Bitter flavour in dairy products. I. A review of the factors likely to influence its development, mainly in cheese manufacture

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Review article

Bitter flavour in dairy products. I. A review of the factors likely to influence its development, mainly in cheese manufacture

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Summary — Following a definition of bitterness and a mention of the link between the bitterness of peptides and their content of hydrophobic amino acid residues, a detailed description is given of the numerous factors likely to influence the development of bitter off-flavour defect in dairy products with emphasis on cheese. Factors such as milk quality, pH, psychrotrophic bacteria, fat and mineral content, the cheese manufacturing process including the cooking temperature, salt concentration and manner of draining the curd, the acidity or the pH of cheese, and sanitary processing and hygienic packaging conditions are less important than bacterial proliferation in the cheese vat and the type and amount of starter and rennet used. The major roles played by these last three factors are better understood since the development of the aseptic vat technique. Finally, possible ways are mentioned of keeping the concentration of bitter peptides below the threshold level for bitter taste detection.

bitterness / dairy product / cheese / peptide / hydrophobicity

Résumé — L'amertume dans les produits laitiers. I. Une revue des facteurs susceptibles d'influencer son apparition, principalement au cours de la fabrication fromagère. Après avoir défini l'amertume et mentionné la relation existant entre l'amertume des peptides et leur contenu en acides aminés hydrophobes, les nombreux facteurs susceptibles d'influencer l'apparition du défaut d'amertume dans les produits laitiers sont décrits en détail. Les facteurs tels que la qualité du lait, son pH, son contenu en bactéries psychrotrophes, en matière grasse et en minéraux, le mode de fabrication du fromage et la température de cuisson, l'acidité ou le pH du fromage, les conditions sanitaires de transport du lait, de fabrication et d'emballage du produit fini sont moins importantes que la prolifération bactérienne dans le bac à fromage et que le type et la quantité de ferment et de présure utilisés. La compréhension du rôle joué par ces 3 derniers facteurs a été rendue possible avec l'avènement de la technique de fabrication aseptique du fromage. Pour terminer, sont mentionnés d'éventuels moyens de conserver la concentration en peptides amers au-dessous du seuil de détection de l'amertume.

amertume / produit laitier / fromage / peptide / hydrophobicité

INTRODUCTION

Taste buds, which are located in papillae on the tongue, can detect the characteristic taste of food determined by the balance of the primary (sweet, sour, salt, bitter and umami) and/or secondary (astringent, metallic and hot) tastes (Nishimura and Kato, 1988; Cheftel *et al,* 1977). Umami is the characteristic taste of monosodium glutamate and 5'-ribonucleotides.

Sometimes desired and regarded as a typical flavour note (*eg,* in grapefruit) and in some cases indesirable and objected to as off-flavour (*eg,* in cheese), bitterness of foodstuffs may be caused either by naturally occurring bitter compounds or by products from chemical reactions that occur during storage or processing.

Characterised by a pleasant and slightly sweet taste, milk is very susceptible to flavour defects from a variety of sources (absorption of flavours, bacterial contamination and chemical reactions such as lipolysis (Charalambous, 1986)), and unless properly heat-treated and refrigerated, it has a very short shelf-life. For centuries cheese-making has been the only means of preserving the major constituents of milk, and among the variety of cheese types some can be considered as products with real long-term storage capabilities. The proteolytic activity of starter cultures produces bitterness in cheese. By this process, caseins are broken down into peptides of low molecular weight (~1400 Da) with bulky hydrophobic groups towards the C-terminal end of the peptide (Matoba and Hata, 1972). Peptide bitterness, caused by the hydrophobic property of the amino acid side chain, can be predicted from the rule proposed by Ney (1971, 1979).

Bitterness in yoghurt has been shown to be caused by the proteolytic activity of *Lactobacillus bulgaricus* during storage (Renz and Puhan, 1975). Gelation and the occurrence of bitter flavour in UHT milk are caused by heat-stable proteases and lipolytic enzymes produced by psychrotrophic bacteria. However, milk processing temperature is critical; while appreciable amounts of lipase may be inactivated by heating milk at 64 °C for 10 s, heat treatments required to inactivate proteases also denature the milk proteins (Charalambous, 1986).

The greatest contribution to the intensity of cheese flavour has been shown to be present in the water-soluble fraction (McGugan *et al,* 1979). Bitterness is most intense in that fraction and has been attributed to medium sized (tri- to hexa-) peptides (Biede and Hammond, 1979) resulting from the enzymic digestion of casein. Beta-casein may be the source of bitter peptides produced by the proteinase(s) present in the cell wall of starter bacteria (Exterkate, 1976), while chymosin (rennet) may produce bitter peptides from all casein components (Exterkate, 1983).

Following the development of technology for the acceleration of the cheese ripening process (Fox, 1988–1989), development of low fat cheeses, improved shelf-life, transformation of milk to new food products and alteration of milk components by use of recombinant DNA techniques (Muysson and Verrinder Gibbins, 1989), the dairy industry will have to find ways to avoid this potential defect of bitterness.

From the literature surveyed it is surprising to find that bitterness, although reported for all dairy products, has been studied most extensively in Cheddar and Gouda cheeses. This research has identified 20 factors related to bitter off-flavour which will be described in detail in this review. It is understandable that most of the text will be devoted to bitterness in cheese.

BITTERNESS–HYDROPHOBICITY

Bitterness, a flavour defect which is encountered quite frequently in Cheddar and Gouda cheeses, results from the accumulation of bitter-tasting peptides formed by the action of proteolytic enzymes on casein (Fryer, 1969; Sardinas, 1972; Sullivan and Jago, 1972; Sullivan *et al,* 1973; Stadhouders, 1974; Stadhouders and Hup, 1975; Lawrence *et al,* 1976; Creamer, 1978). The bitter peptides have special chemical properties which enable them to interact with the taste buds at the back of the tongue to give the sensation of bitterness.

The existence in the bitter peptides of 2 bitter taste determinant sites which can bind to the bitter taste receptor has been proposed by Okai (1977), who also postulated that the hydrophobic residue of a peptide acts as a first binding site and in the presence of the second site, called the stimulating site, a bitter taste is detectable. However, the mechanism of the second site has not yet been resolved (Ishibashi *et al,* 1987).

The concentration of the bitter peptides in the cheese must exceed a certain threshold level before bitterness can be detected. The overall level of bitterness in cheese might be determined finally by the relative rates at which bitter peptides are formed and broken down to non-bitter products (Jago, 1974).

Early on it was suggested, based on the study of Emmons *et al* (1960b) on 132 cheeses, that cheese bitterness was due to unhydrolysed peptides. These authors obtained a correlation coefficient of 0.90 between the TCA-soluble N/amino N ratio, which is an estimate of the average number of amino groups per peptide in the TCA soluble extract, and the level of bitterness.

It is now accepted that the bitter flavour produced during enzymic hydrolysis of casein is due to particular types of peptides (Fujimaki *et al,* 1970). Ney (1971) was the first to propose the Q-hypothesis, a semi-quantitative relationship between the amino acid composition of a peptide and its bitterness. According to Ney (1979), no particular single amino acid or sequence was needed to impart the bitter taste. However, Japanese workers (*eg,* Shinoda *et al,* 1985, 1986a, b) proved later on, by synthesizing bitter peptides and several analogs, that the nature of the terminal amino acids and their steric parameters have some significance in the intensity of bitter taste.

BITTER FLAVOUR DEVELOPMENT IN CHEESE

Cheese-making, which is an important way of preserving the nutritive components of milk, is practised in various parts of the world. That explains why many different cheese varieties have been developed. However, the results obtained using one cheese variety with a particular milk coagulant cannot be extrapolated to another cheese variety or cheese made with a different milk coagulant. Modification of the cheese-making conditions and parameters (choice of starter, rennet concentration, cooking temperature and salt addition (Harwalkar and Elliott, 1971; Creamer, 1978) has helped in the study of cheese bitterness.

Bitter peptides are released from the casein molecules primarily by the action of the rennet and bacterial proteinases; a contribution may also be expected from bacterial peptidases, which may reduce the size of peptides initially too large to give a bitter taste (Sullivan and Jago, 1972; Sullivan *et al,* 1973). The production

of bitter peptides during the process of enzymic digestion is not always unfavourable; indeed, a bitter taste is one of the important components of cheese taste quality (Shinoda *et al,* 1985; 1986b). However, when bitter peptides accumulate, their concentration may exceed the flavour threshold for bitterness and can limit acceptance of the cheese, which will then be rated bitter (Visser *et al,* 1983b, c). It should be noted that this threshold is considered to increase with the age of a cheese because of the increase in other flavour components (Creamer, 1979).

Bitterness has long been recognised as a major defect in Gouda (Visser *et al,* 1983b) and Cheddar cheeses (Emmons *et al,* 1962a; Richardson and Creamer, 1973; Hamilton *et al,* 1974; Crawford, 1977; Champion and Stanley, 1982; Edwards and Kosikowski, 1983), but it also occurs in Camembert and similar types of cheeses produced in France (Pélissier *et al,* 1974; Mourgues *et al,* 1983). This defect has also been reported in Swiss mountain cheese (Guigoz and Solms, 1974), Butterkäse (Huber and Klostermeyer, 1974), Japanese yeast-ripened cheese (Kaneko and Yoneda, 1974), Gorgonzola (Delformo and Parpani, 1986) and Cottage cheese (Sandine *et al,* 1972), indicating the universality of this problem, which is a complicated defect affected by many factors. In cheese varieties other than Cottage, bitter flavour development has been associated with proteolysis (Stone and Naff, 1967).

Proteolysis in cheese can be considered synonymous with the degradation of the caseins to peptides and amino acids. Although analysis of the casein sequences reveals that they contain many hydrophobic fragments which are related to the formation of a bitter taste during the hydrolysis of these proteins, identification of bitter peptides isolated from whole casein and from cheese has shown that they originate mostly from α_{s1}- and β-casein. It seems reasonable that α_{s1}- and β-caseins with high average hydrophobicities of 1.17 and 1.33 kcal/residue, are potential sources of bitter peptides on hydrolysis (Visser, 1977c). Until now, no reports on the identification of bitter κ-casein fragments in cheese have appeared, although Visser *et al* (1975) have shown that rennet or purified chymosin could generate bitter peptide material from para-κ-casein.

The C-terminal portion of β-casein has an extremely bitter taste (Visser *et al,* 1983b; Shinoda *et al,* 1986a) and is the principal source of bitter peptides in Gouda cheese (Visser *et al,* 1983b, c). In Cheddar cheese, β-casein is highly resistant to proteolysis while α_{s1}-casein is extensively degraded; the main reason for this resistance to proteolysis is the formation of β-casein polymers that are not readily hydrolysed (Phelan *et al,* 1973; Creamer, 1975).

Beta-casein was earlier suggested as being the main source of bitterness in dairy products (Sullivan and Jago, 1972). Indeed, strains which produce bitterness in cheese degrade β-casein at a faster rate than non-bitter strains (Sullivan and Jago, 1972); moreover, processes used for the preparation of partly digested dairy products from whole casein will of necessity give rise to bitter tasting products unless they are capable of reducing the peptide representing the sequence 53–79 of β-casein to very small fragments (Clegg *et al,* 1974). However, the occurrence of bitter defects was observed to be rare in goat and ewe cheeses compared to cow cheeses; cow's milk contains more α_{s1}-casein, hydrolysates of which are more bitter than those of β-casein, suggesting that the smaller the amount of α_{s1}-casein, the less bitterness that develops in the cheese (Pélissier and Manchon, 1976).

Bitter peptides produced from casein by the action of rennet (or chymosin), as well

as proteinases from the cell wall of certain starter bacteria, are degraded by the action of proteolytic enzymes from the cytoplasmic membrane of bacterial cells, either in concert with enzymes from the cytoplasm or without their aid. It is known that salt strongly influences the net formation and degradation of bitter peptides (Visser, 1977b; Visser *et al,* 1983a). Although many studies report on bitterness in cheese made with various rennet substitutes, the action of exopeptidases from microbial rennets may help in preventing the occurrence of bitterness, and in accelerating the cheese ripening process (Mälkki *et al,* 1978).

THE ASEPTIC TECHNIQUE

Before the development of the aseptic vat technique (Mabbitt *et al,* 1959), the bitter flavour defect had been observed by numerous workers (Moir, 1930; Price, 1936; Raadsveld, 1953), some of whom had been able to show specific causes for it. Strict bacteriological control of the cheese during making was not exercised at that time, and it was generally believed, from the results of cheese-making experiments that starter was the main agent involved in the maturation of Cheddar cheese, while other organisms, in particular lactobacilli, were found to be necessary for the production of Cheddar flavour. However, according to Fryer (1969), the results of such relatively uncontrolled experiments might justifiably be ignored.

Aseptic curds are obtained using a technique of artificial milk acidification (Mabbitt *et al,* 1955) by the addition of δ-gluconic acid lactone in place of starter (Visser, 1977a; Stadhouders *et al,* 1983). General information concerning the role of the different ingredients used for cheese manufacture and the general mechanism of protein breakdown during cheese ripening (LeBars *et al,* 1975; Desmazeaud and Gripon, 1977) have been obtained from these aseptic curds.

Aseptic manufacture of cheese has been performed principally with Cheddar and Gouda cheeses. In the former case, research was mainly directed towards the study of the typical Cheddar flavour (Reiter *et al,* 1967; McGugan *et al,* 1968; Reiter and Sharpe, 1971). The aseptic technique of cheese manufacture has conclusively demonstrated the central role of starter lactococci in the development of Cheddar cheese flavour, and that different flavour intensities are related to different starters (Reiter *et al,* 1967). The contribution of starter lactococci to bitterness production was also studied by Lowrie *et al* (1974) under controlled bacteriological conditions. It has been concluded, from the study of the rate of acid development during Cheddar cheese manufacture, that the excessive proteolysis which accompanies rapid acidification does not result in the development of bitter flavour (O'Keeffe *et al,* 1975).

To study aspects of cheese ripening, aseptically made cheeses in which the protein is decomposed during ripening through the separate and combined actions of rennet, starter bacteria and milk protease must be used (Gripon *et al,* 1975; Visser, 1977a; Visser and de Groot-Mostert, 1977).

Studies conducted by Visser (1977b, c, d), Visser and de Groot-Mostert (1977) and others in which normal aseptic, aseptic starter-free, aseptic rennet-free, and aseptic rennet and starter-free Gouda cheeses were manufactured, have shown that rennet and bitter starter bacteria were able, separately, to produce bitterness in cheese, although the starter bacteria appeared to do this far more specifically than did rennet by breaking down para-casein in peptides of low molecular weight (MW).

Cheeses did not develop bitterness when non-bitter starters were used. It was also observed that higher levels of free amino acids accumulated in aseptic rennet-free and normal aseptic cheeses when non-bitter starters were used instead of bitter ones.

Rennet, which is responsible not only for coagulation but also for some aspects of the subsequent ripening (Scott, 1972; Visser, 1977c), has been shown to liberate peptides of high and low MW but only very small amounts of amino acids (AA). In normal cheeses, starter peptidases hydrolyse peptides produced by the action of the rennet leading to the accumulation of AA and low-MW (< 1400 Da) peptides. Small amounts of AA and low-MW peptides are also liberated by milk protease.

An experiment conducted by Scott (1972) on Cheddar cheese has indicated that the breakdown of milk constituents was present in the cheese in spite of irradiation destruction of bacteria. Although this treatment produced an intense rancid/bitter flavour, it has shown that once enzyme systems are present in the curd, living organisms are not necessary for the degradation of milk constituents, as occurs in cheese during ripening.

FACTORS LIKELY TO INFLUENCE BITTER OFF-FLAVOUR DEVELOPMENT IN DAIRY PRODUCTS

Milk quality

Dairy products possessing good flavour and shelf-life cannot be consistently produced when made from raw milk of poor organoleptic and bacteriological quality (White *et al,* 1978).

The feeding of good-quality silage to cows usually results in milk of good quality (Polansky, 1989), while feeding beet and turnip tops and roots to cows has been found to cause a bitter flavour in the milk (Bassette *et al,* 1986). When bitter sneezeweed *(Helenium amarum)* – a warm-season annual widely distributed in the eastern and southern United States – is eaten by cows, it imparts a bitter taste to the milk and renders it unpalatable (Smith, 1989). A single dose of 500 g or more of green *H amarum* to a lactating cow was required to cause appreciable bitterness in milk (Ivie *et al,* 1975). This off-flavour was found to be very persistent and did not disappear from one milking to the next.

The intensity and persistence of feed and weed flavours in milk are a function of the time interval between feeding and milking, ventilation in the barn, soil fertility, and of the physical condition of the cow. Besides the feeding (Delbeke and Naudts, 1970), the composition of milk also varies according to the season (Lawrence and Gilles, 1971), the stage of lactation (Okigbo, 1986), the breed (Richardson and Creamer, 1973), the health of the animal (Sorokina and Karagueuz, 1978) and even the individual (Reiter and Sharpe, 1971).

It is generally suggested that milk with poor rennet coagulation properties should not be used for the manufacture of cheese owing to a reduced yield of cheese with a high moisture content and a very bitter flavour (Okigbo, 1986). Significant quantities of casein cleavage products are to be expected in bulk milk supplies containing substantial amounts of such milk. However, casein cleavage products are also present in normal milk, for example in mid-lactation milk, but in limited quantities, with time, as more plasminogen is activated and microbial flora increases, higher concentrations of such products accumulate (Visser *et al,* 1983b).

Milk as produced in the mammary glands is considered to be sterile but mi-

crobial contamination occurs during passage through the udder. Flavour defects such as bitterness, resulting from this microbial contamination in raw and pasteurised milk, can occur at any stage of production and processing (Bassette *et al,* 1986).

Psychrotrophic bacteria

Psychrotrophic bacteria are present in most raw milk supplies and can grow readily at refrigeration temperatures, producing proteolytic and lipolytic enzymes. The results obtained by Gebre-Egziabher *et al* (1980) and many others (*eg,* Richardson and Newstead, 1979; Dousset *et al,* 1988) have shown that all psychrotrophs were hydrolysing casein in raw and processed milk, thus causing bitterness problems.

During cold storage of milk at the farm and at the dairy before processing, the growth of acid-producing bacteria is suppressed while psychrotrophic bacteria, after a certain lag period, start to develop. Several psychrotrophic bacteria (*eg, Pseudomonas fluorescens* P_{26}, B_{12} and B_{52}) especially if they reach a cell population of 10^6/ml, may produce extracellular lipases and proteases that can survive the heat treatments encountered in pasteurisation (73 ± 1.0 °C for 16 s) and in the UHT processing of milk (135–150 °C for 3–10 s), and cause the development of bitter taste (Shipe *et al,* 1978; Dousset *et al,* 1988). Both bacterial and native (alkaline) milk proteinases can hydrolyse casein and produce bitter off-flavour in milk and milk products with an extended shelf-life (White and Marshall, 1973b; Richardson and Newstead, 1979; Visser, 1981; Torrie *et al,* 1983; Dousset *et al,* 1988). White and Marshall (1973a) have shown that contamination of raw milk having a bacterial count of < 33/ml inoculated with 160 cells/ml of *Pseudomonas fluorescens* P_{26} produced significant proteolysis and bitter off-flavour in UHT pasteurised milk stored at 4.4 °C for up to 20 days. However, even in the absence of post-pasteurisation contaminants, off-flavours may be encountered after pasteurisation of raw milk containing large populations of psychrotrophs due to: 1) end-products of microbial metabolism; 2) constituents of large numbers of heat-inactivated and lysed bacterial cells; 3) heat-stable microbial enzymes; 4) presence and growth of thermoduric psychrotrophs (Patel and Blankenagel, 1972).

A lipase and several esterases occur naturally in milk, the lipase attacks the milk triglycerides, releasing free fatty acids and forming mono- and diglycerides which are responsible for the bitter flavour that occasionally accompanies lipolysis (Lebedev and Umanskit, 1981; Choisy *et al,* 1984; Bassette *et al,* 1986). Dibutyrin is the bitter flavour principle in homogenised raw milk. Since the growth of lactic streptococci is depressed in lipolysed milk, it is possible that slow lipolysis which occurs in milk held in bulk tanks could produce enough acids to slightly inhibit the growth of lactic cultures and, as a result, the flavour and odour of milk may be altered and the surface and interfacial properties modified (Jensen, 1964). In order to help the milk to recover its physico-chemical and biochemical properties, Desmazeaud (1983) has proposed the storage of good bacterial quality milk for 12–15 h at 10 °C in the presence of 0.1–0.2% of starter.

McKellar (1981) was the first to report an attempt to associate bitterness development in milk with proteolysis measurement. He found that proteolysis ranging from 0.289–0.554 and from 0.499–0.746 µmol of trichloroacetic acid-soluble free amino groups/ml for UHT and pasteurised milk, respectively, was necessary for significant off-flavour development.

Bitter flavour which frequently developed under commercial handling of Cottage cheese was associated with increases in soluble nitrogen and psychrophilic bacteria. Also, much of the White cheese offered in Egyptian local markets is bitter and highly acidic due to raw milk heavily contaminated with lactobacilli (Abo-Elnaga, 1974). Similarly, Greek Teleme cheeses made with raw milk stored for 5 days were unacceptable for body and texture and were rejected because of rancidity, unclean flavour and bitterness due to the presence of psychrotrophic bacteria (Kalogridou-Vassiliadou and Alichanidis, 1984). Other workers (Chapman *et al,* 1976; Law *et al,* 1976; Hicks *et al,* 1986) have found similar off-flavours in Cheddar cheese made from cold-stored milk.

Treatment and pH of the cheese milk

As early as 1930, Moir observed that cheese made from pasteurised milk develops a bitter flavour, a defect more pronounced for milk flash-pasteurised at 85 °C than at 74 °C (20–30 s). As mentioned by Stadhouders (1962), cheese-making from over-pasteurised milk is still not allowed in the Netherlands; indeed, by this process more rennet is retained and the cheese has a greater chance of becoming bitter. This was confirmed for Gouda and Cheddar cheeses produced from milk of low pH (pH 6.25) (Stadhouders and Hup, 1975). The pattern of proteolysis is altered during maturation; consequently, there is more intensive breakdown of the α_{s1}-casein and increased bitterness in the Cheddar cheese (Creamer *et al,* 1985).

The lowering of the initial pH of cheese milk happens when: a), the milk is of inferior quality and acid formation has taken place (Okigbo *et al,* 1985); b), the milk is pre-ripened with starter; or c), a large amount of starter has been used. A solution to this problem may be achieved by reduction of rennet levels (Banks, 1988) and manufacture of Cheddar cheese according to the method of Banks *et al* (1987), which includes incorporation of denatured whey protein into the curd. However, the development of high quality Cheddar cheese flavour was impaired by this technique.

Bacteriophage proliferation in the cheese vat

The presence of antibiotics or bacteriophages seriously inhibits the starter activity and prevents or reduces the development of bitterness (Stadhouders, 1974).

Bacteriophage contamination during making, at levels which restricted starter growth in the final stages of manufacture without markedly affecting acid production, had a striking effect on cheese flavour, especially the intensity of bitterness (Lowrie *et al,* 1972), and was responsible for longer cheese-making times on subsequent days (Lawrence and Gilles, 1973; Lowrie, 1977). The cheeses manufactured under controlled bacteriological conditions both with the normally bitter strain *Lactococcus lactis* subsp *lactis* ML_8 or the slow acid-producing strain *Lactococcus lactis* subsp *cremoris* AM_2 at a low cooking temperature developed an intense bitter flavour; however, this defect was almost entirely eliminated from cheese made in the presence of bacteriophage (Lowrie *et al,* 1974). Since significant bacteriophage contamination may occur without apparent effect on acid production during cheese-making, instances of such bacteriophage infection must be widespread in commercial cheese-making operations, even when so-called "phage-tolerant" or "phage-resistant" starters are used (Lowrie, 1977).

Many years ago, cheese of good flavour, as well as complete control over bacteriophage could be obtained by rotation of carefully selected pairs of starters (Lawrence and Gilles, 1973; Gavron *et al,* 1982). The information that bacteriophage does not replicate in non-growing cells which still retain the capacity to ferment lactose to lactic acid would appear, as suggested by Hillier *et al* (1975), to form a basis for the control of phage proliferation in cheese factories.

Fat content

Cheddar cheese made from pasteurised milk with a reduced fat content (1.5 ± 0.1%) has been found to develop an increasingly acid and bitter flavour (Deane and Dolan, 1973). On the other hand, this off-flavour defect was also observed in Italian cheeses such as Crescenza, Gorgonzola and Robiola when they were manufactured from milk with a high fat content (Delformo and Parpani, 1986).

Hydrolytic cleavage of fatty acids from milk fat by the lipase, which is secreted by microbial contaminants in milk, can produce bitter off-flavour (Bassette *et al,* 1986), as discussed before.

Choice of starter

As observed by Lawrence and Gilles (1969), the most important role in bitterness is played by the starter. Tests by Hansen *et al* (1933) indicated that addition of more than 4% of a culture of *Streptococcus paracitrovorus* to raw or pasteurised cheese milk resulted in cheese with a bitter flavour and open texture. This was also true of *Steptococcus citrovorus* when an inoculum of 1–10% was used, regardless of the kind of milk (raw or pasteurised). Addition of 0.1 to 0.5% of a culture of *Streptococcus liquefaciens* to the regular starter resulted in cheese with a flavour so bitter that it became unpalatable (Deane, 1951). Similar results were obtained by Yates *et al* (1955), who made Cheddar cheese with cultures of *Streptococcus faecalis* var *liquefaciens* previously isolated from raw milk cheese or whey. Marked proteolysis and bitterness resulted in cheese prepared with pasteurised cheese milk, added *Streptococcus liquefaciens* and a lactic starter (Tittsler *et al,* 1948). Outgrowth and survival characteristics of starter bacteria during cheese ripening have been implicated in the development of Cheddar cheese flavour as well as of bitter flavour (Perry and McGillivray, 1964; Reiter *et al,* 1967; Martley and Lawrence, 1972; Lowrie *et al,* 1974).

For many years starter lactococci have been classified as bitter or non-bitter according to whether or not they consistently produce bitterness in Cheddar cheese (Emmons *et al,* 1962a; Lawrence and Gilles, 1969). Two simple methods for detecting bitter strains have been proposed by Klimovsky *et al* (1970). The first method consists of evaluating the flavour of curds of skim-milk and of skim-chalk-milk made with rennet in concentration of 0.7 g/l of milk following incubation for 24 h and 7 days at 26 °C with the strains being tested. Selected strains were used in the manufacture of Kostroma, a Russian cheese. There was a perfect correlation between bitterness in the cheese and bitterness in the 7-day test. The second method is based on the ability of lactic acid bacteria to form peptides rapidly and to produce glutamate slowly in milk, following addition of rennet, knowing that the most bitter strains consistently produce less glutamate than non-bitter strains, independently of the milk or rennet used. Hillier *et al* (1975)

have shown that the estimation of bacterial growth in milk containing 1% yeast extract, at pH 6.3 and 37.5 °C, by a rapid absorbance method, provides a simple means of identifying strains likely to produce high or low cell populations in the curd and hence, either a bitter or non-bitter cheese. Certain strains of *P roqueforti* and *P caseicolum* have been shown to be responsible for the development of bitterness in Camembert cheese (Adda *et al*, 1982). According to the findings of different workers (Martley and Lawrence, 1972; Sullivan and Jago, 1972; Choisy *et al*, 1978) a fast method is proposed, based on the determination of the starter growth rate, proteolysis and proline-iminopeptidase activity, to characterise *lactococcus* strains for their capacity, if any, to develop bitterness in cheese.

Bitter and non-bitter strains are supposed to differ only in their resistance to the cooking temperature applied in Cheddar cheese production (≈ 38 °C). At this cheese cooking temperature, since the growth of the non-bitter strains is repressed while that of the bitter strains is not, a high final total population of starter organisms in the curd, irrespective of strain, has been thus shown to be the major factor producing bitterness in cheese (Lowrie *et al*, 1972). The viability of starter cultures in milk incubated at different temperatures has been used (Sullivan *et al*, 1974) to indicate the potential of individual strains to produce bitter or non-bitter cheese at specific cooking temperatures. It has been observed (Phillips, 1935) that for 3 different cultures of *Lactococcus lactis* subsp *lactis*, when the pH value of the cheese was ≤ 5.00 at 4 days, bitter flavour subsequently developed. As suggested by Harris and Hammer (1940), if a *Micrococcus* is to be employed in making Cheddar cheese from pasteurised milk, it should be selected on a strain rather than on a species basis because of the differences between cultures apparently belonging to the same species. As reported by Klimovsky *et al* (1970), *Lactococcus lactis* subsp *lactis* and *Lactococcus lactis* subsp *cremoris* strains may give rise to bitterness in Cheddar cheese and also in Dutch-type cheeses. Martley and Lawrence (1972) have also come to the conclusion that it is unwise to regard ML_1, a strain of *Lactococcus cremoris*, as a typical non-bitter starter because this strain gives slightly bitter and other characteristic off-flavours, variously described as "burnt" or "malty". Similarly, Lawrence *et al* (1972) stated that the strain of starter used appeared to be the only factor controlling the acceptability of Cheddar cheese and the development of off-flavours such as bitterness when normal commercial cheese-making conditions were used. Their finding is not in full agreement with the conclusions of Reiter and Sharpe (1971) from trials with cheese made under carefully controlled conditions in an aseptic vat, that the chemical composition of the milk (which may be influenced by the feed of the cows and the effect of the native milk enzymes, *ie* lipases and proteases) and the bacteriological quality of the cheese milk may also influence the development of cheese flavour.

The culture characteristics play a more decisive role in bitter flavour production than the type of milk-clotting enzyme (Nelson, 1974). Cheeses made with "slower" starter strains (ML_1, E_8, TR, R_1, US_3, SK_{11}, AM_1 and AM_2 of *Lactococcus lactis* subsp *cremoris* (Exterkate, 1976)) have been found to exhibit good flavour, quite free from bitterness, while those made with "faster" starter strains (HP, C_{13}, KH, FD_{27} and Wg_2 of *Lactococcus lactis* subsp *cremoris* (Exterkate, 1976)) exhibited bitterness. The starter strains HP and Wg_2 were found to be capable of producing bitter cheeses on their own, without any interaction with

rennet (Visser, 1977b). When used as supplemental starters, strains of *Streptococcus faecalis* were found unsuitable for Cheddar cheese manufacture because they produced more defective flavours than did their controls. However, 2 strains of *Streptococcus durans* (15–20 and 9–20) could be considered for supplemental use as commercial starters because they significantly and consistently produced cheeses with fewer defects than their controls, which were manufactured only with lactococci (Jensen *et al*, 1975). As observed by Lawrence *et al* (1976), *Lactococcus lactis* subsp *cremoris* strains are less likely than *Lactococcus lactis* subsp *lactis* strains to give fruity and bitter defects in Cheddar cheese. The central role of starter bacteria in the ripening of Gouda cheese has been established by Kleter (1976), who found that it was possible to make Gouda cheese with a normal ripening process and normal organoleptic properties when no bacterial enzymes other than those from a proper starter *Lactococcus lactis* subsp *cremoris* were active in the cheese. *Lactococcus lactis* subsp *lactis* C_2 has the potential to produce bitter cheese based on its growth in milk at the normal cooking temperatures for Cheddar (Sullivan *et al*, 1974; McKay and Baldwin, 1978). Improvement of *Lactococcus lactis* subsp *lactis* C_2 as a potential Cheddar cheese starter, by genetic manipulation, reduced the proteolytic activity of the stabilised transductants as compared to the parent. This resulted in a reduction in the formation of bitter flavour in Cheddar cheese (Kempler *et al*, 1979).

Lactic starters prepared from non-bitter strains such as *Lactococcus lactis* subsp *lactis* INIA 12, may be employed to minimize the risk of bitterness in cheese varieties where fast acid production is not essential for the manufacturing process, *ie* Spanish Burgos cheese manufactured from pasteurised milk, but without inoculation with lactic starters (Chavarri *et al*, 1988). However, as reported by Nuñez *et al* (1982) there is no specific commercial starter for the manufacture of Manchego cheese in Spain, and bitter flavour defects due to the use of imported starters have been frequently observed.

Lowrie *et al* (1972) have concluded that culture strains which produce little or no bitterness and good cheese flavour exhibit one or more of the following characteristics: 1), a low rate of cell division at normal cheese cooking temperatures; 2), poor survival in cheese during curing; 3), low proteolytic activity; 4), high acid phosphatase activity (Dinesen, 1974; Nelson, 1974).

Behaviour of bitter and non-bitter strains

Lawrence and Gilles (1969) observed that cheese was seldom bitter when non-bitter strains were used as starter, regardless of the amount of rennet, salt or moisture present, or the rate of acid production in the curd, or the final pH of the cheese. However, when bitter strains were used, the cheese was invariably bitter except when the salt-in-moisture levels and the pH of the cheese were relatively high. They also found (Lawrence and Gilles, 1971) that the bitterness of cheese manufactured with the bitter strains was directly proportional to the amount of rennet used. This was in contrast to cheese manufactured with the non-bitter strains, since a 2- or 3-fold increase in the amount of rennet used did not produce a bitter cheese (Jago, 1974).

As mentioned by Exterkate and Stadhouders (1971), bitter and non-bitter strains of lactococci differ in their growth characteristics: bitter strains grow rapidly under normal cheese-making conditions and reach high populations in the cheese curd prior to salting; on the other hand, the multiplication of non-bitter strains is inhibit-

ed at the normal cooking temperature in cheese-making. According to their results, all strains must therefore be potentially capable of contributing directly to the formation of bitter-flavoured components in cheese.

If the starter population density in the cheese at salting is allowed to become too high, however, flavour defects such as bitterness or fruitiness are produced and detract from or mask cheese flavour (Lawrence *et al,* 1976). The role of starter may therefore be merely to provide a suitable environment which allows the elaboration of cheese flavour (Lowrie *et al,* 1974).

As reported by Dunn and Lindsay (1985), even if many strains of lactococci (*eg*, ML_8) produce bitter peptides in more aged cheeses, they are preferred to non-bitter strains because of their efficiency in the cheese-making process. Indeed, these latter strains tend to be sensitive to salt and exhibit poor survival in cheese. It is then advisable, in order to avoid the development of off-flavours and bitterness, to select the bacterial population of a starter on the basis of its proteolytic activity and its rate of lactic acid production over the entire range of temperature and pH occurring during manufacture and maturation of cheese (Emmons *et al,* 1962a).

Bitterness in cheese and degradation of bitter peptides

From their experiments, Emmons *et al* (1960a, b) concluded that bitterness in Cheddar cheese is closely associated with the strain of starter culture. Their results suggested that bitterness was due to a deficiency, in the strains used, of proteolytic enzymes capable of hydrolysing bitter primary breakdown products of the cheese protein. According to the nitrogen content of Cheddar cheese samples (Emmons *et al,* 1962a), strains that produced bitter cheese hydrolysed bitter-tasting peptides less extensively than strains that produced non-bitter cheese. Jago (1962) came to a similar conclusion, *ie* that the differences between bitter and non-bitter strains were due to the inability of bitter strains to hydrolyse the bitter peptides produced by rennet. It was suggested that bitterness may result from the formation of pyrrolidone carboxylic acid (PCA) at the N-terminal end of a hydrophobic peptide derived from casein during proteolysis. Later, Sullivan and Jago (1970a, b) claimed that the possession of a pyrrolidone carboxylyl peptidase by non-bitter, but not by bitter starters, was the critical difference between strains in their ability to degrade bitter peptides. This enzyme specifically hydrolyses the peptide bond joining PCA residues to the remainder of peptides and proteins. This was further correlated by the detailed investigation of Exterkate and Stadhouders (1971) who showed that the enzyme activity was not only present, but was higher, in cell extracts from bitter strains than from non-bitter strains.

When the amount of bitter peptides present in bitter as well as in non-bitter cheeses exceeds the threshold value for bitter taste perception, the cheese is rated bitter (Visser *et al,* 1983b). Two reasons why the concentration of bitter peptides may never exceed the threshold at which bitterness can be detected in cheese made with non-bitter strains were reported by Lawrence *et al* (1972): a), non-bitter starters would be expected to degrade high MW peptides at a slower rate since they exhibit less proteolytic activity in cheese than bitter starters (Martley and Lawrence, 1972); and b), non-bitter strains may have a peptidase activity greater than that of bitter strains. The conclusion of Gordon and Speck (1965) that a bitter strain was more proteolytic than a non-bitter one in milk cul-

ture was later asserted to be a non-general feature of all bitter and non-bitter starter strains (Martley and Lawrence, 1972). All the bitter strains are capable of producing bitter peptides from casein by the action of their specific cell wall proteinase(s) (Mills and Thomas, 1980; Exterkate, 1983). However, slow variants of these strains *ie*, those which give no off-flavours under normal conditions (Lawrence and Pearce, 1968) have lost this ability. Whole cells of *Lactococcus lactis* subsp *cremoris* HP can produce bitter peptides only from whole and β-caseins and degrade only chymosin-generated bitter peptides from α_{s1}- and (para)-κ-casein (Visser *et al,* 1983c).

Reduction of bitterness in cheese

As shown by Sullivan *et al* (1973), the power of each strain to remove the bitter taste is directly related to its ability to hydrolyse the bitter peptides to amino acids. A strain of *Pseudomonas fluorescens*, VTTE 8.7, originally isolated from soil and able to produce a complex of endo- and exopeptidases, has been found to prevent, in the presence of a microbial rennet, the formation of bitter taste in Edam cheese (Mälkki *et al,* 1976, 1978). Indeed, this strain produces proteinases and peptidases able to convert, under laboratory conditions in culture flasks, 60–90% of the milk proteins to free amino acids; therefore, the breakdown of bitter compounds will allow a modification of taste properties (Mälkki *et al,* 1979). Knowing that bitter starters lack the protease enzyme able to degrade bitter peptides, Sood and Kosikowski (1979) suggested that addition, at low levels, of microbial proteases such as fungal protease 31000 from *Aspergillus oryzae*, microbial protease P-53 of *Bacillus subtilis*, or lipase-MY from *Candida cylindracea*, to the bitter starter may lead to cheeses free of bitterness. According to Lawrence *et al* (1976), the use of cultures containing slow-coagulating variants (Stadhouders, 1974) for Gouda cheese, and the use of strains that cannot grow at 38 °C (Lowrie and Lawrence, 1972; Martley and Lawrence, 1972; Jago, 1974) for Cheddar cheese would seem to be logical approaches to the prevention of bitterness. A reduction in the maximum starter population or the deliberate infection of the starter with homologous phage was shown by Martley (1975b) to result in a marked reduction or elimination of bitterness and in a great improvement in the overall flavour acceptability of Camembert cheese. A better flavour, with a lower incidence of bitterness was also noticed for Gouda cheese manufactured with thawed single strain lactococci concentrates inoculated directly to the cheese milk (Martens, 1974).

The starter culture can be altered by inclusion of a high level of mutant strains (Prt^-) with a substantial deficiency in surface-associated proteinase and with the same peptidase activities as the parent cell (Kamaly and Marth, 1988). Because bitter flavours are related to proteinase positive (Prt^+) cell activity, use of cultures containing a high proportion of proteinase negative (Prt^-) cells may represent a logical approach for reducing bitterness development in cheese (Mills and Thomas, 1980, 1982; Richardson *et al,* 1983). A non-bitter Cheddar cheese has thus been produced using a high level (45–75% Prt^- cells), of variants of *Lactococcus lactis* subsp *lactis* (C_2) Lac^-Prt^- used to increase the normal starter population of experimental cheeses without increasing the rate of acid production during the manufacturing process. The absence of bitterness in the final product has then indicated that the intracellular peptidase system of the added mutant strain was efficient in degrading bitter peptides (Grieve and Dulley, 1983). A decrease in bitterness in Cheddar was

also obtained for cheese manufactured with starters containing Prt⁻ variants of *Lactococcus lactis* subsp *cremoris* and *Lactococcus lactis* subsp *lactis* (Monnet *et al,* 1986). Bitter cheese will no longer be produced when an originally bitter strain in which the "slow" variants, or those who have lost the ability of forming bitter peptides from casein, have grown to a large proportion of the culture (Mills and Thomas, 1980; Stadhouders *et al,* 1983) is used as a starter. Exterkate (1976) suggested that the presence of proteolytic activity P_{II} was correlated with bitter peptide production; non-bitter peptide producers such as *Lactococcus lactis* subsp *cremoris* strain ML_1 produced only P_I and P_{III}. It was later demonstrated by Hugenholtz *et al* (1984) that strain ML_1 contains the same proteolytic system as the bitter peptide producing strains Wg_2, HP and C_{13}. As this strain excretes most of its proteases into the surrounding medium, the proteases do not remain in the curd and do not contribute to bitter peptide formation during cheese ripening.

Starter-pairing

It is now recognised that the use of certain starters results in bitterness in cheese and that pairing of "bitter" starters with "non-bitter" starters markedly reduces bitterness in Cheddar (Lawrence and Pearce, 1968; Creamer *et al,* 1970) and Gouda (Visser, 1977b) cheesemaking.

Emmons *et al* (1961, 1962a, b) studied bitterness in pasteurised milk cheese as influenced by different combinations and proportions of paired strains of *Lactococcus lactis* subsp *cremoris* in the starter. They found that the level of bitterness decreased as the proportion of non-bitter strains in the starter culture increased. Non-bitter cheese was made by combining a bitter and a non-bitter strain in suitable proportions as starter in the vat. In a similar way, Creamer *et al* (1970) reported the undesirability of using "fast" single strain starters (HP, P_2, ML_8), unless these are paired with a "slow" starter (AM_2) in order to reduce the bitterness to an acceptable level; however, the bitterness of the starter cannot be suppressed entirely by this pairing. The very significant decrease in bitterness observed when the mixed starter was used can be totally explained as a dilution effect of the bitter starter by the non-bitter starter. Experiments have clearly shown that the proteolytic activity of bitter strains is different from that of non-bitter strains (Stadhouders, 1974), and that the interaction between one strain and another may be important (Reiter and Sharpe, 1971). The suitable pairing of such strains can thus contribute to considerably reduce bitter flavour in cheese.

Factors implicated in starter activity and in starter failure

The temperature of storage has been found to be critical for starters. They remain active for only a few weeks at –20 °C, but lose no activity at –40 °C when stored for 6–8 months, a period which would be long enough for successful commercial use. Freezing in liquid N_2 (–196 °C) is now the most common practice in dairying countries. Even if storage of starter concentrate at –40 °C results in a slightly lower level of cell survival compared to storage at –196 °C, the difference is unlikely to be significant when concentrates are used to inoculate mother cultures and bulk starter vessels, or even for direct vat inoculation in the case of Dutch-type cheeses (Lawrence *et al,* 1976).

The most important extrinsic factors implicated in starter failure are antibiotics and

bacteriophage attack. Since starter bacteria are sensitive to very low concentrations of antibiotic residues, milk containing antibiotics should not be accepted or used for the manufacture of cheese. However, Gavron *et al* (1982) were able to manufacture non-bitter Gouda cheese in the presence of antibiotics, and bitter cheese in the absence of antibiotics by using group N streptococcus strains which had previously been made resistant to penicillin and bacteriophage, and which were stable with regard to both lactose metabolism and proteinase activity.

An alternative method of slow and continuous acidification in cheese-making by substituting δ-gluconic acid lactone (GAL) for the starter organism was developed by Mabbitt *et al* (1955). This technique was also used (O'Keeffe *et al*, 1975) in Cheddar cheese manufacture to simulate the pH development pattern of starter cheese. These latter workers observed a very marked increase in proteolysis caused by a rapid early acid development in the cheese, and concluded that bitterness was not obviously connected with the level of proteolysis; in fact the excessive proteolysis which accompanies rapid acidification does not result in the development of bitter flavour. The contribution of rennet (or rennet substitutes) to the ripening process may be assessed by the use of aseptic cheeses in which acidulation is simulated by this technique (Visser, 1977a; Stadhouders *et al*, 1983).

Cheddar cheese manufactured under controlled bacteriological conditions using GAL in place of starter was sharp or bitter (Perry and McGillivray, 1964; Reiter *et al*, 1967), and Gouda cheese did not develop any cheese taste or flavour at all. However, after some months of ripening the latter became bitter (Kleter, 1976).

Amount of rennet and starter added to the cheese milk

According to Stadhouders (1962), 3 main factors control the concentration of rennet in cheese: 1), the quantity of rennet used in cheese-making; 2), the manner in which the curd is washed and dried (with short drying and washing times, more rennet remains in the cheese); 3), the pH of the milk and curd during cheese-making. When the pH of the curd is much below the normal pH of milk (pH 6.5–6.6), the casein retains more rennet, which is then found in larger quantity in the cheese.

The rennet concentration used in Cheddar cheese-making has an important influence on the development of bitterness, especially when temperature-insensitive strains are used (Scott, 1972; Mills and Thomas, 1980). Lawrence *et al* (1972) have shown that with these strains (AM_1, AM_2), increased rennet levels gave more bitter cheese. As found by Lawrence and Gilles (1971), bitterness scores were directly proportional to the level of rennet used with the fast lactococci starters (HP, ML_8, Z_8, BA_1, E_8 and ML_1). However, no increase in bitterness was observed with 2- to 3-fold increases in rennet used with the slow streptococci starters (AM_1, AM_2 and US_3).

Bitterness in Mozzarella cheese made by direct acidification (Keller *et al*, 1974) and in a Japanese yeast-ripened cheese (Kaneko and Yoneda, 1974) may be avoided by reducing the amount of rennet.

Since the relative clotting power of rennet is not constant during the normal dairying season (Lawrence and Gilles, 1969, 1971), the level of rennet used throughout the season could be varied according to its clotting power. This would save money by

reducing the amount of rennet used, and would also lower the incidence of bitterness in Cheddar cheese when certain "fast" starters are used. Furthermore, it is still possible to reduce the level of rennet normally used (70–85 g per 450 kg of milk) when the setting temperature is raised by 0.63–1.26 °C.

The rennet retention is linearly related to the amount of rennet added to the milk (Visser, 1977a). Retention is greatly increased with decreasing milk pH and with greater starter activity (Stadhouders and Hup, 1975; Lawrence *et al,* 1984). A study by Vassal and Gripon (1984) on Camembert-type soft cheeses, made with *Lactococcus lactis* subsp *cremoris* and *Penicillium caseicolum* as surface flora, has led them to conclude that the pH of the milk at renneting modified only slightly the quantity of rennet retained in the curd, whereas the quantity of rennet had a much greater influence. In fact the amount of retained rennet varied almost in a linear manner but did not affect the development of bitterness.

The significance of both the starter strain and type of rennet used in the development of the bitter flavour defect in the manufacture of Cheddar cheese was shown by Lawrence *et al* (1972): 1), the calf rennet concentration determined both the acceptability and the intensity of bitterness when the "faster" strains of starter were used; 2), at any given calf rennet concentration the strain of starter used determined the flavour and acceptability of the resulting cheese.

Research in New Zealand has clearly established that if starter and non-starter growth is controlled so as not to reach levels that produce off-flavours and if as little rennet as possible is used, the flavour which develops in Cheddar cheese is likely to be acceptable to most consumers. It is probable that a similar situation exists in all cheese varieties (Lawrence *et al,* 1983). The rennet activity depends on its concentration in the cheese (Stadhouders, 1974) and to a great extent on the salt content (Stadhouders, 1962; Fox and Walley, 1971). There is more opportunity for the chymosin to be active in the interior of cheeses which are brined or rubbed with salt than in cheeses of which the curd is mixed with salt before pressing.

Based on the results obtained with aseptically-made Gouda cheeses, Visser (1977b) concluded that rennet alone has the potency to produce bitter peptides and, if retained in high concentrations, to make the cheese very bitter. It was also observed that the contribution of rennet to the development of bitterness in Cheddar cheese is not as important as in Gouda cheese (Stadhouders and Hup, 1975; Visser, 1977b).

Milk ultra-filtration (UF) can be used in cheese-making to produce a retentate with total solids at a suitable level; the retentate is thus a concentrate of protein and fat, and the permeate is a solution of lactose and minerals (Mortensen, 1984). Creamer *et al* (1987) manufactured Cheddar cheese from milk concentrated 5-fold by UF, and observed that despite the relatively high residual rennet concentration in the product (up to 33 RU/1 000 g cheese), bitterness was not detected. Rennet activity appears to be somewhat inhibited in UF Cheddar cheese. Such inhibition is likely to be due to whey proteins (Creamer *et al,* 1987). Moreover, incorporation of whey proteins in the UF cheese-making process also has an effect on the characteristics and yield of product and then limits the extension of the range of UF cheeses on the market (Lelievre and Lawrence, 1988).

Mistry and Kosikowski (1986a, b) have shown that starter concentration is important when retentates are used as bulk starters; beyong 1% starter, higher yield in-

creases were possible, but bitterness developed in the cheese stored for 4 months at 10 °C. In the manufacture of traditional Cheddar, the rates of acid development and moisture expulsion are critical. Therefore, the amount of normal starter culture added to the cheese milk cannot be greatly increased without affecting the quality of the final product. Lowrie *et al* (1974) have shown that a high viable starter population in curd leads to the development of bitterness. However, Grieve and Dulley (1983) have demonstrated that high starter levels do not necessarily produce bitterness and, in their experiments, have even produced Cheddar cheese with superior flavour scores. In order to increase the normal starter population of experimental cheese without increasing the rate of acid production during the manufacturing process, they used frozen mutant concentrates of a *Lactococcus lactis* subsp *lactis* C_2 Lac^- Prt^-.

Calf-rennet substitutes

Supply shortages and variations in the quality of rennet from young calves spurred efforts to discover suitable rennet substitutes (Dinesen, 1974). Since most of the proteinases will coagulate milk under suitable conditions, many proteolytic enzyme preparations of animal, plant and microbial origin have been assessed as replacements for calf rennet in the manufacture of many standard types of cheese. Very substantial numbers of people in the world are opposed to the use of certain animal secretions in cheese production on grounds of religion, morality, or diet. All these factors have contributed to the worldwide search for animal rennet substitutes of microbial or plant origin. Enzymes from the fig (ficin), pineapple (bromelain), papaya (papain), fungi (*Endothia parasitica*, and *Mucor miehei*, *Mucor pusillus*) or bacteria (*Bacillus subtilis*, *B cereus*) have been and are used as coagulants in the cheese process. Unfortunately, many of the rennet substitutes have undesirable side-effects, *eg,* bitter flavour. Any substitute for calf rennet must not only coagulate milk, but must have low proteolytic activity and produce cheese with acceptable flavour and rheological characteristics (Quarne *et al,* 1968; Edwards and Kosikowski, 1970; Sardinas, 1972; Scott, 1972; Hofi *et al,* 1976; Rao *et al,* 1979).

Calf rennet, which contains chymosin (rennin) as the main enzyme component, is well known as the best milk coagulant. While in the Netherlands no type of rennet other than calf rennet is allowed for cheese-making without special exemption (Visser, 1981), the use of substitutes from animal, plant or microbial origin has become a more general practice in several countries.

Animal origin

Cheddar cheese made using only porcine pepsin tends to have a bitter flavour when fully ripe (Scott, 1972). However, the use of porcine pepsin in rennet mixtures may be of advantage because it contributes strongly to clotting, while it is largely inactivated under the subsequent processing conditions. Chymosin is more specific than pepsin, and therefore exhibits a more limited proteolytic behaviour (Visser, 1981). Hence, in admixture with rennet, porcine pepsin has a limited proteolytic action during cheese ripening; this avoids the development of flavour defects such as bitter taste.

In a small number of studies, use of commercial and pure chicken pepsin preparations in the manufacture of Cheddar cheese did not affect the cheese-making

conditions, yield or cheese composition. However, the final product underwent faster proteolysis, more intense flavour, off-flavours and bitterness and was softer than cheese made with calf rennet (Stanley *et al,* 1980; Green *et al,* 1984).

Plant origin

As early as 1956, Windlan and Kosikowski used papain for Cheddar cheese manufacture. Unfortunately, bitterness was severe enough to make the cheese unfit for consumption.

A vegetable rennet extracted from *Withania coagulans* berries has been used to prepare Cheddar cheese from cow and buffalo milks. The former cheese was slightly bitter while that made from buffalo milk was inferior in quality and had a bitter flavour (Singh *et al,* 1973).

A coagulant obtained from the flowers of *Cynara cardunculus* L and used traditionally in Portugal for the manufacture of ewe cheese of Serra and Serpa types, has been assessed in the manufacture of Camembert and Gruyère cheeses. Unfortunately, these cheeses developed very pronounced bitterness (Barbosa *et al,* 1976).

Microbial origin

Microorganisms represent a large, inviting reservoir of potential animal rennet substitutes. However, the majority of microbial rennets are much too proteolytic for cheese-making. Replacement of chymosin by microbial rennets influences cheese ripening: proteolysis of α_{s1}- and β-caseins is increased and retarded, respectively, as observed by Christensen *et al* (1989) who suspected the type of rennet used in the cheese manufacture to have an effect on bitter flavour development in the final product. The bitter taste, often associated with increased proteolysis, may result from a different pattern of casein breakdown and an increased breakdown of whey proteins (Schulz and Thomasow, 1970).

The criteria that a microbial rennet preparation must meet in order to substitute satisfactorily for animal rennet are the following: 1), the preparation must effectively coagulate milk without excessively hydrolysing the resulting curd during or at maturation; 2), it must be non-toxic and devoid of antibiotic activity, as well as free of pathogens; 3), preferably, it should be readily water-soluble and possess acceptable colour and odour; 4), it should exhibit reasonable shelf-life (Sardinas, 1972).

Without giving any details, Windlan and Kosikowski (1956) reported that when rennet-like enzymes from microbial sources were used at optimum concentration, smooth curds resulted. Bitter flavours in either curd or Cheddar cheese were relatively insignificant.

Edam, Gouda and Cheddar type cheeses made with microbial rennets obtained from *Mucor pusillus Lindt* and *Bacillus polymyxa* were bitter. Kikuchi and Toyoda (1970) attributed this off-flavour to the inherent characteristic of the microbial milk clotting enzymes and concluded that microbial rennets could replace the conventional calf rennet in cheese-making if aspects of manufacture were modified to suit microbial rennets.

Lawrence *et al* (1972) found that both Rennilase and Meito microbial rennets, obtained from *Mucor miehei* and *Mucor pusillus* var *Lindt*, respectively, produced considerably less bitterness in Cheddar cheese manufactured with a bitter strain (*Lactococcus lactis* subsp *cremoris* strain HP) than did calf rennet. However, not all microbial rennets were alike in this respect, since a rennet obtained from *En-*

dothia parasitica produced bitter cheese even with a non-bitter starter (*Lactococcus lactis* subsp *cremoris* strain AM_2). On the other hand, a "somewhat bitter" flavour appeared more frequently in Gouda made with the microbial rennets, Rennilase and Fromase, than with calf rennet (Martens, 1973).

Microbial rennet substitutes such as Meito (from *Mucor pusillus*), Suparen (from *Endothia parasitica*) and Milcozyme (from *Bacillus polymyxa*) were also used in comparison with rennet for the manufacture of Cheddar-type, Tilsit (a Polish semi-hard cheese) and Kortowski cheeses. Cheeses made with Suparen and Meito were slightly bitter, while Milcozyme was found to be unsuitable for cheese manufacture (Reps *et al,* 1974).

Bitter flavour developed during aging of Cheddar cheese manufactured with milk clotting enzyme of *Rhizopus oligosporus* (Rao *et al,* 1979) a recombinant chymosin purified from *Escherichia coli* K_{12} (Hicks *et al,* 1984) or as a rennet substitute. However, further cheesemaking studies conducted with dilute enzyme preparations yielded to a non-bitter Cheddar cheese which did not retain excessive amounts of protease (chymosin) (Hicks *et al,* 1988). Chymosin prepared with the aid of genetically engineered microorganisms such as *E coli*, *Aspergillus niger* and *Kluyveromyces lactis* has been shown to be identical to the natural enzyme and to produce different types of cheeses (Cheddar, Colby, Edam, Gouda, etc) which were indistinguishable from normal cheese (made with natural chymosin) in regard of cheese yield, texture, smell, taste and ripening (Teuber, 1990). Since bitterness is more significant in long-hold cheese varieties such as Cheddar, then many potential rennet substitutes which render cheese bitter will have to be rejected.

Bacterial rennets from *Endothia parasitica*, *Mucor miehei*, and *Mucor pusillus* have proved sufficiently suitable for large-scale commercialisation, even if bitterness does appear particularly in some long-hold cheeses. Of these rennet substitutes, the first 2 had a greater proteolytic action on β-casein than calf rennet (Edwards and Kosikowski, 1970). Knowing that a coagulant which does not excessively hydrolyse β-casein is suitable for the production of cheeses of good quality without bitterness, Kobayashi *et al* (1985) evaluated the milk clotting enzyme from *Irpex lacteus* (IR) as a calf rennet substitute in Cheddar cheesemaking. Although IR cheese showed a slightly higher extent of proteolysis in comparison to the calf rennet control during ripening, it did not develop a bitter taste even after 6 months of ripening. IR is thus a promising rennet substitute for cheesemaking.

The use of mixtures of equal parts of calf rennet and microbial rennet (*Mucor miehei* protease) in Ras cheese-making caused bitterness in this Egyptian hard type cheese which persisted until the fourth month and disappeared thereafter (Mahran *et al,* 1976).

Use of peptidases (*eg*, *Pseudomonas* peptidase) capable of hydrolysing bitter peptides has been suggested as a possible mechanism for preventing the occurrence of bitterness, thus enabling the wider use of microbial rennets. This option has been tried for Edam cheese manufactured with a commercial rennet from *Mucor miehei;* cheeses were not bitter and proteolysis was more rapid in the experimental than in the control cheese (Mälkki *et al,* 1978).

Addition of fungal acid proteinases with pH optima close to the pH of cheese has been shown to cause excessive proteolysis, leading to bitterness (Law and Wigmore, 1982b).

Addition of proteolytic enzymes or heat-treated cells to the cheese milk or cheese slurry to accelerate cheese ripening

The influence of commercial mixtures of proteolytic and lipolytic microbial and animal enzyme preparations added directly to cheese curd and cheese slurries to accelerate cheese ripening has been studied by many workers (Fox, 1988–1989). When microbial acid proteases were added to Cheddar cheese slurries, it led to strong bitterness; however, blending of individual lipases with proteases or peptidases before addition to cheese blends or slurries created pronounced cheese flavour with less bitterness and rancidity (Kosikowski and Iwasaki, 1975). Similarly, the incorporation of fungal protease 31000 from *Aspergillus oryzae*, microbial protease P-53 of *Bacillus subtilis*; or MY-lipase from *Candida cylindracea* into slurries of salted Cheddar curds did not cause the development of flavour defects (Sood and Kosikowski, 1979); it seems that as soon as bitter peptides were formed they were hydrolysed into still smaller peptides and free amino acids.

Different results were obtained when the same food-grade enzyme preparations were added to Cheddar cheese curds as powders mixed with the salt (Law and Wigmore, 1982c; Fox, 1985). Acid proteinases invariably yielded bitter cheeses with an unacceptable soft, crumbly and greasy texture (Law and Wigmore, 1982a). Neutral proteinase when added at only 0.001–0.002% (w/w) produced medium-flavoured cheese at 12 °C in 2 instead of 4 months; addition of neutral proteinase at higher concentration resulted in bitter cheese. When Rhozyme P_{II}, a neutral protease from *Aspergillus oryzae*, was added to the milled curd during the manufacture of Cheddar cheese, proteolysis and bitterness increased with storage time and enzyme level (from 0.01–0.1% of curd weight) (Fedrick *et al*, 1986). These authors also found that Colby cheese prepared with a very low amount of Rhozyme P_{II} developed mature cheese texture without bitterness. Cheddar cheese exhibited moderate proteolysis with extreme bitterness when it was treated with Subtilisin A, the *Bacillus licheniformis* alkaline proteinase, to accelerate ripening (Law and Wigmore, 1982a).

Bitterness was absent when peptidases from *Pseudomonas fluorescens* VTTE 8.7 were added to Edam-type cheese in conjunction with a commercial *Mucor miehei*-type rennet. The same enzyme preparation from *Pseudomonas* prevented bitterness and enhanced the ripening process of Cheddar type cheeses made with calf rennet (Mälkki, 1978; Mälkki *et al*, 1978, 1979). A pronounced bitter flavour developed in Ras cheese (an Egyptian hard cheese) when Maxazyme, a proteolytic and lipolytic enzyme preparation, was added to the curd before moulding to increase the rate of ripening (El Soda *et al*, 1985).

Addition of germinated barley extract, Neutrase (an extracellular *Bacillus subtilis* neutral proteinase) or a combination of both enzyme preparations to the Cheddar cheese curd yielded, in all cases, a bitter flavour defect. Although Frey *et al* (1986) found this off-flavour defect to be more important in the cheese made with Neutrase alone, some workers (Law and Wigmore, 1982b; Fedrick *et al*, 1986) observed that optimum acceleration of Cheddar cheese ripening using Rhozyme P_{II} a neutral fungal protease, seemed to be accompanied by higher levels of bitterness than is the case with Neutrase. Development of a bitter taste was also observed during the ripening of a Swedish hard type cheese treated with Neutrase to accelerate the casein breakdown. It was shown that the

simultaneous addition of heat-treated (67 °C for 10 s) cells of *Lactobacillus helveticus* could eliminate the bitter taste by accelerating the breakdown of peptides in the Swedish full-fat (Ardö and Pettersson, 1988) or low-fat (Ardö *et al,* 1989) hard cheeses. Addition of Rulactine (a metalloprotease from *Micrococcus caseolyticus* with properties close to those of Neutrase) to Saint-Paulin cheese milk also promoted early proteolysis and enhanced bitterness development (Alkhalaf *et al,* 1987). However, encapsulation of Neutrase into multilamellar liposomes partially avoided bitterness. Indeed, encapsulation of proteinase into liposomes has prevented further lowering of pH at draining by slowing down the proteolysis process which was excessive for the free proteinase, and thus produced less peptides used as substrates for microbial proteinases and peptidases to yield small peptides and amino acids (Lawrence *et al,* 1983; Fox, 1988). Furthermore, the enzymes retained in the curd have been progressively released in the cheese matrix after pressing. This slow but gradually accelerated proteolysis has then helped to prevent the accumulation of bitter peptides in the cheese. From these results and those obtained by Law and Wigmore (1983) on the action of exopeptidases, Alkhalaf *et al* (1988) came to the conclusion that entrapment of both proteinase and exopeptidase within reverse-phase evaporation vesicle liposomes could eliminate the potential defect of bitterness. The presence of exopeptidase could thus enhance the degradation of bitter peptides which released low molecular weight peptides and amino acids.

In Gouda cheese, Bartels *et al* (1985a, b) found that a strain of *Lactobacillus helveticus,* when heat-treated at 70 °C for 18 s or freeze-shocked (Kim *et al,* 1987), had a similar debittering property when added as a starter adjunct (2%) to milk. The same phenomenon, but to a lesser extent, was observed when heat-shocked whole cells of *Lactobacillus bulgaricus* were added. The reduction in bitterness intensity observed in several trials by Bartels *et al* (1985a, b; 1987a), and which apparently resulted from peptidase activity of the added cells, disagrees with the observations of El Soda *et al* (1982). Indeed, the latter workers observed that incorporation of cell-free extracts from *Lactobacillus helveticus, Lactobacillus bulgaricus* or *Lactobacillus lactis* subsp *lactis* into Cheddar cheese curd enhanced bitterness. In the study of Bartels *et al* (1987a), reduction of bitterness in cheese by certain lactobacilli was related to lower concentrations of bitter peptides, separated by gel permeation chromatography. In contrast, bitterness was not reduced by addition of heat-shocked *Streptococcus thermophilus* 110 or *Lactobacillus bulgaricus* subsp *jugurti* ATCC 12278. According to El Abboudi *et al* (1991), addition of heat-shocked cells of lactobacilli to the cheese milk before renneting could contribute to the reduction of bitterness in accelerated ripening Cheddar cheese.

Accelerated ripening and reduced bitterness were noticed in Cheddar cheese prepared with Lac^- mutant strains, isolated from *Lactococcus lactis* subsp *lactis* C_2, added as a starter adjunct to milk (Dulley *et al,* 1978). However, when a cell-free extract from *Lactobacillus casei* was added to the curd during the manufacture of a Cheddar-type cheese, the ripening was accelerated but, unfortunately, bitter flavour developed rapidly (El Soda *et al,* 1981).

Before the study of Dulley (1976), attempts to accelerate cheese ripening were based mainly on the premise that proteolysis must be increased. However, Dulley (1976) observed that extra proteolysis may not be a necessary prerequisite for flavour development. Knowing that the products of

proteolysis are normally present in excess, he thought about converting them into flavour components by adding enzymes to the cheese in order to accelerate the ripening process. For that purpose Cheddar cheese slurries, ripened for about 1 week at 30 °C, were added to the cheese curd. Used as a source of enzymes and microorganisms, the Cheddar cheese slurries were shown to shorten the ripening time of Cheddar cheese by about 1,5 month without the development of excessive bitterness.

Use of commercial starter lactococci in conjunction with *Lactobacillus* strains isolated from Cheddar cheese shortened the cheese ripening process (Lee *et al,* 1990b) and also led to an improvement of Cheddar flavour (Lemieux *et al,* 1989). However, certain strains of *Lactobacillus casei-pseudoplantarum* and *Lactobacillus casei-rhamnosus* caused bitterness in the cheese (Lee *et al,* 1990a).

The manner of draining the curd

Extension of the contact time between the curd and the whey reduces the curd's buffering capacity by increasing its lactose retention and its loss of phosphorus. Cheddar cheese made from such curd becomes highly acid at maturity and this leads to the development of bitterness (Czulak *et al,* 1969).

The production of bitter components by incubation of a bitter and a non-bitter strain of *Lactococcus lactis* subsp *cremoris* in skim milk and in various media, with and without casein, was studied by Harwalkar and Seitz (1971). They observed that when curd was separated from whey after 18 h ripening, further incubation resulted in considerably greater bitterness in curd than in whole ripened milk; this could be due to increased casein and cell density in the curd.

Cheese manufacturing process

As early as 1932, Riddet *et al* concluded that it was possible to modify the cheese flavour simply by changing some details in the manufacturing process. Such changes produce curd of a different nature, and since the curd is the raw material of the ripening process, the whole course of the biochemical changes which subsequently occurs may be modified. A typical example of a change in the manufacturing process which induces cheese modifications is a variation in acidity at any stage of the process. Type of acid and pH at curd formation affect moisture, mineral content and firmness of Mozzarella cheese made by the direct acidification procedure. In this process, there is no addition of lactic starter cultures, the pH of milk and curd is adjusted by various acidulants (hydrochloric, phosphoric, or lactic acids) to a value which remains constant during whey syneresis (Keller *et al,* 1974). It was also reported by Quarne *et al* (1968) that Pizza cheese made by direct acidification using calf rennet developed bitterness.

Stadhouders and Hup (1975) have shown that the cumulative formation of bitter peptides was due either to the use of certain starters or to some method of cheese-making which resulted in too high a level of rennet retained in the cheese (the lower the pH and the lower the cooking temperature, the higher the level of rennet retained in the cheese (Holmes *et al,* 1977; Stadhouders *et al,* 1983; Creamer *et al,* 1985)).

Many workers have observed that the manufacturing conditions during cheese-making markedly affect cheese flavour,

and more specifically undesirable defects such as bitterness (Emmons *et al,* 1960a, Lawrence and Gilles, 1969; Delbeke and Naudts, 1970; Lowrie *et al,* 1972; Jago, 1974; O'Keeffe *et al,* 1975; Visser *et al,* 1983b; Berdagué and Grappin, 1988).

Cooking temperature

The final concentration of starter bacteria in the cheese and salt-in-moisture levels are known to be altered by the cheese-making cooking temperature (Crawford, 1977). Moreover, the rate of cell division during cooking has been found to be a decisive factor in bitter flavour production (Dinesen, 1974; Nelson, 1974).

Barton (1957) found that the entire production of the culture organisms used as starters in the manufacture of Cheddar cheese from pasteurised milk is governed by the rate of cooking and the final temperature reached. Although the classification of culture strains as "bitter" or "non-bitter" is arbitrary, it is more specifically related to cooking temperature used during cheese-making (Lowrie and Lawrence, 1972). In all the experiments conducted by Lowrie *et al* (1972), Cheddar cheese was bitter when the standard cooking temperature, reduced from 37.8 to 33.3 °C, permitted either bitter or non-bitter strains to grow to a high population in the curd. Conversely, when starter growth was limited, bitterness was absent or of reduced intensity in the cheese. These observations led to the conclusion that starter bacteria had a direct role in the development of bitterness in Cheddar cheese. Later, Jago (1974) suggested that the formation of bitter peptides could proceed at a much faster rate than their degradation to non-bitter products. According to Nelson (1974), slower strains exhibited higher rates of cell division and yielded a bitter cheese (Lowrie *et al,* 1974) when used in experimental cheese manufacture in which the cooking temperature was lower than normal. On the other hand, a lower rate of cell division was recorded for faster growing strains used at a higher cooking temperature than normal; the resulting cheese exhibited low levels of bitter flavour.

The cooking temperature used during the manufacture of Gouda cheese was suspected by Stadhouders (1974) to control the amount of rennet retained in the curd and thus to influence the development of bitterness in the cheese. At cooking temperatures above 35 °C (35–39 °C) less rennet was found in Gouda cheese, which then had a reduced bitter flavour (Stadhouders and Hup, 1975). However, according to Martley (1975a) Stadhouder's suggestion does not seem to apply to Camembert cheese.

Ripening temperature

As reported by Law *et al* (1979), maturation temperature was the most important factor in determining the flavour intensity of Cheddar cheese made with *Lactococcus lactis* subsp *cremoris* NCDO 924 or 1986, either in enclosed (excluding non-starter flora) or open vats. Cheeses ripened at 13 °C for 6 months developed a stronger flavour with less bitterness than those ripened at 6 °C for 9 months, irrespective of the starter or vat used.

In contrast, Gouda cheese had a more bitter flavour when ripened at 16 than at 6 °C (Stadhouders and Hup, 1975). Normal ripening temperatures for Gouda cheese being 12–13 °C, the risk of bitterness occurring was found to be greater at temperatures of 15.5 and 18 °C (Stadhouders *et al,* 1983).

Acidity or pH of cheese

Excessive acid production by the cultures used as starters is associated with bitterness (Scott, 1986). Phillips (1935) proposed to establish a relation between acidity and the development of bitter flavour at the different stages of Cheddar cheese processing. His preliminary results seemed to associate an increased bitterness in cheeses which develop acidity greater than pH 5.00 at 4 days after making. Besides, Czulak (1959) found bitter flavour most frequently in Cheddar cheese having a pH value of less than 5.0 in the first week. Dawson and Feagan (1960) showed a definite peak of bitterness within a pH range of 5.2–5.3, decreasing at greater and lower pH values. Similarly, Emmons *et al* (1962a) confirmed a dependence of bitter flavour on cheese pH and found it to be similar to the effect of pH on protein breakdown which leads to the formation of a large pool of polypeptides, including the bitter-tasting ones. They also showed that some starter strains which produced bitterness were not greatly affected by pH while others were very significantly affected.

According to Czulak *et al* (1969), when a high lactic acid level is attained rapidly, the curd does not cheddar well. Thus, cheeses from "fast-acid" vats have a low pH, a sour and bitter flavour, a crumbly body and pale colour. However, Lawrence and Gilles (1969) concluded that a high rate of acid production *per se* does not result in bitter cheese. From their results it is clear that the pH of a cheese at 14 days is not in itself very significant with respect to potential bitterness, it is of great importance only when the "salt-in-moisture" levels lie between 4.30% and 4.90%.

The results of Sullivan *et al* (1973) suggested that pH is a determining factor in the removal of bitterness by individual starter streptococci at the pH of ripening Cheddar cheese (≈ 5.0). Similarly, Nelson (1974) reported that, in Canadian Cheddar manufactured with single strains of *Lactococcus lactis* subsp *cremoris*, bitter flavour was greater in low (pH 5.63) than in high pH (pH 6.41) cheese.

Salt concentration

A close relationship between a low salt content of Cheddar cheese and a characteristic bitter flavour was mentioned as early as 1940 by Tuckey and Ruehe. However, more recently Lawrence and Gilles (1969), in agreement with the results of Stadhouders (1962), observed a decrease in bitterness in Cheddar at salt levels greater than 4.90%, and more retention of the added salt in curd salted at a low acidity than at higher acidity. These observations were confirmed by Phelan *et al* (1973) and by Golding (1947) for Cheddar cheese prepared under conditions of high moisture content at salt concentrations < 1.6%. These conditions led to extensive proteolysis, causing bitter and putrid flavours. A slight bitterness in flavour of Kaskaval, an Egyptian cheese, was also recognised by Moneib and Safwat (1972) for a salt concentration of < 4%. However, this defect was not present when the salt level was between 4–5%. In the same way, the results of Keller *et al* (1974) suggested that development of bitterness could be inhibited by increasing the salt content or by developing a mechanism for infusing the centre of Mozzarella cheese blocks with NaCl. As observed by many workers (Stadhouders and Hup, 1975; Stadhouders *et al,* 1983; Visser *et al,* 1983a, b), salt also reduces the intensity of the bitter flavour of Gouda cheese; indeed, the higher the salt content, the lower the score for bitter flavour.

Being able to retard the appearance of bitter flavour in cheese and to influence the formation/degradation of bitter peptides (Stadhouders *et al,* 1983; Visser *et al,* 1983c), salt must have a direct impact on the proteolytic activity of the cell wall and of the intracellular enzymes (Visser *et al,* 1983a). From the studies of Stadhouders *et al* (1983) on Gouda cheese, it was concluded that salt inhibits the formation of bitter peptides rather than masking the bitter flavour.

Since growth of lactococci was markedly inhibited at salt-in-moisture levels > 4.90%, Lawrence and Gilles (1969) suspected the effect of salt to be more specific on starter, and showed that high salt-in-moisture levels reduce the incidence of bitterness in cheese manufactured with a bitter starter. This was due to the inhibition, by NaCl, of the degradation of β-casein by the rennet and bacterial proteinases (Fox and Walley, 1971; Sullivan and Jago, 1972; Thomas and Pearce, 1981). Exterkate (1983) confirmed the essential role played by salt in the degradation of bitter peptides by the protoplast enzymes of the starter bacteria. Salt may act on the cell wall and membrane structures, in particular those of bitter strains (thus impairing the accessibility of starter enzymes), or have a direct inhibitory effect on various proteolytic enzymes (Lawrence and Gilles, 1969; Visser *et al,* 1983c). Salt-in-moisture levels played also a direct role in the development of bitterness in sterile buffaloes skim milk by altering the final concentration of *S faecalis* subsp *liquefaciens.* Growth of this organism was thus shown to be restricted at salt levels of 8% (Hegazi, 1989).

According to Stadhouders (1962), rennet activity is optimal at pH 5.2 and at a salt concentration of ≈ 3%. However, this activity decreases sharply as the salt concentration is increased to 5%. Since the solubility of paracasein is increased by the presence of salt, the substrate is then more readily available to the enzyme, and this may be responsible for the stimulating effect of salt on the rennet activity (Stadhouders, 1962). Fox and Walley (1971) have shown that the proteolysis of β-casein by rennet or by pepsin was significantly reduced by 5%, and completely inhibited by 10% NaCl. Increments in salt concentration from 1 to 5% were also shown to inhibit the breakdown of β-casein by *Lactococcus lactis* subsp *cremoris* bitter strain HP (Sullivan and Jago, 1972). In contrast, the rate of proteolysis of α_{S1}-casein was found to be maximal in the presence of 5–10% NaCl (Fox and Walley, 1971). The choice of brining method greatly determines the initial salt concentration in the interior of the cheese and may therefore influence the protein degradation pattern during the ripening process (Visser, 1981).

Mineral content

Mortensen (1984) reports that ultrafiltered (UF) Quark (a non-ripened cheese) production was initially hampered by bitterness problems, which were due to the high mineral content in the retentate. Bitterness has also resulted from the presence of excess minerals in UF Cast Feta, a ripened cheese variety in which factors other than proteolysis are important for product characteristics. However, the high salt content and the addition of lipase could effectively mask the bitterness defect (Lelievre and Lawrence, 1988).

Surface moulded soft cheeses (Camembert type)

As long as its growth is not limited, *Penicillium caseicolum* appears to be the princi-

pal factor responsible for bitterness of soft body cheese such as Camembert. There are 2 ways to control the growth of this organism: 1), incubation of cheeses in a slightly ammoniacal atmosphere during the first days of ripening leads to a faster increase of the pH in the outer layer; and 2), some strains of *Geotrichum candidum*, when present in cheese milk (0.25% of the inoculum), have similar effects in reducing *Penicillium* growth, acid proteinase action and bitterness (Mourgues *et al*, 1983; Ribadeau-Dumas, 1984; Vassal and Gripon, 1984).

A study of the neutral volatile compounds in 5 different types of surface ripened cheese (Maroilles, Livarot, Pont-L'Évêque, Époisses and Langres) has shown the cheese to be bitter when indole produced from the degradation of tryptophan by the bacteria was present at a concentration of 10^{-3} ppm (Dumont *et al*, 1974).

Sanitary and hygienic packaging conditions

Bitterness in cheese during the maturation process has been reported to result from overheating of milk during pasteurisation (Riddet *et al*, 1932). However, since the introduction of rigid hygiene and pasteurisation standards, the incidence of the bitter defect has still been widespread. Bitterness cannot therefore be explained entirely by the sanitary and hygiene conditions (Czulak, 1959).

As mentioned above, the development of bitterness in creamed Cottage cheese has been found to be primarily due to growth at refrigeration temperatures of psychrophilic bacteria. However, the destruction of these microorganisms and the elimination of bitterness can be achieved by the pasteurisation of the skim milk and Cottage cheese dressing (Stone and Large, 1968).

When 20% abnormal milk (from cows suffering from mastitis) was added to the cheese milk, the resulting cheese was bitter, having an unclean taste and smell and a short consistency (Sorokina and Karagueuz, 1978).

It has also been reported that poor milk-collecting and transformation hygiene, contaminated feeds or antibiotics injected into the cow might result in bitterness in cheese; thus, Gorgonzola was found to be bitter when manufactured with milk contaminated by penicillin (Delformo and Parpani, 1986).

Packaging conditions may also be responsible for the presence of bitterness in dairy products. In fact, Kosikowski and Brown (1973) have shown that gas packaging (carbon dioxide and nitrogen) was successful in preventing yeast and mould growth on creamed Cottage cheese and thus in reducing the bitter off-flavour.

CONTROL OF CHEESE BITTERNESS

The concentration of bitter peptides must exceed a certain threshold level for the bitterness to be detected in cheese. However, this threshold level has been found to be higher in old than in young cheeses. Control of bitterness in cheese, therefore, hinges on keeping the concentration of bitter peptides below the threshold level for bitter taste, either by decreasing the rate of formation of the bitter peptides, and/or by increasing the rate of their degradation to non-bitter products (Jago, 1974).

In practice, the control of bitterness in cheese can be achieved by a combination of methods which include: 1), limitation of the starter cell population; 2), maintenance

of high-salt-in moisture levels; 3), starter pairing; 4), alteration of the starter culture; 5), avoidance of the use of excess rennet.

Some 20 years ago, a deficiency of the cheese grading system was pointed out; cheese made with extended time in the whey could be of acceptable quality when graded at an early stage, but yet develop serious defects on maturing. Since then standardisation of the pH or acidity of either the whey or the curd alone is not sufficient; it is also necessary to control the rate of acid development and the time the curd is held in the whey (Czulak *et al,* 1969).

Manufacture of cheese by more sophisticated methods (*eg* addition of denatured whey proteins) may often lead to a bitter defect in the final product. Since denatured whey proteins have a stimulatory effect on the proteolytic processes and organoleptic properties of cheese, starters with strains capable of hydrolysing bitter peptides should be used to produce high-quality cheeses with added denatured whey proteins (Krasheninin *et al,* 1974).

Addition (at the 3% level) of sweet cow's whey to cheese milk before pasteurisation in order to increase the water holding capacity of buffalo cheese and the extent of peptide hydrolysis in slurries resulted in the disappearance of the bitterness usually found in buffalo cheese (Tuckey and Al-Fayadh, 1985).

Many means have been tried to accelerate cheese ripening and to replace the calf rennet due to supply shortages and variations in quality. However, a bitter defect is often noticed in the product. A controlled method for accelerated cheese ripening achieved without upsetting the flavour balance would therefore give large cost savings to the cheese industry and satisfaction to the cheese research workers. The freeze-shock treatment of lactic acid bacteria which enables addition of large numbers of whole cells to the milk seems to be a promising method for increasing proteolysis and flavour development in cheese (Bartels *et al,* 1987b). Lactobacilli species and especially *Lactobacillus helveticus* can be applied successfully to cheeses such as Gouda to enhance flavour development at initial stages of ripening without detrimental effect on the cheese-making procedures and cheese quality (Bartels *et al,* 1987a, b). Use of *Lactobacillus casei-casei* L2A in conjunction with the starter lactococci was shown to shorten the ripening period and to improve the Cheddar flavour (El Abboudi, 1990; Laleye *et al,* 1990). However, when compared to other *Lactobacillus* strains used in Cheddar cheese manufacture (Lemieux *et al,* 1989), *Lactobacillus casei-casei* L2A did not produce bitter off-flavour and was then suggested to possess debittering enzymes.

It is recommended to periodically test individual cow's milk to detect poor chymosin-coagulation characteristics; at the same time abnormal milk, which is poorly coagulated by chymosin, would also be detected. Selective breeding of lactating cows could guarantee the exclusion of poor chymosin-coagulating milk from bulk milk supplies (Okigbo, 1986).

Good hygiene and storage of milk at temperatures below 4 °C would help to limit the growth of *Pseudomonas* before UHT treatment and thus prevent the gelation of the product before the expiry date (Bassette *et al,* 1986; Dousset *et al,* 1988). Ultrasound imaging is a promising non-destructive technique by which microbiological deterioration of UHT base material for soft ice-cream caused by *Bacillus cereus* and *Staphylococcus aureus*, and of UHT milk by *Pseudomonas fluorescens* (Ahvenainen *et al,* 1989a, b, c) can be easily detected. This would be a new way to verify the microbiological sterility of the product.

CONCLUSION

Because of the great variety of technological parameters and the extremely complex system of both enzymes and substrates present in the dairy products and especially in cheese, the understanding of the cheese bitter flavour defect is still of present interest. Many studies aiming to elucidate the cause of this off-flavour defect have been considered invalid because they were carried out in relatively uncontrolled conditions. The development of the aseptic vat technique has opened a door to research on the bitterness defect by allowing the problem to be divided into parts which can be investigated separately or in appropriate combinations. This kind of study, while bringing light on the role of individual enzyme systems in bitter flavour development in cheese would possibly lead to a better insight into the mechanism of cheese ripening in general and, more fundamentally, would help to understand the basics of the cheese industry in the 21st century. Indeed, understanding the basics would allow cheese-makers to modify the composition, flavour and physical properties of cheese without creating undesirable side effects.

The second part of this review will be more concerned about the mechanism of formation of bitter peptides, their isolation, identification, composition and structure. A final point will describe ways of masking or inhibiting the bitter off-flavour defect in dairy products, more specifically in cheeses.

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