.45
Cheddaring: start_____						
end _____						
Milling						
Salting						
Pressing			36			5.05

Yield: Number of cheeses:3 Mass: 10,936 kg

Analyses: % Fat: 31,2 Moisture: 41,0 % Salt: 1,98 pH: 5,08

CHEESE MANUFACTURING RECORD

EXPERIMENTAL CHEESE B

Type of Cheese: GOUDA Date: 29/8/98

Milk: % Fat: 3.6 Acidity (%) 0,15

Starter: Type CHN 22

Item/Action	Quantity	% Fat	Temp (°C)	Acidity		
Time				%	pH	Time
Milk	100 ℓ	3,6		0,15	6,6	
Starter 0,75%	750 ml					
Cheese milk	100 ℓ		30,8	0,15	6,6	1.25
Colouring 1,25 ml/100 ℓ	10 ml		30,8			1.25
$CaCl_2$ 50 ml/100 ℓ	50 ml		30,8			1.25
Rennet 3 ml/100 ℓ	30 ml		30,8			1.25
Cut			30,3	0,11	6,4	2.20
First removal of whey			30,3			2.45
Water added			30,1	0,10	6,4	2.50
Start heating			30,1			2.55
Acid development: 1						
2						
3						
End heating			37,6			3.00
Remove half of whey			37	0,10	6,3	3.35
Final whey removal			37	0,13	6,15	3.55
Cheddaring: start_____						
end _____						
Milling						
Salting						
Pressing			37			4.10

Yield: Number of cheeses: 2 Mass: 10,938 kg

Analyses: % Fat: 31,6 Moisture: 40,9 % Salt: 2,0 pH: 5,05

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CHEESE MANUFACTURING RECORD

CONTROL CHEESE C

Type of Cheese: GOUDA   Date: 1/9/98

Milk: % Fat: 3.6   Acidity (%) 0,15

Starter: Type CHN 22

Item/Action		Quantity	% Fat	Temp (°C)	Acidity %	Acidity pH	Time
Milk	100 ℓ	3,6			0,15	6,67	
Starter	0,75%	750 ml					
Cheese milk							
Colouring	1,25 ml/100 ℓ			31			2.45
$CaCl_2$	50 ml/100 ℓ			31			2.45
Rennet	3 ml/100 ℓ			31			2.45
Cut				31	0,12	6,4	2.45
First removal of whey				31			3.20
Water added				31	0,10	6,4	3.35
Start heating				30			4.00
Acid development:	1						
	2						
	3						
End heating				36			4.40
Remove half of whey				36	0,11	6,2	5.25
Final whey removal				36	0,12	6,2	5.55
Cheddaring: start_____							
end _____							
Milling							
Salting							
Pressing				36			6.15

Yield: Number of cheeses:3   Mass: 10,9 kg

Analyses: % Fat: 31   Moisture: 40,8   % Salt: 1,88   pH: 5,1

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CHEESE MANUFACTURING RECORD

EXPERIMENTAL CHEESE C

Type of Cheese: GOUDA   Date: 1/9/98

Milk: % Fat: 3.6   Acidity (%) 0,15

Starter: Type CHN 22

Item/Action	Quantity	% Fat	Temp (°C)	Acidity %	Acidity pH	Time
Milk	100 ℓ	3,6		0,15	6,67	
Starter 0,75%	750 ml					10.45
Cheese milk	100 ℓ					10.45
Colouring 1,25 ml/100 ℓ	10 ml		31			10.45
$CaCl_2$ 50 ml/100 ℓ	50 ml		31			10.45
Rennet 3 ml/100 ℓ	30 ml		31			10.45
Cut			31	0,12	6,5	11.35
First removal of whey			31			12.28
Water added			31	0,11	6,5	12.43
Start heating			31			1.23
Acid development: 1						
2						
3						
End heating			37	0,105	6,3	2.53
Remove half of whey			37	0,13	6,1	3.53
Final whey removal			36			4.15
Cheddaring: start_____						
end _____						
Milling						
Salting						
Pressing			36			4.30

Yield: Number of cheeses:3   Mass: 11,2 kg

Analyses:% Fat: 31   Moisture: 41,2%   Salt: 1,68   pH: 5,1