G. Kleter and F. M. W. Visser

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen

Cheese ripening studied by strictly aseptic techniques of cheesemaking

I Methods for strictly aseptic making of cheese and effect of some bacteria on its ripening
by G. Kleter

II Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese
by F. M. W. Visser

Centre for Agricultural Publishing and Documentation

Wageningen – 1977

ISBN 90 220 0654 9

G. Kleter graduated on 11 November 1977, and F. M. W. Visser graduated on 25 November 1977 as Doctor in de Landbouwwetenschappen at the Agricultural University, Wageningen, the Netherlands, on a thesis with the same title and contents.

Abstract

Kleter, G. & F. M. W. Visser (1977) Cheese ripening studied by strictly aseptic techniques of cheesemaking. I. (G. Kleter) Methods for strictly aseptic making of cheese and effect of some bacteria on its ripening. II. (F. M. W. Visser) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. Agric. Res. Rep. (Versl. landbouwk. Onderz.) 870, ISBN 90 220 0654 9, (viii) +56 p., 3 figs, 2 tables, 132 refs, Eng. and Dutch summaries. Chapter I and II also published as doctoral theses Wageningen, both with and without articles.

To study the influence of different enzymes on ripening of cheese, such as Gouda, a method was developed for making cheese strictly aseptically, including equipment for aseptic cheesemaking and aseptic milking of selected cows. In aseptically drawn milk only heat-labile micrococci and coryneforms were present. Nutrients needed for their growth were determined. Starter bacteria proved essential for the ripening process of Gouda cheese, including breakdown of protein, and production of taste and flavour substances, but strains differed widely. Lactobacilli proved to be not necessary for ripening and the strains tested caused off-flavours.

A method for the aseptic manufacture of cheeses, free either from rennet or from rennet and starter, is described, allowing the action of starter bacteria and milk protease to be studied without the interference of rennet. These cheeses, together with aseptic starter-free cheeses, were used to elucidate the contribution of rennet, starter bacteria and milk protease to breakdown of protein and to development of bitterness and cheese flavour during ripening of Gouda cheese. Their combined action was studied in normal aseptic cheeses, allowing the estimation of possible interactions. Different amounts of enclosed rennet and different starter cultures, either 'bitter' or 'non-bitter', were used in the cheeses. Proteolysis was characterized by different analytical methods.

Free descriptors: aseptic cheesemaking, cheesemaking equipment, rennet-free cheese, starter-free cheese, rennet- and starter-free cheese, Gouda cheese, cheese ripening, rennet, starter bacteria, milk protease, lactobacilli, proteolysis, lipolysis, bitter flavour, cheese flavour, (bitter) peptides, amino acids, gel electrophoresis of cheese, udder, aseptically drawn milk, micrococci, staphylococci. *Corynebacterium bovis*, heat-resistance, bacterial growth.

Preface

The Laboratory for Dairy Science and Technology at Wageningen has an extensive programme of research on cheese ripening. Professor Ir E. A. Vos initiated the approach of making cheese strictly aseptically. Under his supervision the one author (G. Kleter) developed equipment for making cheese, such as Gouda, strictly aseptically from milk of selected cows. He examined the influence of certain added bacteria on ripening of such cheese. The other author (F. M. W. Visser) used the same equipment and developed ways of studying the activities of different enzymes separately under aseptic conditions. He examined the contribution of enzymes from rennet, starter bacteria and milk singly and in combination on ripening of Gouda cheese. Both authors concentrated on protein breakdown, and flavour development during ripening. Each author has submitted a thesis based on this work. This research report contains a general view of their work. This publication contains the summaries of the articles in Netherlands Milk and Dairy Journal.

Contents

Introduction

1 The ripening of cheese

Cheese is a product of great economic and social importance. It keeps long, is nutritious and is enjoyed for its taste and flavour. It is consumed all over the world. Most types of cheese are ripened for some weeks to a year or even more, to develop their consistency and especially the taste and flavour characteristic for the type of cheese. Ripening of cheese is a complicated physical, enzymic and chemical process. The taste and flavour typical for a type of cheese requires a subtile balance between taste and flavour substances (1,2,3). Attempts to shorten ripening time, for instance by adding enzymes or a large number of certain micro-organisms to the milk, have met little success, resulting in imbalance of such a complicated system.

Taste and flavour, and physical changes, which govern the sensoric properties of the cheese, result from enzymic and other chemical processes during ripening. Enzymes involved in cheese ripening have always recieved much attention, since they rather than purely chemical reactions are largely responsible for the changes during ripening. Schormüller (4) has reviewed enzymes that can be involved in cheese ripening. Proteins, fats and carbohydrates in the cheese can all be attacked by the enzymes. The conversions of all three main components are important in relation to the development of characteristic taste and flavour. Decomposition of the lactose especially to lactic acid, is necessary also for the conservation of the cheese. Cheese is in fact a form of preserved milk substances. Breakdown of the proteins governs the physical state of the cheese, usually called the 'consistency'.

Cheese taste and flavour components can each result from different enzymic pathways. For example, ketones and aldehydes result from breakdown of citric acid or fatty acids, the latter especially in soft types of cheese with a surface growth of moulds (e.g. 5) and probably also in Cheddar cheese (e.g. 6). Amino acids result from protein breakdown or are synthesized from amines or amonia (4).

2 Enzymes active during cheese ripening

The raw milk itself contains many enzymes that could be involved in cheese ripening. However recent decades, it has become usual in factories to pasteurize the milk that is used for cheesemaking. This is done for the sake of public health and to avoid cheese defects. With the time-temperature combination usual in pasteurization (15 s 72 °C), certain enzymes are partly or completely destroyed in milk. An example of this is the milk lipase, which is highly active in cheese from raw milk, but not in cheese from pasteurized milk (7). So the presence of the enzymes from the milk itself will be limited in cheese from pasteurized milk. An exception is milk protease (8).

Most types of cheese are made with rennet, used to coagulate the milk. During ripening, the enzymes from rennet still show proteolytic activity (e.g. 9,10,11). The lipolytic activity of enzymes from rennet in the cheese seems negligible (7).

Although pasteurization of milk reduces the microbial flora and microbial enzymes in the milk, for instance lipolytic and proteolytic enzymes from Gram-negative rods (12,13) and also certain micro-organisms itself are heat-resistant. Further after pasteurization of the milk the cheesemaker adds micro-organisms, such as starter bacteria, propionic acid bacteria or moulds. Other micro-organisms enter the cheese milk by contamination after pasteurization. Thus in all types of cheese, even in cheese from pasteurized milk, considerable numbers of different types of micro-organisms and many different microbial enzymes can be found. These microbial enzymes are extremely important in cheese ripening (e.g. 4,14) through decomposition of carbohydrates, proteins and fats.

The conditions in the cheese determine the growth of all these micro-organisms and hence the amount and types of microbial enzymes. The activities of these enzymes, and the enzymes from rennet and milk are also influenced by conditions. There can be interactions between the activity of different enzymes. These aspects have been considered by Schormüller (4). Conditions in cheese depend on several factors such as moisture content, concentration of soluble constituents, pH and redox potential, which in turn are influenced by the composition of milk and especially by the cheesemaking process. The shape of the cheese and the keeping conditions too are important. For these reasons it is not surprising that so many types of cheese exist.

3 How to study the action of different enzymes

Two requirements have to be fulfilled to obtain definite information about the ripening process in cheese. First, we need a system in which the different enzymes can be studied separately and in desired amounts and combinations, without the activity of other enzymes. Secondly, the conditions in this system have to be the same as in cheese itself. These two requirements can be fulfilled by making cheese strictly aseptically.

We in the Netherlands are interested especially in Gouda cheese. Much is known about the ripening of this type of cheese (e.g. 7,15,16,17,18,19,20,21,22,23), although the tests were not under strictly aseptic conditions. A simplified model system cannot be used instead of cheese itself, if also organoleptic properties are among those to be examined. Model systems have also the danger that the conditions may differ from those in cheese. But such experiments have given valuable information (e.g. 24,25,26,27) and they have the advantage that in one experiment a single factor such as pH or NaCl content can be varied to a great extent or even several factors can be varied. However, to draw definite conclusions about the influence of the different enzymes on the ripening of cheese, aseptic cheesemaking in addition to all these researches is necessary as has also been argued by other workers such as Fryer (28) and Lawrence et al. (29).

To make cheese under strictly aseptic conditions, the bacterial count of the raw milk has to be low, preferably even nil, at least after pasteurization. An extremely low level of bacterial enzymes in the milk is necessary, since the ripening of the cheese will

take months. So we paid considerable attention to the milking of the cows and the bacterial quality of the milk. In the cheese, no growth of micro-organisms other than those being studied, is allowed. Every contamination during cheesemaking has to be prevented.

The necessity of an aseptic method has already been emphasized by von Freudenreich (30). His cows were milked under aseptic conditions and an open sterilized cheesevat was used for cheesemaking (31). This method was also used by Boekhout & Ott de Vries (32) and later by Stadhouders et al. (7,33) for Dutch-type cheese. Despite possible contamination during cheesemaking in an open vat, Stadhouders & Veringa (33) reported that some of the cheeses were usable for tests, although no full description of the bacterial quality of the cheese was given.

To study the influence of bacteria on the ripening of Cheddar cheese, aseptic cheese vats have been developed (34,35,36). Although in this way any contamination during cheesemaking could be prevented, the milk used was drawn in the normal way and was therefore not sterile, even after pasteurization. Reiter et al. (37) reported, that fully aseptic conditions were reached when the milk was drawn by cannula, but only very small cheeses of 100 g could be made, and a full description of the bacterial quality of the milk and the cheese is not given.

4 Purpose of the investigations

The purpose of our work, as reported in the five following papers, was twofold:
1. To develop a method for making cheese, such as Gouda cheese, under strictly aseptic conditions, including an aseptic milking technique and equipment for aseptic cheesemaking.
2. To examine the influence of the activity of the enzymes from certain bacteria, which might be important, on the ripening of Gouda cheese.

For the production of Gouda cheese, streptococci (*Streptococcus cremoris* strains), usually with aromaproducing bacteria (*Streptococcus diacetylactis* and/or *Leuconostoc citrovorum*), are used as starter. In cheese produced under normal conditions, there are also bacteria that survive pasteurization and may multiply in the cheese. There are heat-resistant bacterial enzymes, and contaminants after pasteurization of the milk, especially lactobacilli, which normally multiply to very large numbers during cheese ripening. The main question to be answered was, Can starter bacteria give a normal cheese ripening process without the activity of any other bacterial enzymes in the cheese? Besides its scientific interest, the question is also relevant to cheesemaking practice. Sanitary conditions on farms have improved, resulting in less bacteria in raw milk and little opportunity for growth. Moreover there is an increasing interest in closed cheese vats in factories, for better cleaning and disinfection, which could exclude, for instance, lactobacilli.

After completing the two tasks as mentioned above, we hoped to use the method of making cheese under strictly aseptic conditions for further studies on ripening of cheese.

5 Elucidation of the performance of the work

The bacterial count of the milk has to be low, preferably nil, at least after pasteurization. The bacteria still present in the milk have to be prevented from growing in the milk and in the cheese made from it. Antibiotics or other substances preventing bacterial growth may not be added, as the growth of added bacteria and the activity of enzymes might be altered, and sensory tests are unpermissible. The milk cannot be sterilized by heat (e.g. 10 min 120 °C or ultra high temperature treatment) as clotting of the milk and cheese ripening would be altered and a false impression would be obtained of ripening of cheese.

Comparison of samples of milk obtained by aseptic-hand-milking with those obtained by puncture of the reservoir of the udder (38,39,40) have shown that the milk in the upper parts of the udders of clinically healthy cows is usually sterile and is contaminated during milking by the typical flora of the teat canal. So the best way of obtaining milk seemed that developed by Tolle & Zeidler (41). The milk is drawn directly from the reservoir of the udder to avoid contamination in the teat canal. As yet the method has not proved as useful as first claimed (42). A cannula (37) may be used for drawing only small amounts of milk. So only aseptic milking of cows as done by von Freudenreich (30,31) and others (7,32,33) remains feasible. Disinfection of the teat canal before each milking has not been described, as far as we know. In preliminary tests (43) we in our laboratory found that disinfection of the teat canal gave variable results and proved impractical. However, teat-canal bacteria found in milk, are destroyed by pasteurization (30,44,45,46). In factories, milk for cheese is usually pasteurized, so we could do so during our experiments.

For our requirements, the bacterial count of the raw milk obtained by aseptic milking is rather high (47,33). The bacterial count of the milk from individual cows with clinically healthy udders had to be examined in order to obtain raw milk with an extremely low bacterial count. Also the bacterial flora in the aseptically drawn raw milk, the heat-resistance and growth of these bacteria had to be examined, and a method to monitor the cheese made from this milk for absence of these bacteria – in the presence of starter bacteria – had to be found.

For selection of the cows and for aseptic milking, help was given by the Milk Hygiene Research Centre at Wageningen and one of the staff of that centre was co-author of the paper on aseptic milking of cows (Kleter & de Vries, 1974).

To prevent any contamination during cheesemaking, an aseptic cheesemaking equipment had to be designed and constructed. In particular, contamination by lactobacilli must be prevented, since they multiply to very large numbers in cheese. If the action of rennet and possibly heat-resistant milk proteases are studied without the activity of any bacterial enzymes, even an extremely small contamination with any type of micro-organism would risk bacterial growth in the cheese, as the lactose is still present. Therefore the equipment for making cheese under strictly aseptic conditions had to be completely closed and had to withstand sterilization by steam before each cheesemaking. To allow simultaneous experiments with the same milk supply, for instance with inoculation of milk with only starter bacteria and inoculation also with lactobacilli, two aseptic vats were made. The chemical properties of the cheese from the two vats, for instance pH and moisture content, had to be exactly the same at the

beginning of the ripening period.

During ripening, the cheese was monitored microbiologically and chemically, and was submitted to a panel for sensory assessment. The cheese had to be monitored for microbes for two reasons. First, to check for undesired micro-organisms whether strictly aseptic conditions were achieved. Secondly, to count micro-organisms that were being investigated, during ripening in the cheese. The cheese had to be monitored chemically, also for two reasons. First, to check the cheese for the right composition. Secondly, to monitor the ripening process. Development of the technique for making cheese strictly aseptically, including selection of cows, aseptic milking and development and construction of equipment for aseptic cheesemaking, was laborous and time-consuming. Therefore, as a first approach, chemical monitoring of cheese was by simple methods, but had to give as complete a picture of the ripening process as possible.

References

1. F. V. Kosikowski & G. Mocquot. Advances in cheese technology. F.A.O., Rome, 1958, p. 141.
2. H. Mulder. Taste and flavour forming substances in cheese. Neth. Milk Dairy J. 6 (1952) 157.
3. L. A. Mabbitt. The flavour of Cheddar cheese. (Review). J. Dairy Res. 28 (1961) 303.
4. J. Schormüller. The chemistry and biochemistry of cheese ripening. (Review). Adv. Food Res. 16 (1968) 231.
5. J. E. Kinsella & D. H. Hwang. Enzymes of Penicillium roqueforti involved in the biosynthesis of cheese flavor. (Review.) Crit. Rev. Food Sci. Nutr. 8 (1977) 191.
6. J. R. L. Walker. Some volatile compounds in New Zealand Cheddar cheese and their possible significance in flavour formation. J. Dairy Res. 28 (1961) 1.
7. J. Stadhouders. The hydrolysis of cheese fat in relation to the flavour of the cheese. Thesis. Agricultural University Wageningen. Mededelingen Landbouwhogeschool 56, 1956.
8. A. Noomen. Proteolytic activity of milk protease in raw and pasteurized cow's milk. Neth. Milk Dairy J. 29 (1975) 153.
9. N. J. Berridge. Rennin and the clotting of milk. Adv. Enzymol. 15 (1954) 423.
10. R. B. O'Keeffe, P. F. Fox & C. Daly. Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. J. Dairy Res. 43 (1976) 97.
11. F. M. W. Visser. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. III. Protein breakdown: analysis of the soluble nitrogen and amino acid nitrogen fractions. Neth. Milk Dairy J. 31 (1977) 210.
12. F. M. Driessen & J. Stadhouders. Thermal activation and inactivation of exocellular lipases of some Gram-negative bacteria common in milk. Neth. Milk Dairy J. 28 (1974) 10.
13. F. M. Driessen. Eiwitafbraak in stromend gesteriliseerde melk en melkprodukten. Voedingsmiddelentechnologie 9 (1976) No. 21 p. 12.
14. E. F. Gale. The chemical activities of bacteria. University Tutorial Press, London, 1951.
15. W. van Dam. Onderzoekingen over het Edammerkaasrijpingsproces. Versl. Landbouwk. Onderz. 7 (1910) 56.
16. H. A. Sirks. Eenige proeven over den invloed van den reëelen zuurheidsgraad op de rijping van Edammer kaas. Versl. Landbouwk. Onderz. 49 (1943) 221.
17. C. W. Raadsveld & H. Mulder. The influence of the temperature on the ripening of Edam cheese. Neth. Milk Dairy J. 3 (1949) 117.
18. C. W. Raadsveld & H. Mulder. The influence of pH on the ripening of Edam cheese. Neth. Milk Dairy J. 3 (1949) 222.
19. C. W. Raadsveld. Ripening phenomena in cheeses of the Edam type with different water contents and different salt concentrations. Neth. Milk Dairy J. 6 (1952) 342.
20. P. J. de Koning. Papierchromatografisch onderzoek van peptiden en aminozuren geïsoleerd uit kaas. Neth. Milk Dairy J. 14 (1960) 34.

21. J. Stadhouders. De eiwithydrolyse tijdens de kaasrijping. De enzymen die het eiwit in kaas hydrolyseren. Neth. Milk Dairy J. 14 (1960) 83.
22. J. Stadhouders. De eiwitafbraak in kaas gedurende de rijping. Enige methoden om de rijping te versnellen. Neth. Milk Dairy J. 15 (1961) 151.
23. L. A. M. Ali & H. Mulder. Taste and flavour-forming compounds in cheese. The amino acid content of Edam cheese. Neth. Milk Dairy J. 15 (1961) 377.
24. F. A. Exterkate. An introductory study of the proteolytic system of streptococcus cremoris strain HP. Neth. Milk Dairy J. 29 (1975) 303.
25. F. A. Exterkate. The proteolytic system of a slow lactic-acid-producing variant of streptococcus cremoris HP. Neth. Milk Dairy J. 30 (1976) 3.
26. F. A. Exterkate. Comparison of strains of Streptococcus cremoris for proteolytic activities associated with the all wall. Neth. Milk Dairy J. 30 (1976) 95.
27. A. Noomen. Personal communication.
28. T. F. Fryer. Microflora of Cheddar cheese and its influence on cheese flavour. (Review). Dairy Sci. Abstr. 31 (1969) 471.
29. R. C. Lawrence, T. D. Thomas & B. E. Terzaghi. Cheese starters. (Review). J. Dairy Res. 43 (1976) 141.
30. E. von Freudenreich. Ueber einen neuen, in geblähten Käsen gefundenen Bacillus (Bacillus schafferi). Landw. Jb. Schweiz. 4 (1890) 17.
31. E. von Freudenreich. Uber die Erreger der Reifung der Emmenthaler Käse Landw. Jb. Schweiz. 11 (1897) 85.
32. F. W. J. Boekhout & J. J. Ott de Vries. Over de Edammerkaas-rijping. Landbouwk. Tijdschr. 9 (1901) 356.
33. J. Stadhouders & H. A. Veringa. Fat hydrolysis by lactic acid bacteria in cheese. Neth. Milk Dairy J. 27 (1973) 77.
34. L. A. Mabbitt, Helen R. Chapman & M. Elisabeth Sharpe. Making Cheddar cheese on a small scale under controled bacteriological conditions. J. Dairy Res. 26 (1959) 105.
35. K. D. Perry & W. A. McGillivray. The manufacture of 'normal' and 'starter-free' cheddar cheese under controlled bacteriological conditions. J. Dairy Res. 31 (1964) 155.
36. Helen R. Chapman, L. A. Mabbitt & Elisabeth M. Sharpe. Apparatus and techniques for making cheese under controlled bacteriological conditions. Proc. 17th Int. Dairy Congr. (München, 1966) D–1: 55.
37. B. Reiter, Y. Sorokin, A. Pickering & A. J. Hall. Hydrolysis of fat and protein in small cheeses made under aseptic conditions. J. Dairy Res. 36 (1969) 65.
38. J. A. Beech & D. Forbes. Pathogens in the streak canal. Vet. Rec. 77 (1965) 1086.
39. H. J. Heidrich, D. Grossklaus & M. Mülling. Vergleichende bakteriologische Untersuchungen an durch Handmelken und durch Venülenpunktion der Drüsenzisterne gewonnene Milchen. I. Untersuchungen an Vernülenmilchen. II. Vergleichende Untersuchungen an Handmelk- und Vernülenmilchen. Berl. Münch. tierärztl. Wschr. 77 (1964) 83,85.
40. W. Trautmann. Vergleichende bakteriologische Untersuchungen über die primäre und sekundäre Milchflora an durch Handmelken und durch Punktion der Drüsenzisterne gewonnene Proben. Schweiz. Landw. Forsch. 12 (1973) 125.
41. A. Tolle & H. Zeidler. Der kontinuierliche Milchentzug – ein experimentell-chirurgisches Verfahren zur Gewinnung steriler Rohmilch. Milchwissenschaft 24 (1969) 590.
42. H. Grossberger. Zur experimentellen Gewinnung steriler Rohmilch über ein implantiertes Kathetersystem. Thesis. Freie Universität Berlin, Journal-Nr. 759, 1971.
43. R. J. M. le Rütte. Internal report. Agricultural University of Wageningen, Dept. Dairy Science and Technology.
44. T. Gibson & Y Abd-El-Malek. The effect of low-temperature pasteurisation on the bacterial flora of milk. Proc. Soc. agric. Bact. (1940) 19.
45. T. Gibson & Y. Abd-El-Malek. The micrococci of milk. Proc. Soc. agric. Bact. (1941) 31.
46. Y. Abd-El-Malek & A. H. Kirdany. Studies on the bacterial flora of buffalo's and cow's udder milk. Cairo University, Faculty of Agriculture. Bulletin No. 86, 1956.
47. J. Stadhouders & H. Mulder. Fat hydrolysis and cheese flavour. I. The enzymes responsible for the hydrolysis of fat in cheese. Neth. Milk Dairy J. 11 (1957) 164.

Aseptic milking of cows

G. Kleter[1] and Tj. de Vries[2]

[1] Dairying Laboratory, Department of Food Science, University of Agriculture, Wageningen, the Netherlands
[2] Milk Hygiene Research Centre (MOC), Wageningen, the Netherlands

Received: 7 November 1974

Summary

To study the influence of certain special bacteria upon the ripening of Gouda cheese, without the activity of all other micro-organisms, aseptic milking of cows is necessary. Since the bacterial count of the milk from individual cows with clinically healthy udders was found to vary considerably (Table 1), the cows were selected so as to obtain milk which was suitable for our purpose.

With unselected cows, we had an average bacterial count of 138 per ml. This is lower than figures taken from literature, which normally quote some hundreds per ml. Using selected cows, we were able to obtain quantities of about 100 litres of raw milk with a bacterial count which was nearly always < 100, on average 46 and sometimes even only 6 per ml.

In the raw milk no coliforms, other Gram-negative rods or lactobacilli were found. After pasteurization at 72°C for 15 s, no bacteria could be detected, even when portions of 100 ml of pasteurized milk were kept at 20°C for weeks.

Neth. Milk Dairy J. 28 (1974): 212-219

The bacterial flora in aseptically drawn milk

G. Kleter

Dairying Laboratory, Department of Food Science, University of Agriculture, Wageningen, the Netherlands

Received: 7 November 1974

Summary

The bacterial flora in aseptically drawn mixed milk from selected cows (1) has been subjected to further study.

In 8 out of 30 milkings (1) no strict anaerobic micro-organisms could be detected. In 20 milkings only Gram-positive, catalase-positive cocci were found after examination with plate count agar containing 0.1% (w/v) skim milk powder (PCMA). After the addition of 0.5% (w/v) Tween 80 to this medium, coryneforms were also found.

Of 6 milkings 169 strains were aselectively isolated from plates with PCMA with 0.5% (w/v) Tween 80. Of these strains 45 proved to be coryneforms and 124 cocci. The cocci could grow on PCMA, PCMA + 0.5% (w/v) Tween 80, nutrient agar (NA) and NA + 0.5% (w/v) Tween 80. The coryneforms were able to grow on PCMA and NA, both with 0.5% (w/v) Tween 80, but not on PCMA and NA without Tween 80.

Of the 124 isolated cocci strains, 13 belonged to *Micrococcus* species, 106 were *Staphylococcus epidermidis* and 5 *Staphylococcus aureus*. The 45 coryneforms isolated were all identified as *Corynebacterium bovis*. During their examination it was found that the *C. bovis* strains were able to produce acid from glucose in peptone water with Tween 80, but not in peptone water alone, which is normally used for this test.

All isolated strains were destroyed in milk by heating for 30 min at 63°C, starting with an inoculation of millions per ml. Most cocci and all the coryneforms were even killed by a heat treatment of only 5 min at 63°C. Further it was found that a heat treatment of 15 s at 60°C was sufficient to reduce the amount of coryneforms from about 1000 per ml to <1 per ml.

The cocci in the aseptically drawn milk had a lag-phase in this milk, but no bactericidic action could be detected. At 37°C the lag-phase was only a few hours. If the cocci were adapted to the milk, they had no lag-phase at all.

The coryneforms were not able to grow in aseptically drawn, pasteurized (15 s, 82°C) milk.

It is assumed that the bacterial flora present in the udder of clinically healthy cows, and which can therefore be found in aseptically drawn milk, is not in contact with the milk in the udder. After milking, these bacteria have to adapt to the milk, probably without being affected by lactenins.

Neth. Milk Dairy J. 28 (1974): 220-237

Apparatus for making cheese under strictly aseptic conditions

G. Kleter

Dairying Laboratory, Department of Food Science, University of Agriculture, Wageningen, The Netherlands

Received : 16 May 1975

Summary

Enzymes are of great importance to the chemical and physical changes in cheese during the ripening. As there are so many enzymes involved, such as rennet and enzymes from different types of micro-organisms, it is necessary to have a system in which the effect of these enzymes can be studied separately and in the desired combinations, without the results being complicated by the presence of other enzymes.

To comply with this requirement, a new method of aseptic milking of cows (11) and an apparatus for making cheese under strict aseptic conditions, were developed. A description of the apparatus is given. It consists of two completely closed, identical vats equipped with a knive/stirrer and with a capacity of 40 litres each. The vats can be sterilized at 120 °C for 20 min, and everything necessary during the cheesemaking process is already suspended in the vats before sterilization or can be added aseptically during cheesemaking.

Neth. Milk Dairy J. 29 (1975): 295-302

The ripening of Gouda cheese made under strictly aseptic conditions. 1. Cheese with no other bacterial enzymes than those from a starter streptococcus

G. Kleter

Dairying Laboratory, Department of Food Science, University of Agriculture, Wageningen, the Netherlands

Received: 29 November 1976

Summary

The ripening of Gouda cheeses made under strict aseptic conditions and with no other bacterial enzymes active than those from a starter streptococcus has been studied in comparison with that of cheeses made with the same starter under normal conditions.

On the basis of the amount of water-soluble, non-coagulable nitrogen, amino acid nitrogen, acidity of the fat and organoleptic examination of the cheese, it was found that it is possible to make Gouda cheese with a normal ripening process and normal organoleptic properties, when no other bacterial enzymes than those from a proper starter streptococcus are active in the cheese.

Gouda cheese in which only rennet and heat resistant milk proteases but no bacterial enzymes had been active, differed considerably from cheese with starter streptococci. The amino acid content in particular remained at a very low value in the cheese during ripening, and organoleptic examination revealed no cheese taste and flavour at all. In addition, the cheese became bitter after some months of ripening.

It is concluded that the central role of starter bacteria in the ripening of Gouda cheese has been established.

Neth. Milk Dairy J. 30 (1976): 254-270

The ripening of Gouda cheese made under strictly aseptic conditions. 2. The comparison of the activity of different starters and the influence of certain lactobacillus strains

G. Kleter

Dairying Laboratory, Department of Food Science, University of Agriculture, Wageningen, the Netherlands

Received: 10 May 1977

Summary

In this second report about the ripening of Gouda cheese made under strictly aseptic conditions, the activity of various starters and the influence of certain lactobacillus strains is described.

In addition to the results reported earlier (1), a remarkable difference was found between *Streptococcus cremoris* strains in their ability to produce amino acids and other cheese taste and flavour components, and also in relation to the development of cheese taste and flavour defects.

The comparison of aseptically made Gouda cheese with a proper single strain starter and aseptically made Gouda cheese with mixed strain starters from practice showed that protein and fat breakdown in the two types of cheese were about the same. It is stressed that, from the results of this and the previous publication (1), no definite answer can yet be given to the question whether or not mixed strain starters are preferable to single strain starters for the production of Gouda cheese in relation to the ripening process.

The experiments with types of lactobacilli which are normally present in high numbers in Gouda cheese, showed that these bacteria are not necessary for a good ripening process. Their presence may even be undesirable in relation to the development of taste and flavour defects in Gouda cheese.

Discussion and conclusions

1 Selection of cows and bacteriological quality of aseptically drawn milk

For aseptic milking, only cows with clinically healthy udders were used. During a complete lactation, samples of milk from 17 cows were drawn aseptically halfway through a normal milking procedure. The bacterial count (number concentration of bacteria) in milk differed widely from cow to cow. For 6 cows, the median ranged from 15 to 76 ml^{-1}, for 6 cows from 130 to 350 and for 5 cows from 1 100 to 13 000 ml^{-1}. The lowest count in milk from some of the 17 cows was much higher than the highest count from other cows. For our purpose, selection of cows is indeed worthwile (Page I–5 and I–6).

The portions, each of about 100 litres raw milk obtained by aseptic milking of the selected cows through a period of about two years, had an extremely low bacterial count. The lowest count in milk was 6, the highest 145, the median 32 and the mean 46 ml^{-1}. These values were all less than for 11 portions of milk obtained in the same way but without selection of the cows, in preliminary tests.

In the aseptically drawn raw milk, no coliforms or other Gram-negative rods and no lactobacilli were found. After pasteurization, the milk proved to be sterile: after keeping portions of 100 ml for weeks at room temperature, no spoilage occurred and no bacteria could be detected.

Our requirements for the bacteriological quality of the milk that was to be used for making cheese in a strictly aseptic way (Page I–5 and I–6), were met by the extremely low bacterial count of the aseptically drawn raw milk and the sterility of the milk after pasteurization.

2 Bacterial flora of aseptically drawn milk from selected cows

In each portion of about 100 litres of aseptically drawn milk from selected cows, no strictly anaerobic bacteria were found. With plate count agar, only micrococci were found. With plate count agar plus 0.5% (w/v) Tween 80, coryneforms were also detected. The micrococci were not inhibited by the Tween 80, so the latter medium is preferable for counts on aseptically drawn milk.

Of the 169 strains isolated from plates with plate count agar plus 0.5% Tween 80, 13 proved to be *Micrococcus* species, 106 were *Staphylococcus epidermidis,* 5 *Staphylococcus aureus* and 45 *Corynebacterium bovis.* All 169 strains grew on nutrient agar plus 0.5% Tween 80, on which starter bacteria did not grow. With this medium, one can therefore check in cheese that bacteria are absent from the aseptically drawn milk, in the presence of starter bacteria. Such a medium is

especially needed when raw milk is used for aseptic cheesemaking to check that these inevitably present bacteria have not multiplied.

All strains were destroyed (= reduced to less than 5 ml^{-1}) in milk that has been inoculated with several million per millilitre, when heat treated at 63 °C for 30 min. Most strains were even destroyed by a heat treatment of 5 min at 63 °C. These results explain why aseptically drawn milk proved sterile after the pasteurization treatment that is usual for cheese milk (Page I-17), and is equal to 63 ° C, 30 min.

When the aseptically drawn raw milk was kept at temperatures up to 45 °C, its bacterial flora proved to have a lag-phase. At 37 °C the lag-phase was only some hours. At 4 °C even after two days the bacterial count was still the same as directly after milking (in these cases about 20 ml^{-1}).

The bacteria present in aseptically drawn milk must have to adapt to the milk, but between 30 °C and 40 °C the lag-phase is short. So milk should be cooled at milking if the raw milk has to be kept for some time and the bacterial flora must not develop. Cooling during milking was also necessary, when raw milk was used for aseptic cheesemaking.

Bacteria found in the aseptically drawn milk, were not from the milk in the udder of the cow, but were indeed harboured in the teat canal and passed from there into the milk (1,2,3). Otherwise the bacterial count of aseptically drawn milk would not be so low. In fact, the micrococci grew well in the milk after being adapted to it, especially at 37 °C. The coryneforms did not grow in the milk.

Experiments with isolated micrococci showed that, after the lag-phase, these bacteria did not have any lag-phase at all when they were inoculated into fresh aseptically drawn milk. Therefore the lag-phase of the bacterial flora in aseptically drawn milk must be due only to adaption of the bacteria to the milk. Lactenins seem to play no role.

3 Equipment for aseptic cheesemaking

The equipment that we developed to make cheese under strictly aseptic conditions consists of two completely closed identical vats, each with a capacity of 40 litres, to carry out simultaneous experiments, using the same milk supply in both vats. For example, in the one aseptic vat no other bacteria than the starter bacteria are inoculated and in the other aseptic vat no bacteria at all are added to milk.

The tools needed during cheesemaking can be suspended in the vats before sterilization. Materials such as cultures, and Seitz-filtered rennet can be added aseptically during cheesemaking. All manipulations during cheesemaking, can be done entirely aseptically. The equipment was so constructed, that the cheeses made in a parallel experiment, were exactly the same chemically at the beginning of the ripening period but differed bacteriologically as desired.

When raw, the bacterial count of the milk was extremely low and after pasteurization the milk was sterile (Page I-17). In the cheeses, no other bacteria were present than those added after pasteurization of the milk. The cheeses that were made to study the action of rennet and heat-resistant milk enzymes without the activity of any bacterial enzymes were found to be sterile during ripening of the cheese. The moisture content, pH, salt content and fat content of the cheese that we made under strictly

aseptic conditions, proved to be usual for Gouda cheese. Thus the method for making cheese under strictly aseptic conditions meets requirements (Page I–5 and I–6).

4 Influence of bacterial enzymes on ripening of Gouda cheese made under strictly aseptic conditions

Some properties of the cheese, in which no other bacterial enzymes had been active during ripening than those from a proper *Streptococcus cremoris* strain (E8), are summarized in table 1 (mean values of 18 aseptic cheesemaking trials). These figures, including those for taste and flavour, are normal for Gouda cheese (4,5). This was confirmed by comparison of the aseptically made cheese with Gouda cheeses that we made under normal conditions and with commercial Gouda cheese. Thus Gouda cheese can ripen normally, if no other bacterial enzymes are active in the cheese than those from a proper starter streptococcus.

To compare the ripening of cheese without any bacterial enzymes with that of cheese with the enzymes from a starter streptococcus, the starter was replaced by gluconolactone (6) in the one aseptic vat. The bacterial-enzyme-free-cheese differed considerably from the cheese with starter bacteria. Hardly any amino acid was found in the bacterial-enzyme-free-cheese up to six months of ripening and no cheese taste and flavour developed. Thus starter bacteria are essential in the ripening of Gouda cheese.

Figure 1 shows the protein breakdown in cheese from simultaneous experiments with no bacterial enzymes other than those from *Streptococcus cremoris* E8 or *S. cremoris* Wg2, respectively. The amount of amino acid during ripening of the cheese with *S. cremoris* Wg2 as starter is only about half that with *S. cremoris* E8 as starter. Moreover the cheese with *S. cremoris* Wg2 as starter scored much lower by sensory assessment because of less cheese taste and flavour during ripening and bitterness. Thus *S. cremoris* strains differ widely in their ability to produce cheese taste and

Table 1. Properties during ripening of Gouda cheese made aseptically, and with no other bacterial enzymes active than those from *Streptococcus cremoris* strain E8 (mean of 18 cheesemaking trials). Initial mean count of bacteria in the raw milk was 47 ml^{-1}. Almost all cheese samples were free from defects and normal to good in taste and flavour.

Ripening time[1]	Moisture (%)	pH	Fat in d.m. (%)	NaCl in moisture (%)	NCN in % of TN[2]	AN in % of TN[2]	AF[3]	log streptococci per g of cheese
3 d								9.0
9 d	43.5	5.10	50.6	3.9	5.2	1.1		
21 d								6.6
2 m	36.3	5.18			16.3	4.2	0.5	4.9
6 m	34.3	5.22			25.8	8.6	0.7	0.1

1. d = days; m = months.
2. TN = total nitrogen; NCN = water-soluble non-coagulable nitrogen; AN = amino acid nitrogen.
3. AF = acidity of the fat.

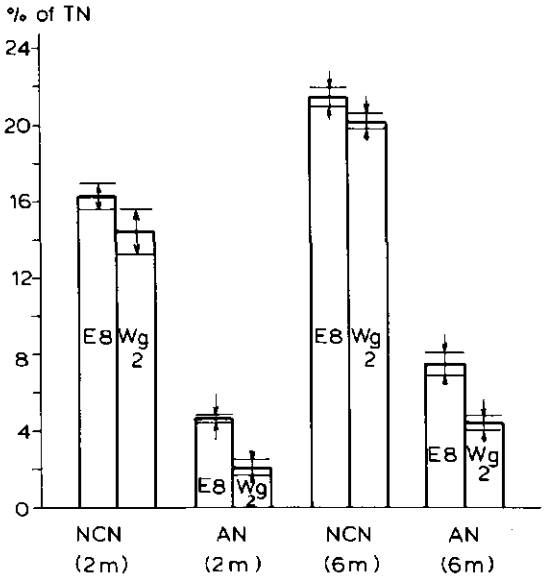


Fig. 1. Protein breakdown in aseptically made Gouda cheese with *Streptococcus cremoris* E8 or *S. cremoris* Wg2 as starter (mean values of two simultaneous experiments; the values of two repeated experiments are indicated by the lines at the end of the bars). TN = total nitrogen; NCN = water-soluble non-coagulable nitrogen; AN = amino acid nitrogen; m = months.

flavour by means, for instance, of amino acids, and also in the formation of taste and flavour defects like bitterness.

Cheeses made with mixed strain starters or with the single strain starter *S. cremoris* E8, differed only to a minor extent in ripening. Protein breakdown and development of cheese taste and flavour were about the same during ripening. Much more research will be needed to confirm whether mixed strain starters are indeed preferable to single strain starters in the production of Gouda cheese (Page I-5), since the differences between a proper single strain starter and mixed strain starters were so small in our experiments. In addition, for the commercial production of cheese other starter characteristics than protein breakdown and taste and flavour development in the cheese, have to be considered like susceptibility to phages, rate of acid production and formation of holes in the cheese.

Little fat was broken down in all the cheeses with no other bacterial enzymes active than those from a starter.

In cheese with bacterial enzymes only from *S. cremoris* E8, breakdown of fat and protein were about the same as with those from cheese with additionally certain lactobacillus strains. The cheese with lactobacilli scored lower for taste and flavour because of defects, especially at 6 months of age.

Thus Gouda cheese can ripen normally if no other bacterial enzymes are active in the cheese than those from a proper starter. In sterile cheese, hardly any cheese taste and flavour develops during ripening. So the enzymes from the starter bacteria are essential for ripening of Gouda cheese. Lactobacilli, which are normally present in large numbers in Gouda cheese during ripening, are not essential for ripening. They may even be undesirable, since they can give taste and flavour defects.

References

1. J. A. Beech & D. Forbes. Pathogens in streak canal. Vet. Rec. 77 (1965) 1086.
2. H. J. Heidrich, D. Grossklaus & M. Mülling. Vergleichende bakteriologische Untersuchungen an durch Handmelken und durch Venülenpunktion der Drüsenzisterne gewonnene Milchen. I. Untersuchungen an Venülenmilchen. II. Vergleichende Untersuchungen an Handmelk- and Venülenmilchen. Berl. Münch. tierärtzl. Wschr. 77 (1964), 83,85.
3. W. Trautmann. Vergleichende bakteriologische Untersuchungen über die primäre und sekundäre Milchflora an durch Handmelken und durch Punktion der Drüsenzisterne gewonnenen Proben. Schweiz. landw. Forsch. 12 (1973) 125.
4. A. M. M. F. Monib. The Calcium-paracaseinate-phosphate-complex under conditions similar to those in cheese. Thesis, Agricultural University Wageningen. Mededelingen Landbouwhogeschool 62 (1962).
5. J. Stadhoudes. De eiwithydrolyse tijdens de kaasrijping. De enzymen die het eiwit in kaas hydrolyseren. Neth. Milk Dairy J. 14 (1960) 83.
6. L. A. Mabbitt, Helen R. Chapman & N. J. Berridge. Experiments in cheesemaking without starter. J. Dairy Res. 22 (1955) 365.

smaakgebreken in de kaas. Wat betreft de eiwitafbraak en de ontwikkeling van

Summary

kaasgeur en -smaak, bleken de verschillen tussen een goed één-stam-zuursel en meer-stam-zuursels gering. Er wordt met nadruk op gewezen, dat bij de vergelijking van de activiteiten van één-stam ten opzichte van meer-stam-zuursels, meer aspecten in acht dienen te worden genomen.

Lactobacillen die normaliter in grote aantallen voorkomen in Goudse kaas gedurende de rijping, bleken niet nodig voor het gewenste rijpingsproces. Verder werd gevonden, dat deze bacteriën zelfs ongewenst kunnen zijn in de kaas, omdat ze smaakgebreken kunnen veroorzaken.

Contents

Introduction

1. Ripening of cheese

The development of a high quality cheese is primarily dependent on a controlled fermentation of the concentrated milk constituents. Although this process starts during manufacture, for most cheese varieties a longer or shorter period of maturation is required before consumption. During this ripening the lactose, the protein and the fat are involved in a complex series of biochemical reactions, resulting in the development of the desired flavour and the optimum physical characteristics.

Gouda cheese is a semi-hard, full-cream cheese variety that can be consumed from about 1 month of ripening onwards, but that is often kept for a longer ripening time up to 1 year and more. Its production is of great economic importance for the Dutch dairy industry. In 1976 the production amounted about 260 000 tons. Although large amounts (about 50%) are exported from the Netherlands, Gouda cheese is produced in considerable proportions in other countries too.

Normal Gouda cheese is manufactured from low-pasteurized cow's milk, using calf rennet to clot the milk. In other countries rennet substitutes are being used increasingly. As a rule mixed-strain starter cultures containing *Streptococcus cremoris* strains together with aroma producing streptococci or leuconostocs are used. Edam cheeses, that have a lower fat content and a different shape, are manufactured in an corresponding way and therefore can be expected to show a ripening process almost identical with that in Gouda cheese.

2. Breakdown of protein

Decomposition of protein during ripening governs the conversion of the rubbery and coarse original curd into a more or less smooth-bodied, homogeneous substance. Especially in soft types of cheese like Camembert, Limburger and Meshanger, these changes in consistency are outstanding (1).

Proteolysis, however, also influences the flavour of the cheese since many breakdown products – amino acids and peptides in particular – are considered to be significant contributors to cheese flavour directly, or to be precursors for other characteristic taste and aroma components (2,3). Moreover they are implicated in certain flavour defects of the cheese, especially bitterness (4,5,6).

Proteolysis, therefore, is a determinative process for the quality of nearly all ripened cheese types, whether a short-ripened soft type of cheese, like Camembert, or a long-ripened hard variety like Cheddar cheese.

Hardly any studies on cheese ripening have included an estimate of the amount of rennet in the investigated cheeses. A more quantitative approach to the role of rennet in cheese ripening is possible only if the amount of rennet acting in the cheeses is known exactly, especially since retention of rennet is extremely sensitive to variation in cheesemaking conditions (54). We always determined the amount of rennet present in our cheeses (66).

Table 1. Survey of the different aseptically made cheeses used during the investigations.

Type of cheese	Proteol. enzyme systems active on ripening
1. 'normal' aseptic	rennet starter bacteria milk protease
2. aseptic starter-free (ASF)	rennet milk protease
3. aseptic rennet-free (ARF)	starter bacteria milk protease
4. aseptic rennet- and starter-free (ARSF)	milk protease

As a control, the cheeses were analysed for components like moisture, fat, salt and pH and were checked for the absence of unwanted micro-organisms. The growth and survival of starter bacteria was monitored during cheesemaking and ripening too.

Proteolysis was monitored during the ripening in different ways, characterizing both the primary casein components and the smaller breakdown products. The amount of breakdown products was estimated in a soluble-nitrogen fraction and the molecular weight (MW) distribution of the peptides in this fraction was analysed by gel filtration. The amount and composition of free amino acids in cheese – the ultimate products of proteolysis – were monitored as well. The data on breakdown products and on primary attack of α_{s1} and β-casein (obtained by polyacrylamide gel electrophoresis) reflected well the 'extent' and 'depth' of proteolysis.

To monitor the development of bitterness and cheese flavour, the different cheeses were submitted to a taste panel.

Bitter peptides were extracted from the cheeses and analysed by means of gel filtration.

References

1. L. de Jong. Protein breakdown in soft cheese and its relation to consistency. 1. Proteolysis and consistency of 'Noordhollandse Meshanger' cheese. Neth. Milk Dairy J. 30 (1976) 242.
2. H. Mulder. Taste and flavour forming substances in cheese. Neth. Milk Dairy J. 6 (1952) 157.

3. E. W. Evans & L. A. Mabbit. The flavour of Cheddar cheese. Bienn. Rev. natn. Inst. Res. Dairy. (Reading, GB) (1974) 59.
4. V. R. Harwalkar & J. A. Elliot. Isolation of bitter and astringent fractions from Cheddar cheese. J. Dairy Sci. 54 (1971) 8.
5. C. W. Raadsveld. Bitter compounds from cheese. Proc. 13th int. Dairy Congr. (1953)2: 676.
6. B. C. Richardson & L. K. Creamer. Casein proteolysis and bitter peptides in Cheddar cheese. N.Z.J. Dairy Sci. Technol. 8 (1973) 46.
7. J. C. Mercier, F. Grosclaude & B. Ribadeau-Dumas. Primary structure of bovine caseins. A review. Milchwissenschaft 27 (1972) 402.
8. J. Stadhouders & G. Hup. Factors affecting bitter flavour in Gouda cheese. Neth. Milk Dairy J. 29 (1975) 335.
9. F. A. Exterkate. An introductory study of the proteolytic system of Streptococcus cremoris strain HP. Neth. Milk Dairy J. 29 (1975) 303.
10. H. B. Castberg & H. A. Morris. Degradation of milk proteins by enzymes from lactic acid bacteria used in cheesemaking. A review. Milchwissenschaft 31 (1976) 85.
11. R. H. Schmidt, H. A. Morris, H. B. Castberg & L. L. Mc.Kay. Hydrolysis of milk proteins used in cheesemaking. J. agric. Fd. Chem. 24 (1976) 1106.
12. R. C. Lawrence, T. D. Thomas & B. E. Terzaghi. Reviews of the progress of dairy science: cheese starters. J. Dairy Res. 43 (1976) 141.
13. J. R. Dulley. Bovine milk protease. J. Dairy Res. 39 (1972) 1.
14. E. H. Reimerdes & H. Klostermeyer. Milchproteasen 1. Micellenassoziierte Protease-Anreicherung und Hydrolyse von Caseinen. Milchwissenschaft 29 (1974) 517.
15. A. Noomen. Proteolytic activity of milk protease in raw and pasteurized cow's milk. Neth. Milk Dairy J. 29 (1975) 153.
16. J. Schormüller. The chemistry and biochemistry of cheese ripening. Adv. in Food Res. 16 (1968) 231.
17. H. A. Sirks. Eenige proeven over den invloed van den reëelen zuurheidsgraad op de rijping van Edammer kaas. Ministerie van Landbouw en Visserij, The Hague. Versl. landbouwk. Onderz. 49 (1943) 221.
18. S. Poznanski & J. Rymaszewski. Proteolysis during the ripening of Edam cheese with the participation of some bacteria strains Part 1. Changes in particular nitrogen fractions. Milchwissenschaft 20 (1965) 14.
19. R. B. O'Keeffe, P. F. Fox & C. Daly. Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. J. Dairy Res. 43 (1976) 97.
20. L. K. Creamer. Protein breakdown in Gouda cheese. N.Z.J. Dairy Sci. Technol. 5 (1970) 152.
21. G. v.d. Berg & E. de Vries. Bovine rennet for the manufacture of Gouda cheese. Report of the Netherlands Institute for Dairy Research (1976) R103, Ede, Netherlands.
22. L. de Jong. A quantitative electrophoretic method of studying cheese ripening. Neth. Milk Dairy J. 29 (1975) 162.
23. P. M. D. Foster & M. L. Green. A quantitative gel filtration method for analysis of the proteinaceous fraction of Cheddar cheese. J. Dairy Res. 41 (1974) 259.
24. M. Bachmann, J. Meuli & F. Baumgartner. Die Gelfiltration als Hilfsmittel zur Charakterisierung des Eiweissabbaues bei der Käsereifung. Schweiz. Milchwirtsch. Forsch. 6 (1976) 7.
25. J. C. Gripon, M. J. Desmazeaud, D. Le Bars & J. L. Bergère. Etude du rôle des micro-organismes et des enzymes au cours de la maturation des fromages 2. Influence de la presure commerciale. Lait 548 (1975) 502.
26. K. R. Nath & R. A. Ledford. Growth response of Lactobacillus casei variety casei to proteolysis products in cheese during ripening. J. Dairy Sci. 56 (1973) 710.
27. L. A. M. Ali. The amino acid content of Edam cheese and its relation to flavour. Commun. Agric. Univ. Wageningen, Netherlands 60 (1960) 1.
28. B. Reiter, Y. Sorokin, A. Pickering & A. J. Hall. Hydrolysis of fat and protein in small cheeses made under aseptic conditions J. Dairy Res. 36 (1969) 65.
29. L. K. Creamer & B. C. Richardson. Identification of the primary degradation product of α_{s1}-casein in Cheddar cheese. N.Z.J. Dairy Sci. Technol. 9 (1974) 9.
30. J. Stadhouders. De eiwithydrolyse tijdens de kaasrijping. De enzymen die het eiwit in kaas hydrolyseren. Neth. Milk Dairy J. 14 (1960) 83.

Method for the manufacture of rennet-free cheese

F. M. W. Visser

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen, The Netherlands

Summary

A method is described for the aseptic manufacture of cheese, which excludes any effect of rennet on the ripening process. By reducing the calcium plus magnesium content of the milk below 0.100 % by means of ion exchange, normal renneting could proceed without clotting. After completing the enzymatic phase of rennin action, the enzyme was inactivated completely by pasteurization of the milk at 72 °C for 20 seconds. By cooling the milk to 4 - 5 °C calcium could be returned to the milk without clotting. Clotting was obtained by dielectrical warming to 30 °C.

The milk had to be adjusted to a calcium plus magnesium content of about 0.160 % and a pH of 6.30 - 6.34 to avoid on the one hand flocculation of the milk at 4 - 5 °C, and on the other hand to get a homogeneous, firm gel on warming. The gel obtained was used for the manufacture of rennet-free cheese. In this cheese the role of starter bacteria and milk protease on the ripening can be studied.

The final cheeses prepared by this method showed normal starter development and calcium content.

Neth. Milk Dairy J. 30 (1976): 41-54

Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 1. Description of cheese and aseptic cheesemaking techniques

F. M. W. Visser

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen, the Netherlands

Summary

To study aspects of cheese ripening, aseptically made cheeses were used in which the protein is decomposed during ripening through the separated and combined actions of rennet, starter bacteria and milk protease. This paper describes the aseptic cheesemaking techniques used and the average composition of normal, rennet-free, starter-free and rennet- and starter-free aseptic cheeses. The pH, moisture, salt and fat contents resembled those of Gouda-type cheese and, when starter was used, the calcium and inorganic phosphorus also showed normal levels.

The losses of calcium phosphate and the rennet retention in the curd proved to be very much dependent on the chemical acidification procedure used in the starter-free cheesemaking process. Extremely high rennet levels and low calcium and phosphorus contents were observed in starter-free cheeses acidified early in the process, according to the frequently used Mabbitt method (9). A method of chemical acidification is described that overcomes these disadvantages to a large extent.

The rennet contents of all cheeses are reported.

The outgrowth and survival of seven different starter strains in aseptic Gouda-type cheeses were followed. Characteristic differences were observed.

The next paper will present results on proteolysis, bitterness and cheese flavour.

Neth. Milk Dairy J. 31 (1977): 120-133

Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 2. Development of bitterness and cheese flavour

F. M. W. Visser

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen, the Netherlands

Summary

The development of bitterness and cheese flavour was studied in aseptically made, Gouda-type cheeses in which the actions of rennet, starter bacteria and milk protease were separated (23). At the same time different aspects of bitterness development were studied in normal aseptic cheeses, such as the influence of varying amounts of rennet both with 'bitter' and 'non-bitter' starter cultures.

It was observed that

– rennet, when present in the concentration found in normal Gouda cheese, is capable of producing bitter starter-free cheeses after some months of ripening. The application of larger amounts of rennet makes the bitterness appear earlier and be more intensive;
– 'bitter' starters, as contrasted to 'non-bitter' starters, are able to produce a distinctly bitter rennet-free cheese;
– milk protease on its own does not contribute significantly to bitterness in our Gouda-type cheeses;
– mutual stimulation in the formation of bitter peptides by rennet and starter bacteria is not necessarily an important factor;
– non-bitter' starters have the power to degrade considerable quantities of bitter peptides to non-bitter products in our cheese. 'Bitter' starters seem to possess this property to a far less extent;
– the growth of a starter to high cell densities in the cheese does not appear to be the determinative factor for the occurrence of bitter flavour in Gouda cheese;
– neither rennet nor milk protease contribute noticeably to the development of cheese flavour;
– 'non-bitter' starters develop important levels of cheese flavour in rennet-containing cheeses; distinct cheese flavour was formed without the help of rennet. 'Bitter' strains have far less potency to do so.

A mechanism for the development of the bitter defect in Gouda cheese is discussed.

Neth. Milk Dairy J. 31 (1977): 188-209

Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino acid nitrogen fractions

F. M. W. Visser

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen, the Netherlands

Summary

Protein breakdown was studied in aseptically made Gouda cheeses in which the action of rennet or starter bacteria during the ripening could be eliminated. By comparing the results of the analyses of these cheeses with those of normal aseptic cheeses, it was possible to estimate the contributions of rennet, starter bacteria and milk protease to the production of soluble-N compounds and free amino acids. For a more quantitative approach the rennet contents of the cheeses were determined.

Rennet appears to be responsible for the greater part of the soluble-N production in Gouda cheese, but the starter bacteria and – to a lesser extent – milk protease also make contributions. The latter two were shown to be capable of producing soluble-N compounds in cheese without the help of rennet. In normal cheeses, no distinct mutually stimulatory or inhibitory effects on soluble-N liberation appear to occur between the enzyme systems.

Determinations of amino acid N and gel filtration of soluble-N compounds showed that rennet liberates peptides of high and low molecular weight (MW) in particular, but only very low amounts of amino acids. On the contrary, starter bacteria without rennet predominantly accumulate lower-MW (<1400) peptides and — dependent on the culture used – more or less significant levels of free amino acids. Milk protease on its own liberates amino acids and low-MW peptides but only in small amounts.

In normal cheeses, with the enzyme systems acting together, the action of rennet clearly stimulates the starter bacteria to accumulate amino acids and low-MW peptides. It could be shown that this was most likely due to the progressive degradation by starter peptidases of the higher-MW products of rennet action.

Free amino acid patterns of the different aseptic cheeses are presented and discussed.

Differences in the residual amounts of rennet proved to account completely for the differences in the rate of proteolysis observed in starter-free cheeses that showed different calcium levels due to the method of manufacture.

In this study both 'bitter' and 'non-bitter' starters were applied. Therefore the observed effects are also discussed in relation to (bitter) flavour development.

Neth. Milk and Dairy J. 31 (1977): 210-239

Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 4. Protein breakdown: a gel electrophoretical study

F. M. W. Visser and A. E. A. de Groot-Mostert

Dairying Laboratory, Department of Food Science, Agricultural University, Wageningen, the Netherlands

Summary

Quantitative polyacrylamide gel electrophoresis (PAE) was used to study the primary breakdown of α_{s1}- and β-casein in aseptically made Gouda-type cheeses. Any action of rennet and/or starter bacteria during the ripening could be eliminated in these cheeses.

In normal aseptic cheeses α_{s1}-casein is attacked rapidly, the degradation being nearly complete after one month of ripening. β-casein is more resistant to proteolysis; after 6 months ripening about 50 % is still intact.

From starter-free cheese experiments it was concluded that rennet – in the concentration as present in Gouda cheese – is completely responsible for the observed degradation of α_{s1}-casein and for the decomposition of β-casein during the first month of ripening. In the longer term starter bacterial enzymes also appear to contribute, especially in the further degradation of β-casein. The results of rennet-free cheese experiments are in agreement with this, since starter bacteria are able to degrade part of the α_{s1}- and β-casein in these cheeses after some months of ripening. This activity is relatively low during the first month of ripening.

No differences were observed in the PAE patterns of cheeses made with different starters – both 'bitter' and 'non-bitter' – neither in the presence nor in the absence of rennet.

From the rennet- and starter-free cheeses it appeared that milk protease is responsible for the formation of the 'minor caseins' from β-casein during the ripening of Gouda cheese. Its total contribution to the α_{s1}- and β-casein degradation, however, is low in relation to that of rennet and of starter bacteria.

The results are also discussed in combination with those of a previous paper (2) in which the soluble-N and amino acid-N fractions were characterized.

Neth. Milk Dairy J. 31 (1977): 247-264

the native milk protease during cheese ripening on its own. The developed method for the preparation of a rennet-free milk coagulum was also used by Noomen (3) to produce curd and a calcium paracaseinate- calcium phosphate complex, both free from rennet and starter, which were used to study the action of milk protease under various conditions.

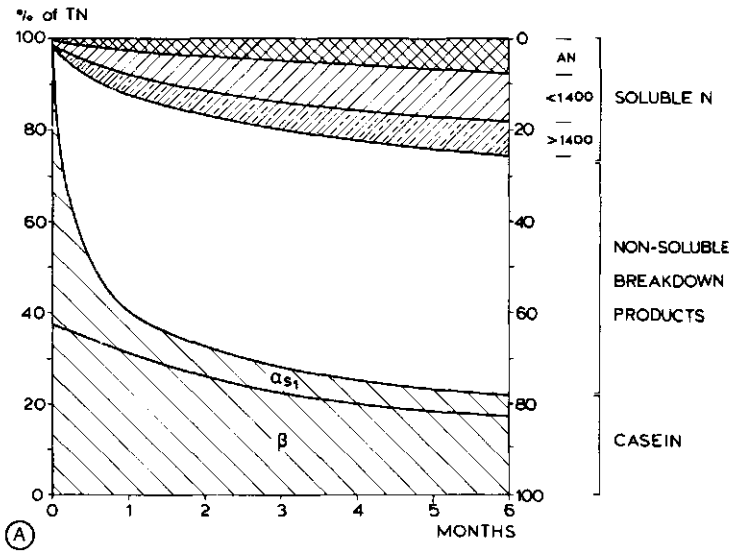
All the cheeses made, both normal aseptic, aseptic rennet-free, aseptic starter-free and cheeses free from rennet and starter, fulfilled the requirement of the absence of unwanted bacteria.

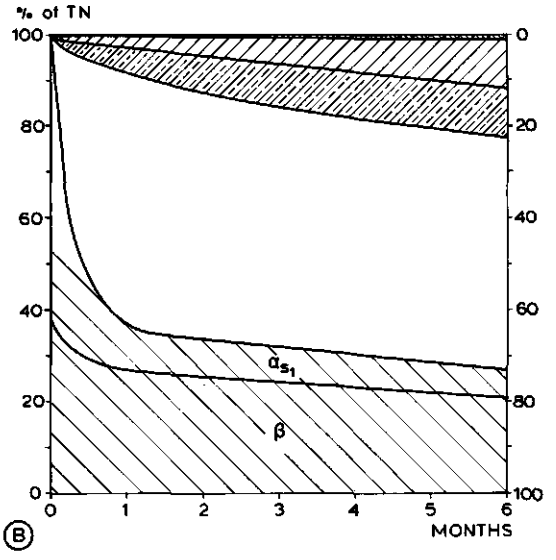
2. Rennet, starter and milk protease in protein breakdown

The experiments with normal aseptic cheeses, using different starter strains and amounts of rennet showed that the enclosed amount of rennet in the cheese distinctly influences the rate of casein attack and the accumulation of soluble-N compounds but not the accumulation of free amino acids. However, the strain of starter strongly influences liberation of amino acid N but only to a minor extent the production of soluble N. The pathway and rate of the primary casein attack is not at all affected by using different starters.

Both the primary degradation of casein – as determined by the quantitative polyacrylamide gel electrophoresis – and the production of soluble N can be regarded as characterizing the 'extent' of protein breakdown, and the accumulation of free amino acids and small peptides the 'depth' of it. So these results suggest that rennet influences the 'extent' of proteolysis in cheese and starter bacteria the 'depth'.

In a normal aseptic Gouda cheese, in which rennet, starter bacteria and milk protease act together, and that contains a normal amount of rennet, α_{s1} -casein is quickly and intensively broken down. Within the first month of ripening, most of the original α_{s1} -casein has vanished. β-Casein is attacked more slowly, after 6 months of ripening about half still being intact. In Figure 1a this breakdown and the accompa-





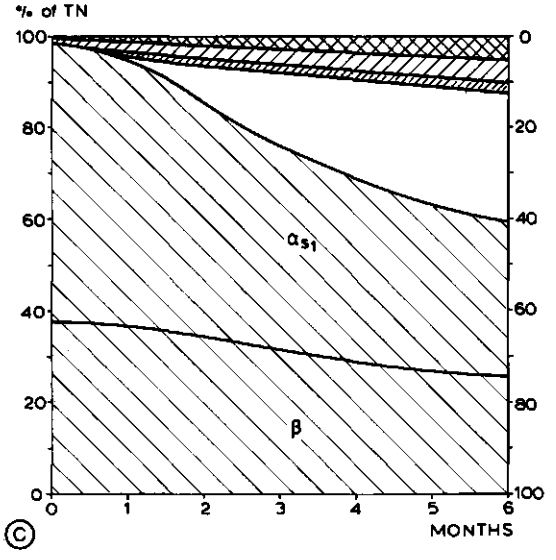


Fig 1. Breakdown of casein and development of soluble-N fractions during the ripening of different aseptically made cheeses. A: normal aseptic cheeses. B: aseptic starter-free (ASF) cheeses. C: aseptic rennet-free (ARF) cheeses. Soluble N is subdivided into amino acid N (AN), peptides with mol.wt. < 1400 and breakdown products with mol.wt. > 1400 α_{s1}-casein plus β-casein together are supposed to make up total nitrogen (TN). For A and C, the average values obtained in cheeses made with 'non-bitter' starter are entered. The normal aseptic and ASF cheeses both contained normal amounts of rennet.

nying accumulation of free amino acids, smaller peptides and larger soluble products is summarized. The soluble-N fraction in this cheese is constituted mainly of smaller products and represents about a third or a quarter of the total amount of breakdown products during ripening. Large amounts of insoluble breakdown products must be produced in the cheese. Although we did not estimate this insoluble fraction directly, including mostly products of high molecular weight, its proportion is calculable from the other data. The small fraction of para-κ-casein was not measured in our cheeses; others (4,5) reported that it is not likely to be broken down during ripening. For ease, it is assumed that the protein in cheese is constituted only of α_{s1} -and β-casein.

To estimate the respective roles of rennet, starter bacteria and milk protease in cheese proteolysis, breakdown of protein during ripening of aseptic starter-free (ASF), aseptic rennet-free (ARF) and aseptic rennet- and starter-free (ARSF) cheeses was monitored. A broad survey is given for ASF and ARF cheeses in the Figures 1b and 1c.

In the ARSF cheeses a rather small but detectable amount of free amino acids and other soluble products is formed on ripening. By gel electrophoresis some attack of α_{s1} - and β-casein can be recorded after extended ripening times. Since the 'minor'caseins (γ,R,TS) – always detectable in ripened Gouda cheeses (6) – are also normally produced in the ARSF cheeses, milk protease was directly responsible. Milk protease apparently contributes to protein breakdown in Gouda cheese to a minor degree. Consequently, any clear proteolytic action observed in the ASF or ARF cheeses can be largely attributed to the action of rennet and starter bacteria, respectively.

Figure 1b shows that in ASF cheeses, containing a normal amount of rennet, the same breakdown of α_{s1} -casein and nearly the same breakdown of β-casein is observed as in normal aseptic cheeses. The primary breakdown products are also identical. Considerable but somewhat smaller amounts of soluble N are formed in the ASF cheeses. The composition of this soluble fraction, however, differs from that in normal cheeses. Only small amounts of free amino acids were detected and the larger products (mol. wt. $>$ 1400) are relatively more important in the ASF cheeses.

Figure 1c shows that the rate of casein attack in the ARF cheeses is far less. Only after 1 month of ripening are α_{s1} - and β-casein degraded to some extent. Nevertheless considerable amounts of soluble N, especially amino acids and smaller peptides, are liberated. Starter bacteria can apparently degrade paracasein in cheese without rennet. Different starter strains ('bitter' and 'non-bitter') cannot be distinguished by casein attack or soluble-N production but can be by amount of amino acid N produced. In general the 'non-bitter' starters produce considerable larger amounts of free amino acids than the 'bitter'starters. In the patterns of individual amino acids, however, there are only slight differences.

In conclusion, rennet is completely responsible for the primary degradation of α_{s1} - and β-casein in Gouda cheese during the first one or two months of ripening.

Thereafter the proteinases of starter bacteria and to a lesser extent milk protease begin to contribute too. Since rennet will break down most of the α_{s1} -casein in the mean time, their contribution is, however, restricted to the further attack of β-casein. On the disappearance of the original α_{s1} -casein the action of the enzymes will also be directed to the degradation of the larger, predominantly insoluble, products.

Although rennet produces the greater amount of the soluble N in normal cheese,

liberating especially peptides of low and high molecular weight, starter bacteria and, to some extent, milk protease contribute too, producing especially amino acids and smaller peptides. From the net contributions of rennet, starter and milk protease, mutual stimulation or inhibition cannot be considerable in total production of soluble N in normal cheese. The combined action of the three enzyme systems in normal cheese however does influence the composition of soluble-N compounds. Far larger amounts of free amino acids and smaller peptides are liberated than can be accounted for by separate action. The starter bacterial peptidases progressively degrade the (soluble) products of rennet action of higher molecular weight. Obviously the peptidase system of starter bacteria has a certain qualitative or quantitative overcapacity relative to the proteinase system. Differences in amino acid patterns of ARF cheeses from those of normal aseptic cheeses also indicate some rennet/starter interaction.

The production of free amino acids in cheese is almost exclusively due to starter bacteria. Rennet only seems to have an indirect, stimulating capacity, while milk protease contributes only slightly.

Generally speaking proteolysis in Gouda cheese bears the stamp of rennet action for the 'extent' of breakdown and of starter bacterial enzymes for the 'depth'.

3. Rennet, starter and milk protease in development of bitterness

Normal aseptic, ASF, ARF and ARSF cheeses were made with different amounts of rennet and different starters. In normal aseptic cheeses, four starters (E8, AM1, AM2 and Bos) always produced a good flavour without bitterness and three other starter strains (HP, Wg2 and Z8) consistently produced bitter cheeses. The starters were strains of *Streptococcus cremoris,* except Bos.

From all cheeses – both bitter and non-bitter – bitter tasting peptide substances were isolated by extraction. On fractionating these peptides, the bitterness was always detected in a fraction with molecular weights less than 1400. From the non-bitter cheeses, less bitter material was however extracted than from bitter cheeses. In cheese, 16 – 20 times as much bitter substances are needed as in water to achieve an equal bitterness. Obviously a considerable amount of bitter peptides can be formed in cheese before they can be detected sensorially. The presence of cheese flavour did not interfere with the sensation of bitterness.

Figure 2 summarizes some results on the development of bitterness during the ripening of the different cheeses.

The results with ASF cheeses show that rennet contributes directly to the production of bitter peptides in cheese. During ripening, the amount of bitter peptides steadily increases. The moment when and the degree to which the cheese becomes bitter only depends on the amount of rennet enclosed in the cheese. For the amount normally present in Gouda cheese, about 2 – 3 months ripening are necessary before bitterness reaches the threshold value. On further ripening the cheese becomes increasingly bitter. The bitter peptide pool produced by rennet in ASF cheese contains both larger peptides and – in contrast to other cheeses – considerable amounts of oligopeptides.

Since Cheddar cheese contains smaller amounts of enclosed rennet than Gouda

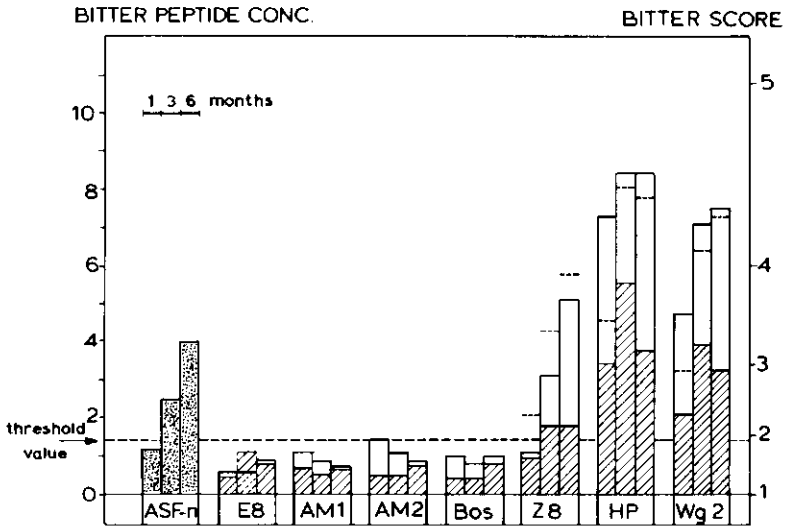


Fig. 2. Bitterness observed in different aseptic cheese types after 1, 3 and 6 months of ripening. Normal aseptic cheeses: blank + hatched area. ASF cheeses: dotted area. ARF cheeses: hatched area. The normal aseptic and ASF cheeses both contained normal amounts of rennet. For the 'bitter' starter strains HP, Wg2 and Z8 the sum of ARF and ASF bitterness is indicated with marks (–). Bitter scores of the panel are transformed into relative concentrations of bitter peptides.

cheese (7), the contribution of rennet to the bitterness in this cheese type will be less, possibly sub-threshold.

The experiments with ARSF cheeses showed that the proteolytic action of milk protease during the ripening of Gouda cheese is not intensive nor specific enough to cause detectable bitterness.

To what extent starter bacteria cause accumulation of bitter peptides during cheese ripening in the absence of rennet depends closely on the starter culture used. The 'bitter' starters HP and Wg2 liberated bitter peptides to such an extent as to cause early and persisting bitterness in ARF cheeses. Since bitterness appeared in these cheeses too. It is a mute point, whether these starters hydrolyse casein specifically obviously produced more directly and specifically from casein than by rennet. When the ASF cheeses had become bitter, far more casein had been attacked and much larger amounts of smaller peptides, the class to which the bitter peptides belong, had been liberated.

The 'non-bitter' starters (E8, AM1, AM2 and Bos) all did not produce detectable bitterness in ARF cheeses, although some bitter fractions were extracted from those cheeses too. It is a mute point, whether these starters hydrolyse casein specifically without the liberation of bitter peptides or the absence of bitterness is only the balance of a normal production of bitter peptides and an intensive immediate breakdown to non-bitter products. The truth may lie between these two ideas.

That 'non-bitter' starters indeed degrade bitter peptides in cheese to substances like amino acids, that are less bitter, if at all, can be deduced from the absence of bitterness in the normal aseptic cheeses. Even the addition of twice the normal amount of

rennet did not lead to bitterness, indicating the capacity of the peptidase system. Obviously the 'bitter' starter bacteria have far less capacity to degrade the bitter peptides from rennet action, since the level of bitterness found in normal aseptic cheeses with 'bitter' starter can be largely explained by the separate productions of bitter peptides by rennet and 'bitter' starters. Mutual stimulatory effects need not play an important role. The decrease in the amount of enclosed rennet in these cheeses indeed results in less bitterness.

Multiplication of starter bacteria to high counts in the cheese is not decisive for the appearance of bitterness in our aseptic Gouda cheeses, though it was for Cheddar cheese (8). In this respect our results agree with those of Stadhouders (7) for normal open-vat Gouda cheese. The selection of reliable 'non-bitter' starter cultures is essential for the production of non-bitter Gouda cheese. Although rennet actively contributes to bitter-peptide formation, the decrease in the retained amount of rennet by adapting the procedure of cheesemaking only comes into play when the starter culture is not optimum in breaking down bitter peptides. It is difficult to relate differences in the protein breakdown of cheeses made with 'bitter' and 'non-bitter' starters to the appearance of bitterness in these cheeses. No differences at all can be detected in the pathway or extent of primary casein attack as analysed by gel electrophoresis. No relation was found in the total production of soluble N, although, in the presence of rennet, there were some differences in composition of the peptide fraction of lower molecular weight. However far more research is needed, especially to identify the (bitter) peptides present in young rennet-free cheeses.

The only consistent and distinct difference observed between 'bitter' and 'non-bitter' starters was a higher production of free amino acids in 'non-bitter' starter cheeses, both in the presence and absence of rennet. 'Non-bitter' starters in cheese are obviously equipped with a more active exopeptidase system than 'bitter' starters, hence, perhaps, their greater capacity to degrade bitter peptides. Indications that also specific differences in the quality of the peptidase system exist between starters were not evident from comparisons of the amino acid patterns in the different cheeses.

4. Rennet, starter and milk protease in development of cheese flavour

By aseptic vat techniques Kleter (9) and others (10) demonstrated that starter bacteria play the central role in the development of flavour in cheeses like Gouda and Cheddar.

Since hardly any cheese flavour developed in our ASF and ARSF cheeses during ripening, milk protease and rennet – even at high concentrations – do not directly contribute to the formation of cheese flavour compounds. The presence of cheese flavour, to varying degree, in normal aseptic cheeses suggested that starter bacteria do produce these compounds, but direct evidence was the presence of cheese flavour in ARF cheeses. Although 'non-bitter' starters in both types of cheese produced distinctly more cheese flavour than 'bitter' starters, starter bacteria are directly involved and determine cheese flavour production. However, the amount of cheese flavour in normal aseptic cheeses is considerably more than in comparable ARF cheeses, so rennet must stimulate flavour production by starter bacteria. A relatively small amount of rennet is obviously sufficient.

The most widely accepted theory on cheese flavour is the Component Balance

between 'bitter' and 'non-bitter' starters were detected in the amount of total free amino acids produced and not in the pattern of individual free amino acids. Milk protease contributed only slightly to proteolysis in Gouda cheese, being responsible for the formation of 'minor' caseins from β-casein and producing small amounts of free amino acids and soluble peptides.

During ripening of Gouda cheese the rennet clearly determined the 'extent' of protein breakdown whereas enzymes from starter bacteria determined the 'depth'. No stimulatory interactions were observed between rennet and starter bacteria in breakdown of paracasein nor in total production of soluble N. However, a clear shift towards smaller soluble products was detected. Peptidases from starter bacteria produced clearly larger amounts of free amino acids and smaller peptides by the presence of breakdown products of rennet action.

The separate actions of rennet and starter bacteria were responsible for the development of the 'bitter' defect in Gouda cheese. During ripening, rennet gradually liberated an increasing amount of bitter peptides, resulting in a distinctly bitter starter-free cheese after 3 months. 'Non-bitter' starters hardly accumulated any bitter peptides in cheese, but they did degrade the bitter peptides produced by rennet. By doing so they prevented matured Gouda cheese from becoming always bitter. 'Bitter' starters could in a highly specific way liberate such amounts of bitter peptides as to cause early bitterness in rennet-free cheese. They had far less capacity to degrade bitter peptides to non-bitter products than 'non-bitter' starters.

Cheeses made with 'bitter' starters always had less intensive cheese flavour than those with 'non-bitter' starters, either in the presence or absence of rennet. Although neither rennet nor milk protease contributed significantly to the production of cheese flavour components, rennet appeared to play an indirect auxiliary role.

Samenvatting

De bijdrage van de enzymen van stremsel, zuurselbacteriën en melk aan de eiwitafbraak en smaakontwikkeling in Goudse kaas

Stremsel, zuurselbacteriën en in mindere mate melkprotease bepalen tesamen grotendeels de afbraak van eiwit tijdens de rijping van kazen van het Goudse type. Deze proteolyse bepaalt in belangrijke mate de structuur en smaak van een kaas. Zowel de ontwikkeling van de gewenste kaassmaak en -geur als het ontstaan van het gebrek 'bitter' ten gevolge van de aanwezigheid van bittere peptiden, hangen ten nauwste samen met de eiwitafbraak.

Om inzicht te krijgen in de rol die elk der genoemde enzymsystemen hierbij speelt, werd tijdens het onderzoek gebruik gemaakt van aseptische kaasbereidingstechnieken die het mogelijk maakten de gescheiden en gecombineerde werking ervan in kaas te bestuderen.

In de 'normale' aseptische kazen is de eiwitafbraak tijdens de rijping het gevolg van de gecombineerde werking van de enzymen van stremsel, zuursel en melkprotease. Om de bijdrage van stremsel vast te stellen, zonder aanwezigheid van zuurselbacteriën, werden kazen bestudeerd waarin tijdens de bereiding de verzuring op chemische wijze werd gesimuleerd. Om de rol van stremsel enigszins kwantitatief te kunnen benaderen, bleek het noodzakelijk de hoeveelheden stremsel in de kaas te bepalen. Het bepalen van de afzonderlijke bijdrage van zuurselbacteriën, zonder de interactie met het stremsel, werd mogelijk door de ontwikkeling van een methode voor de aseptische bereiding van stremselvrije kaas. Door regeling van het calciumgehalte en de temperatuur van de kaasmelk bleek het mogelijk het stremsel, dat werd gebruikt voor de paracaseinevorming, te inactiveren alvorens coagulatie tot een gel optrad. Deze kazen bleken zeer geschikt om de werking van de proteolytische enzymen van het zuursel tijdens de rijping te bestuderen. Door de stremselvrije en zuurselvrije kaasbereidingstechniek te combineren werden kazen verkregen waarin tijdens de rijping nog slechts melkprotease actief was. Daarmee was de mogelijkheid geschapen om de bijdrage van dit enzym tijdens de kaasrijping vast te stellen. Voor de bereiding van de kazen die zuursel bevatten, werden verschillende zuurselcultures gebruikt met het doel de werking van zowel 'niet-bittere' als 'bittere' zuursels te vergelijken. Voor de bereiding van stremselbevattende kazen werden de gebruikte hoeveelheden stremsel sterk gevarieerd.

Tijdens de rijpingsperiode van zes maanden werden de kazen organoleptisch gevolgd om de bitterheid en de kaassmaak vast te stellen. De eiwitafbraak in de verschillende kazen werd gekarakteriseerd met behulp van verschillende analysetechnieken. Kwantitatieve polyacrylamide gelelectroforese werd gebruikt om de afbraak van α_{s1} - en β-caseïne en het ontstaan van de grotere afbraakprodukten te volgen. De hoeveelheid oplosbaar stikstof werd bepaald door extractie en de peptidesamenstelling ervan onderzocht met behulp van gelfiltratie. De hoeveelheid vrije aminozuren en de samenstelling ervan werden eveneens onderzocht. Bittere en adstringerende peptiden

werden uit de verschillende kazen geëxtraheerd en gefractioneerd met behulp van gelfiltratie.

Er kon worden vastgesteld dat stremsel in Goudse kaas grotendeels verantwoordelijk is voor de primaire afbraak van α_{s1} - en β-caseïne tot grote, onoplosbare brokstukken en oplosbare peptiden met een van hoog tot laag variërend molecuulgewicht. Vooral α_{s1}-caseïne wordt zeer snel en volledig afgebroken. Aminozuren worden echter door stremsel nauwelijks gevormd.

Het proteolytisch enzymsysteem van zuurselbacteriën bleek in kaas op een wat langere termijn caseïne te kunnen afbreken onder de gelijktijdige vorming van aminozuren en grotendeels kleine oplosbare peptiden. Duidelijke verschillen wat betreft de eiwitafbraak tussen 'bittere' en 'niet-bittere' zuursels werden slechts gevonden in de totaal geproduceerde hoeveelheid vrije aminozuren.

Aangetoond werd dat melkprotease verantwoordelijk is voor de vorming van de 'minor'caseins uit β-caseïne tijdens de rijping. Bovendien wordt er een geringe hoeveelheid vrije aminozuren en peptiden gevormd.

Wanneer de enzymsystemen tesamen werken, zoals in een normale Goudse kaas, wordt de breedte van de afbraak duidelijk bepaald door de stremselwerking en de diepte door het zuursel. In de primaire afbraak van paracaseïne en de totale aanmaak van oplosbare afbraakprodukten vindt onderling geen duidelijke stimulatie plaats. Er treedt echter wel een verschuiving naar kleinere oplosbare verbindingen op. Zuurselpeptidasen produceerden duidelijk grotere hoeveelheden vrije aminozuren en kleine peptiden door de aanwezigheid van door stremsel gevormde afbraakprodukten.

Tijdens het onderzoek werden de afbraak van de caseïnes en de vorming van de verschillende afbraakprodukten steeds zo kwantitatief mogelijk beschreven.

Bij de ontwikkeling van het gebrek 'bitter' in Goudse kaas blijken de afzonderlijke werkingen van zowel het stremsel als de zuurselbacteriën een belangrijke rol te spelen. Het stremsel produceert tijdens de rijping een gestaag toenemende hoeveelheid bittere peptiden die na drie maanden een duidelijk bittere smaak veroorzaken in de zuurselvrije kaas. 'Niet-bittere' zuursels hopen niet of nauwelijks bittere peptiden op in kaas; ze breken daarentegen juist de door stremsel gevormde grotendeels af. Daarmee verhinderen ze dat een Goudse kaas steeds bitter zou smaken ten gevolge van de stremselwerking. 'Bittere' zuursels kunnen snel en op een zeer specifieke wijze zodanige hoeveelheden bittere peptiden vrijmaken, dat de stremselvrije kaas bitter wordt. Ze bezitten klaarblijkelijk veel minder vermogen tot afbraak van bittere peptiden dan de 'niet-bittere' zuursels.

Zowel in aanwezigheid als in afwezigheid van stremsel blijken kazen bereid met 'bittere' zuursels steeds minder kaassmaak te bezitten dan die gemaakt met 'niet-bittere' zuursels. Hoewel kon worden vastgesteld dat stremsel en melkprotease elk niet bijdragen tot de directe aanmaak van kaassmaak-componenten, blijkt de aanwezigheid van stremsel de kaassmaakvorming door zuursels wel te bevorderen.