Conditions allowing the formation of biogenic amines in cheese

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Abstract

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A study was undertaken to reveal the conditions that allow the formation of biogenic amines in cheese.
The starters most commonly used in the Dutch cheese industry do not have decarboxylative properties. Only if the milk or curd is contaminated with non-starter bacteria, amine formation may be observed. As most of the cheese produced in the Netherlands is made from pasteurized milk, and because recontamination only occurs at very low levels, the concentration of biogenic amines in cheese produced in this country is generally very low.
Actively decarboxylating bacteria were found among the mesophilic lactobacilli and among the Enterobacteriaceae. Enterococci and pediococci are probably not important for amine build-up in Dutch-type cheese.
A study of the kinetics of histamine formation in a Gouda cheese, deliberately infected with a histidine decarboxylating Lactobacillus buchneri strain (St2A, 10^8 cfu/g), revealed that the precursor concentration puts a limit to the histamine content. This explains why toxicologically hazardous quantities can only be found in heavily infected cheese with excessive proteolysis. Histamine degradation does probably not occur in cheese.

De artikelen 2 t/m 6 in dit proefschrift zijn ook gebundeld verschenen als Verslag V272 van het NIZO te Ede.

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STELLINGEN

1. Tyraminevorming in kaas kan slechts bij hoge uitzondering worden toegeschreven aan de activiteit van enterokokken.
   Dit proefschrift.

2. Met de mogelijkheid dat fenylethylamine migraine kan opwekken wordt onvoldoende rekening gehouden.
   Dit proefschrift.

3. De relatie die S. Babu et al. leggen tussen aminevorming in kaas en de decarboxylase-activiteit van *Streptococcus cremoris*, is niet terecht.
   S. Babu, H. Chander, V.K. Batish & K.L. Bhatia, Food Microbiology 3 (1986) 359.

4. Aangezien niet-selectieve MAO-remmers in ons land niet meer worden gebruikt, leveren tyramine bevattende levensmiddelen geen gevaar meer op voor de volksgezondheid.

5. De suggestie van Voigt en Eitenmiller dat aminegehaltes in kaas door bacteriële amine-oxidases beïnvloed worden, berust op de misvatting dat deze enzymen ook onder anaërobe omstandigheden werkzaam zijn.
   M.N. Voigt & R.R. Eitenmiller, J. Food Protection 41 (1978) 182.

6. Ankenbauer et al. hebben te weinig mutanten onderzocht om te kunnen stellen dat de genen die betrokken zijn bij de synthese van pyoverdine door *Pseudomonas aeruginosa*, alle gelocaliseerd zijn in één gencluster.
   R. Ankenbauer, L.F. Hanne & C.D. Cox, J. Bact. 167 (1986) 7.

7. De groei van de secundaire microflora in kaas wordt door Thomas toegeschreven aan kannibalisme. De betreffende bacteriën kunnen echter meer toepasselijk met de term aaseters worden aangeduid.
   T.D. Thomas, New Zealand, J. Dairy Sci. Techn. 22 (1987) 216.

8. Strikte hantering van de nieuwe EG-norm met betrekking tot het vriespunt van melk zal leiden tot het ten onrechte afkeuren van veel onder GMP-omstandigheden gewonnen melk.
   J. Buchberger, Deutsche Molkerei-Zeitung 108 (1987) 1103.

9. De wenselijkheid van een nultolerantie voor *Listeria monocytogenes* in voedingsmiddelen waarin deze bacterie zich niet of slechts in beperkte mate kan ontwikkelen, is onvoldoende beargumenteerd.

10. De opmerking van L.B. James dat het gebruik van relatieve retentietijden bij het integreren van chromatogrammen, afkomstig van aminozuuranalyses, kan leiden tot het niet herkennen van pieken, ten gevolge van variaties in de retentietijden van de betrokken aminozuren, is onjuist.
    L.B. James, J. Chrom. 404 (1987) 321.

11. Aangezien de meerderheid van de surfers bij nadering van een zeilboot toch al terstond hun vaartuigje tot een vlot degraderen en daarmee de doorvaart belemmeren, verdient het aanbeveling de voorrangsregels op het water zodanig te wijzigen dat zij ook de hierbij behorende status krijgen.

12. Gelet op de overwegingen die een rol speelden bij het besluit tot verhoging van de maximumsnelheid op autowegen, zou het van consequent doorgevoerd beleid getuigen indien het wielrenners voortaan werd toegestaan door rood licht te rijden.

Ede, 27 april 1988 H.M.L.J. Joosten

Contents

Voorwoord

Op deze plaats wil ik allen bedanken die hebben bijgedragen aan de totstandkoming van dit proefschrift. Naast de beheerders van het Mesdagfonds, dat dit project financieel ondersteunde, en het bestuur en de directie van NIZO, die mij de ruimte boden om dit werk te voltooien, wil ik vooral Jacques Stadhouders en Pieter Walstra noemen. Zij stonden mij de afgelopen vier jaar steeds terzijde met veel waardevolle ideeën en stimuleerden me ook om mijn bevindingen op papier te zetten. Het kennelijke gemak waarmee jullie mijn pogingen om onzekerheden in het verhaal weg te moffelen dwarsboomden, gaf mij veel vertrouwen in de kwaliteit van het eindresultaat.
Ook Tiny van Boekel ben ik zeer erkentelijk, met name voor zijn grote bijdrage aan het kinetische onderzoek.
Martin Northolt wil ik bedanken voor het mij op weg helpen in de zuivelwereld en Gerard Hup voor het aandragen van resultaten van zijn eigen onderzoek naar aminevorming in kaas.
Cees Olieman heeft een onmisbare bijdrage geleverd bij de ontwikkeling van de analysemethode. Ook zijn medewerkers wil ik danken voor de talrijke malen dat zij mij hielpen weigerachtige apparatuur weer in het gareel te krijgen.
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Voorts dank ik alle medewerkers van de afdeling microbiologie voor de prettige werksfeer, waarin het steeds goed toeven was.

Introduction

Although some cheese varieties are consumed fresh, most require a ripening period. During this time, taste and flavour develop as a result of a set of complex biochemical changes that comprises glycolysis, lipolysis and proteolysis (1-3). The main substrate for the proteolytic enzymes is paracasein and its degradation products range from high molecular weight peptides to smaller peptides and to free amino acids. The greater part of the liberated amino acids is not decomposed any further and therefore their concentrations steadily increase during the maturation stage. In Dutch-type cheese varieties for example, amino acids constitute about 3 % (w/w) of the cheese after one year of ripening, whereas these compounds were virtually absent immediately after manufacturing (4). A relatively small fraction of the amino acids, however, can be converted into other compounds by enzyme-catalyzed reactions such as de- and transamination, desulphurylation, demethiolation and decarboxylation. The latter reaction yields carbon dioxide and primary amines, which are designated biogenic, because they are formed by the action of living organisms. The most important biogenic amines that can be found in cheese are histamine, tyramine, putrescine, cadaverine, tryptamine and phenylethylamine (5,6). Mostly, no or very low levels of these amines are found in cheese, but occasionally high concentrations are encountered.

The presence of biogenic amines in food is considered to be undesirable, because they can give rise to food poisoning. A well known example of this is scombroid poisoning: a histamine poisoning often associated with consumption of fish belonging to the family of Scombridae (7). Histamine containing cheese is sometimes incriminated as a causative agent of this intoxication (8,9). The disease usually has a mild character. Symptoms arise soon after ingestion of the histamine containing food and will mostly last no longer than one day. The most frequently encountered complaints are: rash, urticaria, nausea, vomiting, diarrhoea, hypotension, headache and itching, but individual patients suffering from histamine poisoning usually experience only a few of these symptoms (7).

The other amines can also elicit intoxications. Tyramine can give rise to hypertensive crisis accompanied by a severe headache, but the threshold dose appears to be very high. However, the use of certain drugs (" MAO-inhibitors") can drastically change the toxicity of tyramine and other amines, and in fact even fatal incidents were reported about 25 years ago when these drugs were introduced (10). Because of these serious side effects, the use of MAO-inhibitors has been abandoned.

Phenylethylamine is associated with migraine, while putrescine and cadaverine probably play a role as potentiators of the toxicity of histamine (7,11,12).

In recent years many investigations have been made into the biogenic amine content of various foods (5), and also fundamental research has been performed on decarboxylating enzymes (13,14). However, only a few reports have been published that deal with

the question on how amine formation can actually be avoided in dairy products (15-17): it remains largely unknown under which circumstances massive amine formation can be expected.

The objective of the present study was to investigate which conditions allow the formation of biogenic amines in hard and semihard cheese without a surface flora or internal molds, and especially how very high contents can be explained.

1.1 Outline of the thesis.

Firstly, an analytical method was needed, by which tyramine, histamine, putrescine, cadaverine, tryptamine and phenylethylamine could be determined in cheese. To this end a new HPLC method was developed, which is described in chapter one.

The next step was to investigate which bacteria must be held responsible for the formation of biogenic amines in cheese. It was noticed that amine formation particularly occurred in cheese of poor microbiological quality, indicating that certain non-starter bacteria were probably the cause of this defect. However, the possibility that starter bacteria were also capable of decarboxylating amino acids had to be checked. Therefore cheese was made with six of the most commonly mesophilic mixed strain starters used in the Netherlands: Bos, Ur, Fr8, Fr18, Fr19 and A. Contamination with non-starter bacteria was strictly avoided. Amine contents were measured at regular intervals during ripening. The results of these experiments made clear that the investigated starters lack the capacity to form biogenic amines (chapter two).

In subsequent experiments various non-starter bacteria were examined. Especially from cheese rich in biogenic amines, actively decarboxylating strains were isolated and these were added to pasteurized milk, together with a regular starter. From such milk cheese was made. Indeed, amine formation now took place, although the concentrations found were generally much lower than those which are sometimes found in cheese associated with incidents of food poisoning (chapter three).

As it was realized that there had to be more factors influencing amine formation, new experiments were set up in which again cheese was made from milk deliberately contaminated with decarboxylating bacteria. In these experiments other parameters were varied, especially those which influence the rate of proteolysis and hence the rate of formation of precursor. A combination of the presence of decarboxylating bacteria and excessive proteolysis led to very high amine contents in some cases (chapter four).

As a model system, the formation of histamine in cheese by a histidine decarboxylating strain of Lactobacillus buchneri was studied in detail: both precursor (free histidine) and histamine content were determined during ripening, while the activity of histidine decarboxylase was also measured. The results were used for an analysis of the kinetics of histamine formation in cheese (chapter five).

Chapter 6 gives a survey of the literature about the toxicology of biogenic amines. In this chapter attention is also paid to the amine content of Dutch-type cheese and it is shown that a strict

hygiene during manufacture is an essential factor in minimizing the risk of significant amine formation.
Chapter 1 has also been published in the Journal of Chromatography, chapters 2 to 6 will be published in the Netherlands Milk and Dairy Journal.

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DETERMINATION OF BIOGENIC AMINES IN CHEESE AND SOME OTHER FOOD PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN COMBINATION WITH THERMO-SENSITIZED REACTION DETECTION

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SUMMARY

A simple high-performance liquid chromatographic (HPLC) analysis is described for biogenic amines in cheese and other food products. The sample clean-up consists of a precipitation–extraction step with trichloroacetic acid, which gives recoveries of amines in cheese in the range of 85–105%. The HPLC analysis is performed by reversed-phase ion-pair chromatography, using a ninhydrin-containing eluent, which eliminates the need for an extra reagent pump for the post-column derivatization. Band broadening is minimized by using a poly(tetrafluoroethylene) knitted tube reactor at 145°C. The detection limit for amines is 2 mg/kg cheese and the method is linear for 0.1–4 μg of amine injected. Examples are given of the analysis of amines in cheese, wine and sauerkraut.

INTRODUCTION

During the ripening of cheese, proteins are degraded by enzymes, resulting in an increase of the free amino acid content. Decarboxylation of the amino acids by bacterial enzymes gives rise to the formation of biogenic amines[1–4]. While in most cheeses the content of biogenic amines is low, some have rather high levels. The consumption of large quantities of amines brings about symptoms of intoxication such as headache, nausea, hypo- or hypertension, cardiac palpitation and possibly shock[5,6]. The amines which may be found in cheese are tyramine, histamine, putrescine, cadaverine, tryptamine and phenylethylamine[7–11].

In order to prevent the formation of toxic amounts of biogenic amines in cheese, an investigation to reveal the essential parameters of the production of amines, was commenced. Therefore we needed a simple method for the quantitative detection of biogenic amines in cheese, making possible an automated analysis of many samples.

A variety of methods for estimating biogenic amines in foods has been described[12]. Most of these deal with the detection of only one or two amines[13–16]. Many methods have been developed for the determination of histamine in fish, since his-

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tamine is the causative agent of the food poisoning called "scombroïd fever"[17]. Generally applicable methods for the determination of biogenic amines other than histamine and tyramine are usually based on gas or high-performance liquid chromatography (GLC and HPLC respectively). GLC methods always require a derivatization step to make the amines volatile[18,19]. Derivatization, however, is cumbersome and is difficult to reproduce in the presence of an interfering matrix of compounds in food products like cheese. Pre-column derivatization with, *e.g.*, dansyl chloride, followed by HPLC analysis of the products thus formed, suffers from the same drawbacks.

The separation of underivatized amines by using ion-pair chromatography is a well known HPLC technique. UV detection permits the detection of tyramine and tryptamine at 280 nm, phenylethylamine at 254 nm and histamine at 215 nm. However, 215 nm gives no selectivity and the risk of interfering matrix components cannot be neglected. Putrescine and cadaverine, lacking a suitable chromophore, cannot be detected by UV spectroscopy.

Electrochemical detection of underivatized amines by oxidation of the amino group is a sensitive method but not a very useful one since the oxidation takes place only at pH > 7 and this means that the separation has to be carried out in an alkaline mobile phase, which excludes the use of silica-based columns. The use of a reagent pump has been discarded since electrochemical detection is far too sensitive for shifts of the eluent composition due to pulsation of the pumps. Reversed-phase packing materials, based on organic polymers, should enable the analysis of biogenic amines in the suppressed ion mode at pH > 8. Preliminary experiments with the PRP-1 column, packed with polystyrene, cross-linked with divinylbenzene, were disappointing. Tryptamine and phenylethylamine showed strongly tailing peaks, which could not be suppressed by manipulating the mobile phase.

Post-column derivatization of amines is a widely used technique. With ninhydrin a coloured product is formed[20], while with *o*-phthalaldehyde a highly fluorescent derivative[21] is obtained. A disadvantage of these methods is the requirement of a second pump to deliver the reagent.

Recently, LePage and Rocha[22] described a simple method for the determination of low-molecular-weight aliphatic amines which involved post-column derivatization with ninhydrin. The simplicity is due to the fact that ninhydrin is already dissolved in the mobile phase: the chemical reaction which yields a blue product does not take place until the eluent is heated in a reaction coil (after passage through the column).

The inherent simplicity of thermo-sensitized reaction detection makes this method highly suitable for routine analysis of biogenic amines, requiring in addition to a normal HPLC apparatus only a heated reaction coil. Therefore we have adapted this method for the determination of biogenic amines in cheese and some other food products.

EXPERIMENTAL

Chemicals

Tyramine (TA), histamine (HA), putrescine (PTR), cadaverine (CAD) and tryptamine (TPA) were obtained from Fluka (Buchs, Switzerland), phenylethylamine (PHEA) from Merck (Darmstadt, F.R.G.), ninhydrin and hydrindantin from Pierce

(Rockford, IL, U.S.A.) and sodium dodecyl sulphate (SDS) from Bio-Rad Labs. (Richmond, CA, U.S.A.). Other reagents were obtained from various suppliers.

HPLC equipment

A Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.) and a M440 absorbance detector (detection wavelength 546 nm) were used together with a Perkin-Elmer ISS 100 automatic sampler, equipped with a 1-ml sample loop, and a Sigma 15 B data system (Perkin-Elmer, Oak Brook, IL, U.S.A.). The separations were performed on a radial compression cartridge (10 × 0.8 cm I.D.), custom-packed with Nucleosil C_{18}, 10 μm (Macherey, Nagel & Co., Düren, F.R.G.), in combination with a Waters RCM module. The guard column (3 × 0.3 cm I.D.), was packed with Corasil C_{18} (Waters). The reaction coil consisted of 10 m of PTFE tubing (0.25 mm I.D., volume 0.49 ml) coiled in the form of a twisted figure eight, according to Engelhardt and Neue[23] and heated in an oil-bath to 145°C with a Colora KS ultrathermostat (Colora, Lorch/Württ, F.R.G.).

Mobile phase

Ninhydrin (16 g) and 1.2 g hydrindantin were dissolved in 322 ml dimethyl sulphoxide (DMSO) by sonication for 10 min, followed by the addition of 350 ml of 2.8 *M* sodium acetate buffer (pH 5.00). A 2-g amount of SDS was dissolved in a mixture of 618 ml DMSO and 710 ml water. The two solutions were combined and filtered through a 0.45-μm filter. The mobile phase was stored in a dark bottle and nitrogen was constantly passed through the solution. Under these circumstances it remained stable for at least 1 week. The HPLC system was equilibrated for *ca.* 4 h at 1.0 ml/min. The temperature of the RCM module was kept constant at 29°C by means of a small thermostat. A mixture of DMSO and water (1:1) was used to flush the HPLC system before shut-down.

Extraction of the amines

For the analysis of cheese and chocolate, 45 ml of a 0.07 *M* trisodium citrate solution (45°C) were added to 5 g of the ground sample and homogenized with a "stomacher" for 5 min. A portion (3 ml) of this suspension was mixed with 3 ml of 0.6 *M* trichloroacetic acid (TCA) and centrifuged for 10 min at 10 000 *g* and 4°C in a Sorvall centrifuge. The resulting pellet was resuspended in 3 ml of 0.3 *M* TCA and centrifuged. The combined supernatants were filtered through a 0.45-μm Gelman acrodisc and the volume was adjusted to 10 ml with water. Wine (3 ml) was treated with 3 ml of 0.6 *M* TCA. After centrifugation, the supernatant was filtered and injected. Fish and sauerkraut were prepared for analysis by adding 200 ml of water to 200 g of sample and blending in a household mixer for 3 min. To 3 ml of the suspension were added 3 ml of 0.6 *M* TCA. After centrifugation the supernatant was filtered.

Stock solutions of biogenic amines

Stock solutions containing about 0.2 mg amine per ml in water were kept refrigerated at −20°C. Tryptamine (TPA) solutions were prepared fresh weekly. The calibration mixture was prepared daily by mixing equal volumes of the stock solutions, diluted 1 to 10 in 0.3 *M* TCA solution.

RESULTS AND DISCUSSION

Optimization of the separation and detection

In order to make the method of LePage and Rocha[22] suitable for detection of biogenic amines in cheese, the composition of the mobile phase was adjusted. The composition of the ninhydrin-containing eluent, however, is restricted by the low solubility of hydrindantin: a DMSO content of at least 40% is required. This concentration makes necessary the use of a very non-polar ion-pair reagent in order to get sufficient retention of the amines. A second restriction is the pH dependence of the ninhydrin reaction: the pH should lie between 5.0 and 5.5. Other components of the mobile phase have a limited solubility, and finally the high viscosity of the DMSO–water mixture makes flow-rates of more than 1.5 ml/min impossible. Despite all these limitations we were able to find a set of conditions for a useful separation.

Fig. 1 shows an example of the optimization of the mobile phase. Eluents were prepared with different SDS and DMSO concentrations in such a way as to keep the retention time more or less constant. The effect of increasing the DMSO content can be counteracted by an increase of the SDS concentration. At 44.5% DMSO and 0.5

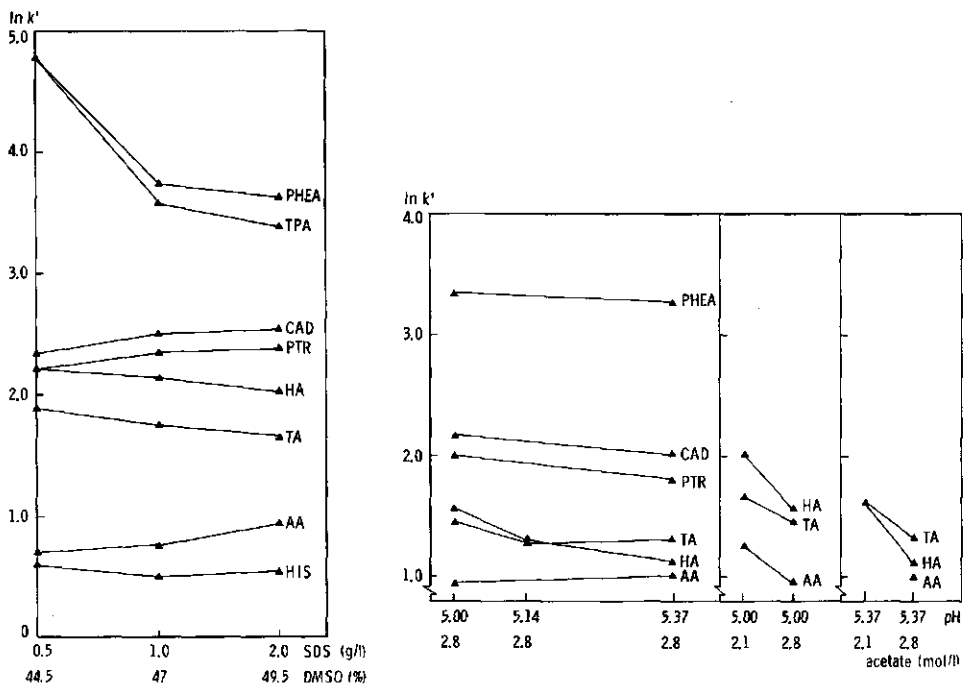


Fig. 1. Dependency of the capacity factor, k', on the mobile phase composition. Samples: mixture of six amines and histidine, 3.3 μg of each compound per ml and a cheese extract. AA = Last eluted amino acid. Mobile phase: pH = 5.00, 2.8 *M*.

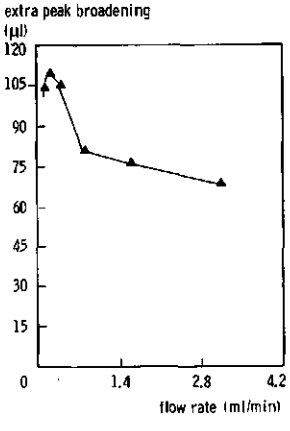
Fig. 2. Dependency of the capacity factor on the pH and molarity of the acetate buffer. Sample: cheese extract spiked with TA, HA, PTR, CAD and PHEA. Mobile phase: DMSO–water (47:53), containing 1 g of SDS per litre.

g SDS/l, there is insufficient separation of putrescine and histamine and of phenylethylamine and tryptamine. With 49.5% DMSO and 2.0 g SDS/l these separations are much better, but this mixture proved to be less useful for cheese samples because of the presence of very large amounts of free amino acids, which interfered with the accurate measurement of the first amine (tyramine) eluted. The mobile phase containing 47% DMSO and 1 g SDS/l proved to be ideal: good separation of all amines and sufficient resolution between tyramine and the amino acids. During the experiments it became clear that the optimum composition was also dependent on the age of the column: after several months of use the DMSO percentage had to be gradually lowered to 43%. This phenomenon is most probably caused by the gradual hydrolysis of the bonded phase.

Another aspect of optimization is shown in Fig. 2. Eluents having small differences in pH and in the molarity of the sodium acetate buffer were prepared. Within the narrow pH range of 5.0–5.4 the degree of protonation of the amino groups of the amines hardly changes, but histamine is very sensitive to small pH shifts, because of the presence of the imidazole group with a pK_a of about 5.0. The 2.8 *M* buffer with pH 5.37 gave a good separation of the first three amines eluted, but analysis of cheese samples caused problems with the resolution of histamine and the amino acids of the cheese extract. Decreasing the molarity of the acetate buffer to 2.1 *M* at pH 5.00 resulted in retention times of more than 2 h for phenylethylamine, which is not a practical proposition, while the resolution between the amino acids and tyramine decreased. Optimum results were obtained with the 2.8 *M* buffer at pH 5.00.

The use of methylcellosolve, which is more toxic than DMSO, gave more problems in keeping the hydrindantin dissolved, while the separation of the amines was less satisfactory than with DMSO.

A reduction of the analysis time by using gradient elution was not possible, since an unacceptable baseline drift was observed. This might be caused by the slow



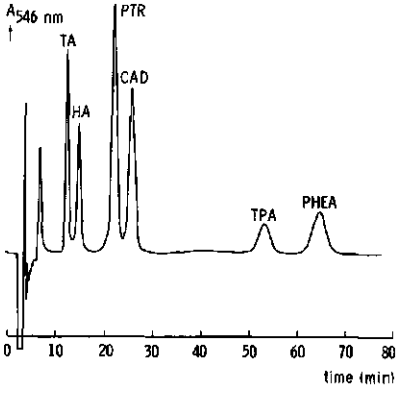


Fig. 3. Extra peak broadening of the knitted tube reactor at different flow-rates of water. A 5 µl volume of acetone was injected into a system with and without the knitted tube reactor.

Fig. 4. Separation of a mixture of biogenic amines, in combination with thermo-sensitized reaction detection with ninhydrin. Detection wavelength: 546 nm. Injected amount: 0.6 µg of each amine. For the column and conditions, see Experimental.

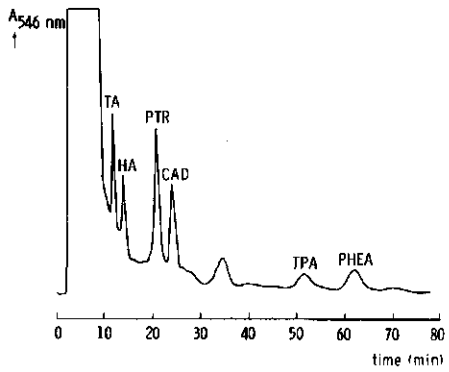


Fig. 5. Chromatogram of a cheese extract, spiked with the amine mixture (each 50 mg per kg cheese). Amino acids are eluted during the first 10 min.

equilibration of SDS with the stationary phase or by desorption of ninhydrin or hydrindantin during the gradient elution.

The band broadening caused by the post-column reaction coil as a function of the flow-rate is shown in Fig. 3 for water. At a flow-rate of 1.0 ml/min an extra band broadening of only 80 μl was measured, having no influence on the resolution. The optimum reaction temperature was found to be 145°C.

Analysis of biogenic amines

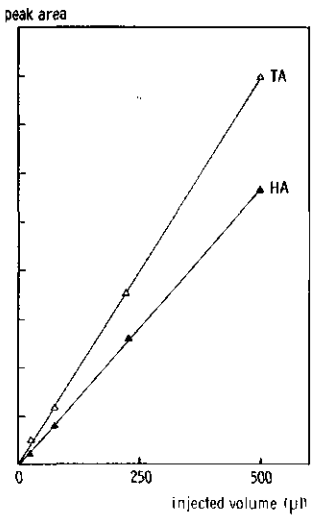
Figs. 4 and 5 show chromatograms of a standard mixture and of a Gouda cheese extract, spiked with a mixture of the amines. An analysis time of 1.5 h is needed for the complete separation of the important amines in cheese. Despite the long retention times of tryptamine and phenylethylamine, these compounds show good peak shapes and they have response factors comparable to those of the faster eluting amines. The rate of the reaction of ninhydrin with amines at 30°C is very low and does not interfere with the analysis. The amino acids are eluted during the first 10 min. Since these are present in very high amounts in cheese, massive peaks are observed on the chromatogram. By lowering the amount of extract injected it is also

TABLE I

RECOVERY OF AMINES BY THE TCA PRECIPITATION METHOD

n = 2. The cheese used contained 5 mg histamine and 5 mg putrescine per kg.

Added amount (mg/kg cheese)	*Recovery (%)*					
	TA	*HA*	*PTR*	*CAD*	*TPA*	*PHEA*
10	140	95	73	100	140	140
50	102	86	86	94	92	94
100	99	93	98	95	100	95
500	93	96	99	97	103	102
1000	92	94	97	95	102	102
2000	93	94	94	94	97	102



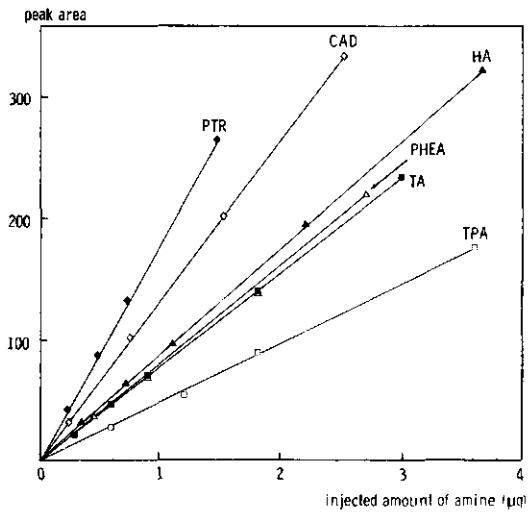


Fig. 6. Peak area as a function of the injected volume (25–500 μl). Sample: mixture of tyramine and histamine (each 3.3 μg/ml). For the column and conditions, see Experimental.

Fig. 7. Linear regression of peak area and the amount of injected amine. Samples of 200 μl were injected, containing 0–4 μg of each amine. Residual standard deviations: TA, 0.3; HA, 0.3; PTR, 2.2; CAD, 0.6; TPA, 5.4; PHEA, 22.6; statistically, the intercepts did not differ from zero.

possible to get an impression of the total amino acid formation in cheese. Histidine (HIS) can be detected selectively by decreasing the content of DMSO to 40% and lowering the molarity of the buffer to 0.7 *M*.

The recovery of amines was studied by homogenizing a three month-old Gouda type cheese with a low amine content ($\leqslant$10 mg/kg). Portions of the suspension

TABLE II

REPEATABILITY

Two cheese samples were fortified with 50 and 500 mg of each amine per kg cheese, respectively. Extracts of these were each injected four times and peak areas were determined. The repeatability of the analysis is expressed as the relative standard deviation (R.S.D.).

Amine concn. (mg/kg cheese)	*Peak area*		*R.S.D. (%)*	*n*
	Mean	*Range*		
TA 50	25.9	25.59–26.27	1.21	4
500	310.4	304.6–315.9	1.58	4
HA 50	21.4	21.03–21.65	1.47	3
500	298.8	292.2–308.0	2.46	4
PTR 50	63.3	62.16–64.35	1.90	4
500	627.9	617.1–641.2	1.92	4
CAD 50	58.1	56.73–59.49	3.36	2
500	465.6	457.1–475.9	2.13	4
TPA 50	14.0	12.24–15.15	9.94	4
500	136.5	133.8–137.5	1.31	4

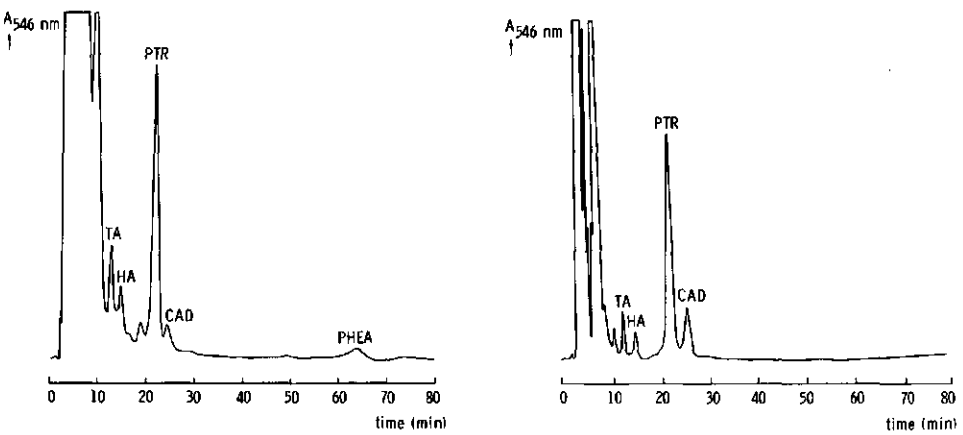


Fig. 8. Chromatogram of a wine extract. This sample contained tyramine (2.7 mg/l), histamine (2.0 mg/l), putrescine (6.9 mg/l) and also traces of cadaverine and phenylethylamine.

Fig. 9. Chromatogram of a sauerkraut extract, containing a large amount of putrescine (210 mg/kg). Tyramine, histamine and cadaverine were also detectable (69, 58 and 79 mg/kg respectively).

were spiked with amines at several concentrations. The results are summarized in Table I. The recoveries of the amines varied between 85 and 105%. Only at the lowest level (10 mg/kg added) was this range larger.

Attempts to make sample preparation easier by reducing the procedure to only one TCA precipitation failed, since recovery rates were about 10% lower. Neither did the addition of 20–40% DMSO to the TCA solution raise the efficiency of the first extraction.

The detection limit for biogenic amines in cheese is 2 mg/kg for each of the amines, which is sufficiently low for toxicological investigations. This low limit is also due to the large sample volume of 200 μl, which could be injected without any change in peak size and shape (Fig. 6). The amines are concentrated at the top of the column probably because they are dissolved in a weaker eluent (0.3 *M* TCA) than the mobile phase. They will not be eluted as long as the surrounding solution does not contain DMSO.

To investigate the repeatability, two Gouda cheese samples, one with a low and one with a high amine content (50 and 500 mg/kg, respectively), were each analysed four times. The results are summarized in Table II. The sample with the low amine content gave a relative standard deviation (R.S.D.)[24] of less than 10%. The sample with the high amine content showed a maximum R.S.D. of 2.5%.

Assuming a linear relationship between the amount of amine injected and its peak area, the residual standard deviation (Syx) was determined. Constant volumes of different diluted standard solutions were injected and the peak areas thus obtained were subjected to linear regression (Fig. 7).

This method was also tested on several other foodstuffs which are known to contain biogenic amines: fish (tuna), wine, sauerkraut and chocolate. Examples are shown in Figs. 8 and 9 for wine and sauerkraut respectively. The detection limit of the amines in sauerkraut is 0.8 mg/kg and in wine 0.3 mg/kg.

CONCLUSION

The described HPLC system permits the determination of biogenic amines in cheese and other foods with good sensitivity and specificity. By means of an automatic injector, this method is very useful for routine analysis.

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Conditions allowing the formation of biogenic amines in cheese. 1. Decarboxylative properties of starter bacteria*

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Summary

Six mixed-strain, mesophilic starter cultures (Fr8, Fr18, Fr19, Bos, Ur and A) were tested for their capacity to cause the formation of biogenic amines in cheese. Cheesemaking was performed under very hygienic conditions using aseptically drawn, pasteurized and bactofuged milk. The amine contents of the cheese were determined after 3, 6 and 12 months of ripening. In addition, the cheese was microbiologically examined. It was found that both biogenic amines and non-starter bacteria were virtually absent.

In another experiment, the formation of biogenic amines was investigated in a Maasdam type of cheese, which was produced under normal conditions. Biogenic amines were not detected in this cheese either.

It was concluded that biogenic amines are not produced in Gouda and Maasdam cheese made from pasteurized milk with sufficient hygienic care.

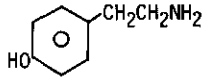
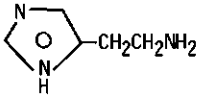
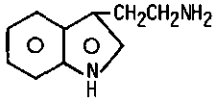
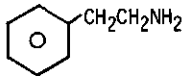
1 Introduction

During the ripening of cheese, casein is slowly degraded by proteolytic enzymes. This mostly leads to a steady increase of the content of free amino acids of which the greater part will not be decomposed further (1, 2). However, some amino acids can be subjected to subsequent breakdown reactions. Decarboxylation is such a reaction. It is catalyzed by specific bacterial decarboxylases and gives rise to the formation of carbon dioxide and an amine (3). These amines are designated as biogenic because they are formed by the action of living organisms. The most important biogenic amines that can be found in cheese are listed in Table 1 together with their precursors.

Usually, cheese does not contain much amines but sometimes very high levels are found. Consumption of cheese rich in amines can give rise to food intoxication. This becomes manifest very shortly after ingestion and the ill-

* This article is part of a series on biogenic amines, which is also distributed as NIZO Research Report V272 (1988).

Table 1. Biogenic amines.

Name	Formula	Precursor
tyramine		tyrosine
histamine		histidine
putrescine	$H_2N(CH_2)_4NH_2$	ornithine
cadaverine	$H_2N(CH_2)_5NH_2$	lysine
tryptamine		tryptophan
phenylethylamine		phenylalanine

ness will usually last for a few hours. Many different symptoms have been reported, of which headache, nausea and urticaria are the most common (4, 5).

The minimum amount of an amine that elicits symptoms of food poisoning is not precisely known, since it may depend on individual susceptibility, simultaneous alcohol consumption, the use of certain types of medicine and potentiation by other amines. As a very rough indication, histamine levels of more than 500-1000 mg/kg (4.5-9.0 mmol/kg) can be regarded as potentially hazardous (6). This is merely based on the amounts found in foods that evoked food poisoning. Even less is known about the threshold level of the other amines.

As the presence of amines in foods represents a potential health hazard, it is important to know what factors affect the contents of biogenic amines of cheese. Therefore the amine formation as well as amine breakdown reactions are to be considered. Some investigators (7) have suggested that decomposition of amines by mono- and diamino oxidases may be important in cheese, but this is unlikely, since oxygen is not available. More effect can be expected from deaminases, which could be active in cheese (2).

Amine formation is caused by bacterial decarboxylases. From earlier stud-

ies by Gale and coworkers (3) much is already known about the decarboxylative enzymes and their way of action. An important conclusion from their work is that only free amino acids can be decarboxylated. It was also found that most decarboxylases can use only one amino acid as a substrate, optimal enzyme activity being found at pH 4-6, that the enzymes are inducible by high substrate levels and that most decarboxylases require pyridoxal phosphate for enzyme action. Consequently, the prerequisites for amine formation in cheese are: the presence of substrate, viz. free amino acid, decarboxylative enzymes and their cofactor and an environment that permits enzyme action. From the literature (1, 8) it is clear that both the amino acid and the pyridoxal phosphate content of most cheese varieties are probably sufficient to allow for at least some amine formation. The physico-chemical conditions in Gouda cheese (pH 5-5.5, ionic strength 0.5-1.0 M) probably permit fairly rapid enzyme action. Therefore we took the capacity of some bacteria to decarboxylate amino acids as the first subject of our investigations.

In other studies (9, 10, 11), many bacteria, also found in dairy products, were grown on specific media and some strains were able to decarboxylate one or sometimes two amino acids. Tyrosine decarboxylase activity, for example, was found in most enterococci, while a strain of *Lactobacillus buchneri* contained histidine decarboxylase (12). Many coliform bacteria can form putrescine and/or cadaverine (13) by decarboxylation of ornithine and lysine respectively. However, the importance of these findings for amine formation in cheese is limited, because the conditions in cheese are quite different from those in the test media used. This may, of course, influence growth and enzyme production.

In this paper we describe experiments with cheesemaking to investigate whether or not starter bacteria can produce biogenic amines. In a subsequent paper we shall describe further experiments to examine the biogenic amine production of non-starter bacteria in cheese. An important aim of this study is to check whether Gouda cheese, produced with sufficient hygienic care, is free from biogenic amines.

Six different cheese starter cultures were used: Fr8, Fr18, Fr19, Bos, Ur and A. All are commercially available mesophilic, mixed-strain starters and together they represent more than 90 % of the starters used for the manufacture of Dutch cheese. In order to prevent contamination with undesired bacteria, cheese was made aseptically. Only bactofuged and pasteurized milk was used. Amine contents and numbers of bacteria were monitored during 12 months of ripening.

In addition, the same analyses were done with a Maasdam type of cheese, which was produced under normal conditions.

2 Materials and methods

2.1 General remarks on aseptic cheesemaking

In order to keep the bacterial contamination of the milk and cheese very low, a number of precautions was taken. The milk was obtained from a farm with a good sanitary reputation. Before milking, the udders of the cows were washed with an iodine-containing soap. After that, they were rinsed with chlorinated water and dried with germ-free towels. All equipment necessary for milking and cheesemaking was sterilized and the brine was pasteurized before use. Furthermore, the cheesemakers were instructed how to avoid contaminaton.

2.2 The milk

A portion of 140 l milk was bactofuged and pasteurized (10 s at 74 °C). 120 l was brought into the cheese vat, from which two 6-kg cheeses were manufactured.

2.3 Cheesemaking

The cheese was made according to the procedure normal for Gouda cheese in 120-l batches. For every 100 l of milk, 600 ml of a fresh starter culture was used. 15 g $NaNO_3$, 25 ml $CaCl_2$ solution (36 g/l) and 22 ml rennet (milk clotting activity 10 800 Soxhlet units) were added. The scalding temperature was 35.5 °C. The time from renneting to brining was 6 hours. Brining took place for 60 hours. After 24 hours of drying the cheeses were coated with a plastic emulsion containing natamycin, and this was repeated several times.

The cheese was stored at 14 °C and 90 % relative humidity for the first three weeks and subsequently at 14 °C and 85 % R.H.

2.4 Starter cultures

Six different starter cultures were used: Fr8, Fr18, Fr19, Bos, Ur and A. The first three starters are B starters, the other three BD starters. B starters contain *Streptococcus cremoris* and *S. lactis* as acid producing microorganisms* and *Leuconostoc cremoris* and *L. lactis* as gas producing organisms. In addition to these microorganisms, BD starters contain *S. diacetylactis**. Each starter culture was prepared by inoculating steamed skim milk (720 ml) with 0.5 % of a fresh culture and incubating for 20 hours at 20 °C.

* The nomenclature of the genus *Streptococcus* has recently been changed (20). *S. cremoris*, *S. lactis* and *S. lactis* subsp. *diacetylactis* are now referred to as *Lactococcus lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *diacetylactis*, respectively. In this publication the old names are used.

2.5 The Maasdam cheese

As well as the six variants of Gouda cheese a Maasdam type of cheese was produced. It was made with the Bos starter culture in combination with thermophilic lactobacilli and propionic acid bacteria (both monocultures). No extra precautions were taken to reduce bacterial contamination. Furthermore, ripening conditions were different: after two weeks at 14 °C, the cheese was stored at 18 °C for three weeks in order to stimulate propionic acid fermentation. Thereafter, the cheese was stored at various temperatures (4-14 °C).

2.6 Sampling of the cheese and preparation of the first dilution

For bacteriological examination, a borer sample of about 8 g was taken, using an aseptic technique. It was added to a ninefold quantity of a 2 % trisodium citrate solution (45 °C) and homogenized with a 'stomacher' for 10 minutes.

For chemical analysis, a sector sample of about 500 g was ground twice after removing the rind. The cut surface of the cheese was covered with plastic emulsion, thus permitting the remainder to be stored further.

Because it was not known whether the amines are homogeneously distributed throughout the loaf of cheese, preliminary experiments were carried out with several lots of Gouda cheese, made from raw milk, obtained from various sources. Samples of different size and weight were taken from each cheese and analyzed. The results showed that the central part of the cheeses contained higher amounts than samples taken close to the rind. A typical example of this is given in Figure 1, representing amine contents of various parts of a three month-old Gouda cheese made from raw milk.

However, if two sector samples from one cheese were analysed, the results were almost equal. Therefore, the sector sampling technique was further used for the test cheeses.

2.7 Bacteriological examination

Portions of 1 or 0.1 ml of a dilution of the milk and cheese samples were mixed with the appropriate media. After solidification of the agar, it was covered with a layer of the same medium, except for the KF streptococcal agar and the plate count agar.

Total aerobic bacterial count. The total aerobic bacterial count was determined with plate count agar (Difco), supplemented with 10 ml reconstituted skim milk per litre, according to NEN 1507. The petri dishes were incubated for three days at 30 °C. Samples of pasteurized milk, prepared from the aseptically drawn milk, were tested in quintuplicate since they contained fewer than 20 cfu/ml.

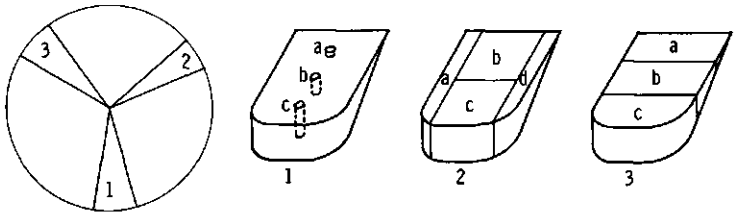


Fig. 1. Distribution of amines throughout a three-month old Gouda cheese made from raw milk.

Sample[2]	Weight	Amine contents[1]			
	(g)	tyramine	histamine	putrescine	cadaverine
1a	6	nd[3]	nd	0.84	1.07
1b	6	nd	nd	0.64	0.46
1c	6	nd	nd	0.58	0.11
2a	136	1.64	0.38	0.71	0.38
2b	137	1.95	0.63	0.75	0.74
2c	50	1.27	0.30	0.48	0.31
2d	127	1.87	0.40	0.53	0.32
(Calculated) average		1.76	0.46	0.65	0.47
3a	115	2.44	0.67	0.80	0.62
3b	202	1.98	0.51	0.68	0.57
3c	180	1.20	0.24	0.32	0.18
(Calculated) average		1.80	0.45	0.58	0.44

[1] Amine contents expressed as mmol/kg. Tryptamine and phenylethylamine were not detected.
[2] 1a, 1b and 1c are borer samples; 2a-3c are parts of a sector sample.
[3] nd: not determined.

The results were statistically analyzed with the Student-T-test. The following differences were found to be significant ($P < 0.05$).

Sector	Tyramine	Histamine	Putrescine	Cadaverine
1			a≠b	a≠b
			a≠c	a≠c
				b≠c
2	a≠b	a≠b	a≠c	a≠b
	a≠c	b≠c	a≠d	b≠c
	b≠c	b≠d	b≠c	b≠d
	c≠d		b≠d	
3	a≠b	a≠b	a≠b	a≠c
	a≠c	a≠c	a≠c	b≠c
	b≠c	b≠c	b≠c	

Coliform bacteria. Violet red bile agar (VRBA, Difco) was used to count coliform bacteria. Incubation took place for 18-24 hours at 30 °C.

Lactobacilli. The number of mesophilic lactobacilli present was estimated by inoculation of TGV 5.4 agar (14). After 5 days of incubation at 30 °C, the colonies were counted. With a microscope it was verified that only rods and no cocci were present.

Enterococci. For the enumeration of enterococci KF agar (Difco) supplemented with 0.01 % TTC (triphenyl tetrazolium chloride) was used. The red colonies were counted after incubation for two days at 37 °C and microscopically checked for enterococcal morphology.

2.8 Chemical analysis

Water content. About 2.5 g of the cheese was dried at 160-165 °C for 30 min and the weight loss determined according to Netherlands Standard NEN 3755.

pH. The pH was measured according to NEN 3775.

Fat content. 3 g of cheese were used for the butyrometric determination of the fat content according to NEN 3758.

Salt content. The chloride content was determined according to the titrimetric IDF method. From this the NaCl content was calculated (FIL-IDF 17:1961).

Amine content. The amine content of the cheeses was determined by the HPLC method of Joosten and Olieman (15).

3 Results and discussion

3.1 Bacteriological examination

The primary aim of the experiments was to find out whether starter bacteria can bring about the formation of biogenic amines in cheese. Many measures were taken to prevent contamination of the milk and cheese with other bacteria.

From Table 2 it can be seen that the raw milk had a very low total count of about 2000 cfu/ml (the Maasdam cheese milk did not receive the same special treatment as the milk used for the other trial cheeses, its total count before pasteurization was 16 000/ml). This, in combination with very hygienic chee-

Table 2. The bacterial flora of the cheese milk and the cheese produced from it[1].

Starter	Sample	Total count[2]	Mesophilic lactobacilli	Entero-cocci	Coliform bacteria
Fr8	raw milk	nd[3]	nd	nd	nd
	bactofuged and past. milk	<0.5	<0	<0	<0
	cheese, 1 day	nd	<1	<1	<1
	cheese, 2 months	nd	<2	<2	nd
	cheese, 6 months	nd	<3	<3	nd
Fr18	raw milk	nd	nd	nd	nd
	bactofuged and past. milk	<0.5	<0	<0	<0
	cheese, 1 day	nd	<1	<1	1.8
	cheese, 2 months	nd	<2	<2	nd
	cheese, 6 months	nd	<3	<3	nd
Fr19	raw milk	3.3	1.6	nd	0
	bactofuged and past. milk	1.1	<0	<0	<0
	cheese, 1 day	nd	<1	<1	<1
	cheese, 1½ months	nd	<3	<3	nd
	cheese, 3 months	nd	<3	<3	nd
	cheese, 6 months	nd	<3	<3	nd
Bos	raw milk	3.3	1.6	nd	0
	bactofuged and past. milk	<0.3	<0	<0	<0
	cheese, 1 day	nd	<1	<1	<1
	cheese, 1½ months	nd	<3	<3	nd
	cheese, 3 months	nd	<3	<3	nd
	cheese, 6 months	nd	<3	<3	nd
A	raw milk	3.0	0.7	nd	1.0
	bactofuged and past. milk	0.8	<0	nd	<0
	cheese, 1 day	nd	nd	nd	<1
	cheese, 1½ months	nd	<3	<3	nd
	cheese, 3 months	nd	<3	<3	nd
	cheese, 6 months	nd	<3	<3	nd
Ur	raw milk	3.0	0.7	nd	1.0
	bactofuged and past. milk	1.1	<0	nd	<0
	cheese, 1 day	nd	<1	nd	<1
	cheese, 1½ months	nd	<3	<3	nd
	cheese, 3 months	nd	<3	<3	nd
	cheese, 6 months	nd	<3	<3	nd
Maasdam	raw milk	4.2	2.5	2.3	2.0
	bactofuged and past. milk	nd	<2	<2	<2
	cheese, 1 day	nd	<3	nd	<3
	cheese, 2 weeks	nd	<3	<3	nd
	cheese, 1 month	nd	<3	nd	nd
	cheese, 6 months	nd	nd	nd	nd

[1] Counts expressed as the logarithm of the number of colony-forming units per ml for milk samples and per gram for cheese samples.
[2] Total count: log number of colonies on PCM agar.
[3] nd: not determined.

semaking, bactofugation and pasteurization, resulted in end products in which the number of lactobacilli, enterococci and coliform bacteria were very low indeed. Coliform bacteria were not present in numbers exceeding 100 cfu/g in the cheese 24 hours after renneting, and since the conditions in Gouda cheese do not permit growth of these organisms except for the first day (16) no further analysis was needed. Enterococci can remain viable much longer (17) and the number of lactobacilli greatly increases during ripening (18), but in all samples the count of enterococci and lactobacilli was lower than 2000 cfu/g. Such small numbers cannot be expected to produce measurable amounts of biogenic amines. These three groups of organisms are the ones most frequently encountered as contaminating flora of Gouda cheese. Other bacteria such as clostridia and propionic acid bacteria are sufficiently suppressed by the use of nitrate, bactofugation and pasteurization and low storage temperature. Pediococci were absent too: there were no colonies of these organisms identified on the TGV 5.4 and KF agar.

This implies that the starter bacteria were the only organisms present in relatively high numbers in these test cheeses. The use of very good quality milk eliminated the possibility that heat stable decarboxylases from other bacteria gained access to the cheese.

The Maasdam type of cheese contained thermophilic lactobacilli and propionic acid bacteria. These were added with the starter culture. Neither strain can grow on the TGV 5.4 medium during the aerobic incubation conditions used.

3.2 Chemical analysis

The amine contents of the six aseptically made cheeses were determined after 3, 6 and 12 months of ripening (Table 3). The amounts found were always very low: tyramine, histamine, cadaverine, tryptamine and phenylethylamine levels were always below the detection limit (which is about 0.2 mmol/kg for the first three amines and about 0.4 mmol/kg for tryptamine and phenylethylamine. Putrescine was the only amine detected: in the one-year-old cheese made with Bos starter, 0.23 mmol/kg was found.

The Maasdam cheese likewise contained only traces of the biogenic amines investigated. After 6 months of ripening only histamine (0.22 mmol/kg) was discovered, the other amines were not detectable.

After 2-6 weeks of ripening the fat, salt and protein content and the pH of the cheeses were determined (Table 4). The aseptically made cheeses were made following the procedure for Gouda, but to minimize growth of bacteria already present and to avoid further contamination, the milk was not standardized. This resulted in cheese with a high fat content. The water content,

Table 3. Amine contents of the test cheese.

Starter	Age of cheese (months)	Tyramine	Histamine	Putrescine	Cadaverine	Tryptamine	Phenylethylamine
Fr8	3	–[1]	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	–	–	–	–
Fr18	3	–	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	–	–	–	–
Fr19	3	–	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	–	–	–	–
Bos	3	–	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	0.22	–	–	–
A	3	–	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	–	–	–	–
Ur	3	–	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	–	–	–	–
Maasdam	1	–	–	–	–	–	–
	3	–	–	–	–	–	–
	6	–	0.23	–	–	–	–

[1] –: less than 0.2 mmol/kg for tyramine, histamine, putrescine and cadaverine; less than 0.4 mmol/kg for tryptamine and phenylethylamine.

Table 4. Some chemical parameters of the test cheeses[1].

Starter	pH	Water (%)	Fat (%)	Salt (%)	Water/solids non-fat	Salt/water (%)
Fr8	5.21	37.0	38.2	2.25	1.49	6.1
Fr18	5.20	37.0	38.8	2.70	1.53	7.3
Fr19	5.17	35.1	38.6	2.34	1.33	6.7
Bos	5.11	36.0	38.1	2.48	1.39	6.9
A	5.25	34.4	38.9	2.39	1.29	6.9
Ur	5.24	34.9	38.3	2.21	1.30	6.3
Maasdam	5.34	44.0	26.0	1.13	1.46	2.6

[1] The analyses were performed after 6 weeks of ripening, except for the Maasdam type of cheese which was analyzed after 2 weeks of ripening.

expressed as water/solids-non-fat was about normal and the salt content, expressed as salt/water was a little higher than is normal for Gouda cheese. The pH can be considered as slightly too low.

4 Conclusion

The results clearly show that the starter cultures investigated cannot form decarboxylases in cheese, at least not enough for significant amine formation. This is in accordance with the results of Antila et al. (19), who also found that amine contents of Edam and Emmental cheese were very low if they were made with modern technology, which almost eliminates contamination with undesirable bacteria.

Theoretically, the possibility exists that amines are formed and subsequently degraded by other enzymes in the cheese. If that really did occur, certain amino acids (the ones that are decarboxylated) would disappear. We found that in a cheese made from milk contaminated with decarboxylating bacteria, the relative amounts of the decarboxylated amino acids are low, while the sum on a molar basis of amine and corresponding precursor amino acid content is virtually the same as in a cheese made without decarboxylating bacteria. Furthermore, in subsequent analyses of ripening cheese containing decarboxylating bacteria, we never detected a significant decline of the amine content (H. Joosten, unpublished results). These findings indicate that amine breakdown processes are probably of limited importance in cheese.

It is also possible that the starter bacteria scarcely formed any amines because the environmental factors such as pH, salt content and temperature were not optimal. Still, this does not interfere with the conclusion that Gouda and Maasdam cheese made from pasteurized milk need not contain biogenic amines. Also, we consider it unlikely that other mesophilic starters used in this country give rise to formation of biogenic amines in cheese. Possible amine formation by other, non-starter bacteria and the influence of environmental factors are the subjects of subsequent papers.

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Samenvatting

H. M. L. J. Joosten en J. Stadhouders, *Omstandigheden waaronder biogene aminen in kaas kunnen worden gevormd. 1. Decarboxylerende eigenschappen van enige zuursels*[1]

Zes mengcultures (Fr8, Fr18, Fr19, Bos, Ur and A) werden onderzocht op hun vermogen om biogene aminen in Goudse kaas te vormen. Hiertoe werd er met ieder van deze zuursels kaas bereid onder zeer hygiënische omstandigheden, uitgaande van aseptisch gewonnen, gepasteuriseerde en gebactofugeerde melk. Na 3, 6 en 12 maanden werd het aminegehalte van de kazen bepaald. Daarnaast werden er bacteriologische onderzoekingen uitgevoerd om na te gaan of er naast de zuurselbacteriën ook andere organismen in de kaas terecht waren gekomen.

Uit de analyses bleek dat er in geen van de kazen biogene aminen gevormd waren. Het aantal niet-zuurselbacteriën in de kaasmelk en kaas was altijd zeer laag.

Bij een ander experiment werd de vorming van biogene aminen onderzocht in een kaas van het type Maasdam. Deze was onder de normale hygiënische omstandigheden vervaardigd. Ook in deze kaas was het gehalte aan aminen zeer laag. Uit deze onderzoekingen werd de conclusie getrokken dat Goudse en Maasdammer geen biogene aminen bevatten, mits de kaas onder hygiënische omstandigheden uit gepasteuriseerde melk wordt bereid.

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Conditions allowing the formation of biogenic amines in cheese. 2. Decarboxylative properties of some non-starter bacteria*

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Summary

In this second article about the formation of biogenic amines in cheese we report on the decarboxylative properties of some non-starter bacteria which could be found in dairy products. This was studied by performing experiments in which cheese was made from milk deliberately contaminated with these bacteria.

The presence of many Gram-negative organisms in the milk, as can be found if the milk is stored for too long, does not lead to amine formation in cheese, at least not if pasteurization is performed properly.

Lactobacilli are probably of great importance for amine formation in cheese. While many strains do not possess decarboxylases, mixtures of strains were found to cause the formation of tyramine and histamine as well as putrescine. A special group, the salt-tolerant lactobacilli, accounted for massive formation of putrescine and cadaverine. Several pure cultures, capable of decarboxylating histidine or tyrosine, were studied in separate experiments.

Enterococci, even though generally considered to be notorious tyramine forming bacteria, were not found to cause this defect in cheese if their number was not higher than 10^7 cfu per gram. If outgrowth took place to $2 \cdot 10^9$ cfu/g, as well as tyramine, large amounts of phenylethylamine were found. But since Dutch cheese hardly ever contains more than 10^6 cfu of these bacteria per gram, enterococci are not important for the formation of biogenic amines.

Representatives of the *Enterobacteriaceae* could cause cadaverine and putrescine build-up, the largest amounts being found when strains with active decarboxylases and exhibiting a low death rate were present in the cheese milk. The other organisms tested, pediococci and *Bacterium proteolyticum* were found to be unimportant for amine formation in Dutch cheese.

In general, the amounts of amines formed were not high: except for the cheeses with an extraordinarily heavy contamination, no amine was detected in higher amounts than 5-6 mmol/kg. Tryptamine was never found and phenylethylamine was only detected in a cheese that contained $2 \cdot 10^9$ enterococci per gram.

Introduction

Formation of biogenic amines may be a problem in the production of fer-

* This article is part of a series on biogenic amines, which is also distributed as NIZO Research Report V272 (1988).

mented foods, including cheese. It was shown earlier (1) that the starter bacteria most commonly used for Dutch cheesemaking cannot form amines in cheese. This was demonstrated by performing experiments in which cheese was made under very hygienic conditions, thereby preventing contamination with non-starter bacteria. Even after one year of ripening the amine contents were still negligible. It was concluded that any formation of biogenic amines must be caused by the action of the adventitious microflora.

As agents of biogenic amine formation, probably only those bacteria that can be present in relatively high numbers in the cheese(milk) need to be considered. For Gouda and Edam cheese, this means that suspicion lies mainly with the non-starter lactic acid bacteria (lactobacilli, enterococci and pediococci) and some Gram-negative bacteria (*Pseudomonadaceae*, *Enterobacteriaceae*).

In this publication we give the results of experiments in which cheese was made from milk which was deliberately contaminated after pasteurization. Most of the bacteria added to the cheese milk were pure cultures of strains isolated from cheese rich in biogenic amines. In general, they were screened for decarboxylase activity in test tubes first. To screen a great number of different bacteria, experiments were also performed with cheese milk contaminated with a mixture of strains. In the most prominent cases of amine formation, the effect of varying the size of the inoculum was also studied.

In general, the experiments were performed in such a way as to provoke amine formation. It must be emphasized that the cheeses described in this publication are by no means representative of normal Gouda or Edam cheese. Only in very exceptional cases were these types of cheese found to contain biogenic amines, and it is for that reason that we chose extreme circumstances for our experiments.

2 Materials and methods

2.1 Cheesemaking

Unless stated otherwise, a portion of about 770 litres of standardized milk was bactofuged and pasteurized (10 s at 74 °C). Four cheese vats were filled with 180 litres each. One batch usually served as a control, to the other vats cultures of different strains of bacteria were added. From each vat, one small cheese (1 kg) and three bigger ones (6 kg) were made in the manner usual for Gouda cheese. The scalding temperature was 35-35.5 °C. The time from adding rennet to brining was about 5.5 hours. The ripening temperature was 14 °C. As a starter, 0.6 % of the B-starter Fr19 was added to the cheese milk.

2.2 Media

The following media were used:

- Brain heart infusion broth (BHI, Difco).
- *Pseudomonas* F agar (Difco).
- KF *streptococcus* agar (Difco), supplemented with 0.01 % TTC (triphenyl tetrazolium chloride).
- Violet red bile agar (VRBA, Difco).
- Plate count agar (PCA, Difco), supplemented with 0.1 % skim-milk powder.
- TGV agar, a very rich medium especially suitable for propagating lactic acid bacteria, as described by Galesloot et al. (2).
- TGV 5.4 agar, due to the acetic acid content and the lower pH selective for mesophilic lactobacilli (3).
- TGV 5.4 + 9 % (w/v) NaCl, for the enumeration of salt tolerant lactobacilli (see text).
- TVC, this medium has the same composition as TGV, except for the glucose being replaced by 5 % (w/v) casamino acids (Difco).
- Modified Niven medium. The original medium as described by Niven et al. (4), was supplemented with Tween 80 (0.1 %), $MgSO_4 \cdot 7H_2O$ (0.02 %), $MnSO_4 \cdot 7H_2O$ (0.005 %) and $FeSO_4 \cdot 7H_2O$ (0.004 %).
- Modified Møller medium (5). The basal medium contained glucose (0.1 %), yeast extract (Difco, 0.3 %), tryptone (Difco, 0.3 %), NaCl (0.5 %), Tween 80 (0.1 %), $MgSO_4 \cdot 7H_2O$ (0.02 %), $MnSO4 \cdot 4H_2O$ (0.005 %), $FeSO_4 \cdot 7H_2O$ (0.004 %) and bromocresol purple (0.006 %). To this, the amino acid of interest was added (0.5 %) and the pH was adjusted to 7.0.

2.3 Cultures

The cultures used for the experiments and their decarboxylative properties in test media are listed in Table 1. Some strains were obtained from the collection of the Netherlands Institute for Dairy Research (NIZO), while others were new isolates from cheese containing high quantities of biogenic amines. *Lactobacillus buchneri* St2A was kindly provided by Dr S. L. Taylor, Madison, USA. The *Pediococcus acidilactici* strains were gratefully received from Dr F. Dellaglio, Piacenza, Italy.

Furthermore, in some experiments mixtures of unidentified strains were added to the cheese milk. These mixtures were obtained by homogenization of agar plates, mostly selective media, containing about 200 colonies per plate. These agar plates were originally intended for microbiological examination of several cheeses rich in biogenic amines. The mixture of salt-tolerant lactobacilli was obtained from cheese brine.

Table 1. Decarboxylative properties of the strains used for the experiments[1].

Name	Source	Decarboxylase for
Lactobacillus buchneri St2A	Emmental	histidine
L. sp. 4720-2	Gouda	histidine
L. brevis 2B5B	Gouda	tyrosine
L. brevis Hem3	Gouda	tyrosine
Streptococcus faecalis H1	milk	tyrosine
S. durans J1	milk	tyrosine
Hafnia alvei LN1	Gouda	ornithine, lysine
Escherichia coli LN2	Gouda	ornithine, lysine
E. coli RC1	milk	ornithine
Bacterium proteolyticum T1	Emmental	not determined
Pseudomonas fluorescens 22F	milk	not determined
Pediococcus acidilactici M301	Italian cheese	not determined
P. acidilactici M324	Italian cheese	not determined

[1] Only if chemical analysis of the test media after incubation revealed the presence of biogenic amines, were the strains assumed to possess decarboxylase activity (see also Section 2.4).

2.4 Selection of decarboxylase-positive strains

There are many reports on the detection of bacteria capable of forming biogenic amines. Often, the rise of the pH accompanying the decarboxylation reaction has been used. This can be detected either by direct measurement with a glass electrode (6) or by means of a suitable indicator in the growth medium, containing the amino acid to be investigated (5, 7, 8). Bromocresol purple and methyl red have been successfully used for this purpose.

Another effect of the decarboxylation reaction is the liberation of carbon dioxide. This can be measured by manometric (9) or by radiometric (10, 11) methods.

Furthermore, the accumulation of amines in the growth medium can be determined by qualitative or quantitative methods (12, 13, 14). We tested the usefulness of several of the above-mentioned methods. In general it was necessary to adapt the composition of the media to meet the specific requirements of the strain. For example, the medium described by Niven (4), developed for the detection of histamine-forming bacteria in fish, does not permit the growth of lactobacilli. Therefore, Tween-80 and several metal sulphates were added (see Section 2.2, Media), however, at best only a very faint colour change was observed. Colour changes were more prominent when liquid media were used. Good results were obtained with a modification of the method of Møller (5). Growth of the tested strain in the basal medium (without amino acid) was indicated by a yellow colour. This colour was also seen in the medium with the amino acid in question, when no decarboxylase was formed. When a certain strain did possess decarboxylase activity, the colour

after incubation was purple. However, a strain was assumed to be decarboxylase-positive only if the chemical analysis of the growth medium also gave evidence of amine formation. This analysis was based on the HPLC method described earlier (15). The sample clean-up consisted of adding 3 ml of 0.6 M trichloroacetic acid to an equal volume of growth medium. The mixture was centrifuged (10 min, 10 000 *g*) and the supernatant was filtered through a 0.45 μm Gelman acrodisc. 20 μl of the filtrate was used for HPLC analysis.

2.5 Cultivation

The lactic acid bacteria were grown in sterilized litmus milk, supplemented with yeast extract (1 %), except for *Streptococcus faecalis* H1, which was propagated in skim milk without yeast extract. An inoculum of 1 % was incubated for 24 hours at 30 °C. These cultures were added directly to the cheese milk.

The salt-resistant bacteria were not propagated separately. Instead, a few grams of sediment from brine known to contain these bacteria were added to the cheese milk.

Bacterium proteolyticum was grown anaerobically for two weeks at 30 °C in TVC medium.

The Gram-negative microorganisms were grown on BHI medium. After 1 day incubation at 30 °C the cells were harvested by centrifugation (10 min, 10 000 *g*, 4 °C) and resuspended in a 0.85 % NaCl solution.

2.6 Microbiological and chemical analysis

Pediococci. As most pediococci grew well on KF medium and on TGV 5.4 medium, a separate counting method was not needed. Their typical morphology (tetrads) allowed us to distinguish these organisms from lactobacilli and streptococci. For determinative purposes tests on catalase and on glucose fermentation (homo- or hetero-fermentative pathway (16)) were also performed.

Salt-tolerant lactobacilli. Growth of these organisms in TGV 5.4 medium was enhanced by adding 4 % NaCl and anaerobic incubation for two weeks at 30 °C. Because non-salt-tolerant species were also present in the examined cheeses, it was necessary to distinguish between these two groups. The addition of 9 % salt suppressed the growth of the latter sufficiently, while the salt-tolerant species were still able to form visible colonies. In experiments with pure cultures of *Pseudomonas fluorescens* use was made of *Pseudomonas* F agar (Difco). Plates were incubated for three days at 17 °C and the number of green fluorescent colonies was determined.

Coliform bacteria. To identify the decarboxylase-positive strains use was made of API 20E strips (17).

Other microbiological and chemical methods were as published earlier (1).

3 Results and discussion

3.1 The influence of Gram-negative bacteria in the milk before pasteurization on the formation of biogenic amines in cheese

Antila et al. (18) demonstrated that the microbial quality of the raw milk greatly affects the formation of biogenic amines in cheese. However, no information was given on the composition of the microflora of the cheese and since the milk used for cheesemaking did not receive a severe heat treatment (20 s at 63 °C), it cannot be excluded that some of the Gram-positive bacteria survived and caused amine formation in the cheese. No answer was given to the question whether the sole presence of large numbers of Gram-negative bacteria in the milk prior to pasteurization could also bring about the formation of biogenic amines.

We set up experiments in which cheese was made from pasteurized milk, originally containing different numbers of Gram-negative bacteria. Apart from the chemical analysis of the cheese, the microbiological load of the cheese was also determined.

Raw milk, with a total count of $5.5 \cdot 10^4$ cfu/ml was pasteurized (not bactofuged) for 10 s at 74 °C. The amine concentrations in the cheese made from this milk were determined after 3, 6 and 12 months of ripening. Another portion of the same milk was stored for two days at 7 °C. During that time, the total count increased to $4 \cdot 10^6$ cfu/ml, mainly due to the growth of Gram-negative bacteria. This milk was then also pasteurized and used for cheesemaking.

In a third experiment, a culture of *P. fluorescense* 22F, that had been incubated in milk for three days at 20 °C, was added to 200 litres of raw milk. This raised the total count to $12 \cdot 10^6$ cfu/ml. Thereafter pasteurization and cheesemaking followed.

The results of the chemical analysis (Table 2) show that in none of the cheeses did any amine formation take place, notwithstanding the high Gram-negative counts before pasteurization. The most likely explanation for the absence of amines is that no bacteria capable of forming decarboxylases gained access to the cheese. The possibility that decarboxylases were actually present in the raw milk, but became inactivated by the heat treatment seems slight, since these enzymes are not formed to any great extent at a pH of 6.5-7.0 (19, 20).

Table 2. Effect of Gram-negative bacteria in the cheese milk before pasteurization on the formation of biogenic amines.

Treatment/addition	cfu/ml[1]	Biogenic amine contents after one year of ripening[2] (mmol/kg)				
		TA	HA	PTR	TPA	PHEA
pasteurization	5.5×10^4	–[3]	–	–	–	–
storage for two days at 7 °C, then pasteurization	4×10^6	–	–	–	–	–
addition of *Pseudomonas fluorescens* 22F, then pasteurization	12×10^6	–	–	–	–	–

[1] Colony count immediately before pasteurization.
[2] TA = tyramine, HA = histamine, PTR = putrescine, CAD = cadaverine, TPA = tryptamine, PHEA = phenylethylamine.
[3] – = <0.2 mmol/kg for TA, HA, PTR and CAD; <0.4 mmol/kg for TPA and PHEA.

In the following sections attention is focused on amine formation caused by infection with non-starter bacteria after pasteurization.

3.2 Decarboxylative properties of lactobacilli

Lactobacilli are very often found in Dutch type of cheese (21). Even if pasteurized milk is used, contamination with lactobacilli is difficult to avoid completely. Since these bacteria can easily multiply in the cheese during the first weeks of ripening, high counts ($>10^7$ cfu/g) may occur (22), particularly in cheese made from raw milk.

Several investigators have studied the decarboxylative properties of lactobacilli (23). Most strains showed no sign of this capacity, but some decarboxylase-positive isolates were found when tested in specific media. Rodwell isolated a Lactobacillus that decarboxylated both histidine and ornithine (24). From an Emmental type of cheese, containing a high concentration of histamine, a histidine-decarboxylating strain of *Lactobacillus buchneri* was isolated (25). Already in 1949, Dacre showed tyrosine decarboxylating activity of a *L. brevis* strain, both in test tubes and in cheese (26).

In one set of experiments, pasteurized and bactofuged milk was inoculated with pure cultures of decarboxylating lactobacilli. In another set of experiments, mixtures of *Lactobacillus* strains were added, obtained from amine-containing cheeses. One mixture was isolated from cheese brine. The cheese was analyzed after 1, 30, 90, 180 and 365 days.

The results in Table 3 clearly show that some strains of lactobacilli can indeed bring about the formation of biogenic amines in cheese. Both *L. brevis* strains, 2B5B and Hem3, gave 5.5-6.0 mmol tyramine/kg after one year of

Table 3. The formation of biogenic amines in cheese by lactobacilli[1].

Exp.	Strain(s)	Density in cheese milk (cfu/ml)	Maximum density[2] in cheese (cfu/g)	Age of cheese (days)	Amine contents (mmol/kg)[3]					
					TA	HA	PTR	CAD	TPA	PHEA
1	*L. brevis* 2B5B	4×10^3	8×10^7	90	1.35	–[4]	–	–	–	–
				180	4.10	–	–	–	–	–
				365	5.55	–	–	–	–	–
2	*L. brevis* Hem 3	5×10^3	7×10^7	90	1.80	–	–	–	–	–
				180	3.95	–	–	–	–	–
				365	6.00	–	–	–	–	–
3	*L. buchneri* St2A	1	9×10^7	90	–	0.75	–	–	–	–
				180	–	1.45	–	–	–	–
				365	–	4.33	–	–	–	–
4	*L. buchneri* St2A	1.5×10^3	20×10^7	90	–	0.85	–	–	–	–
				180	–	1.70	–	–	–	–
				365	–	5.10	–	–	–	–
5	*Lactobacillus* sp. 4720-2	1.4×10^3	10×10^7	90	–	0.40	–	–	–	–
				180	–	1.30	–	–	–	–
				365	–	3.60	–	–	–	–
6	Mixture	9×10^3	13×10^7	90	1.45	–	–	–	–	–
				180	2.60	–	–	–	–	–
				365	4.35	–	–	–	–	–
7	Mixture[5]	5.5×10^3	20×10^7	90	1.45	0.70	1.70	–	–	–
				180	2.35	1.80	2.50	–	–	–
				365	4.60	4.95	4.50	–	–	–
8	Mixture[6]	8×10^3	20×10^7	90	–	0.75	0.70	–	–	–
				180	–	2.15	1.15	–	–	–
				365	0.30	6.00	1.30	–	–	–

9	Mixture of salt-tolerant strains	n.d.	6×10^7	90	–	–	1.70	4.10	–	–
				180	–	–	4.10	13.40	–	–
				365	n.d.[7]	0.45	8.30	>29	–	–

[1] The lactobacilli were added to the cheese milk after pasteurization.
[2] Maximum density of lactobacilli found in the cheese, usually after 1 to 3 months of ripening.
[3] See Table 2, note 2.
[4] See Table 2, note 3.
[5] In addition to the lactobacilli, also 2×10^6 pediococci/g were found after 3 months of ripening.
[6] In addition to the lactobacilli, also 5×10^6 pediococci/g were found after 3 months of ripening.
[7] not determined.

ripening. The addition resulted in maximum numbers of 7 à $8 \cdot 10^7$ cfu/g cheese. The other amines were not detected in significant amounts.

Histamine was found at 3.6-4.5 mmol/kg after one year of ripening in cheese contaminated with *Lactobacillus buchneri* St2A and *L.*4720-2. Here, again, no great differences were detected when using different strains capable of decarboxylating the same amino acid. The size of the inoculum had little effect on the concentration of histamine. Although addition of about 1 bacterium per ml milk resulted in a slightly lower maximum density of these bacteria in the cheese than an addition of $1.5 \cdot 10^3$ per ml, the amount of histamine detected did not differ very much after six months of ripening.

Cheese produced from milk contaminated with mixtures of lactobacilli (Table 3, Exp. 6, 7 and 8), showed formation of tyramine, histamine and also putrescine in various amounts. The mixture of salt-tolerant lactobacilli caused massive formation of cadaverine, more than 29 mmol/kg being detected after one year of ripening, while putrescine (8.3 mmol/kg) was also found. This was accompanied by such gas production that the interior of the cheese showed numerous big holes and cracks. The presence of these bacteria must be regarded as exceptional, unless brine of reduced strength is used (27, 28).

Microbiological examination of the maturing cheese revealed that in some cases pediococci were also present (Exp. 7 and 8). Probably this was caused by the fact that a suspension prepared from all the colonies grown on a TGV 5.4 agar plate was used for inoculation; during the course of these experiments it became clear that this group of bacteria can also grow on TGV 5.4. Nevertheless, lactobacilli were the predominant microflora (apart from the starter bacteria), maximum numbers of $5 \cdot 10^7$-$20 \cdot 10^7$ cfu/g being observed. *Pediococcus* counts ranged from $<10^4$ to $5 \cdot 10^6$ cfu/g. As will be shown in Section 3.5, no indications were found that pediococci can cause amine formation in cheese. It is noted that none of the cheeses contained tryptamine or phenylethylamine.

3.3 Decarboxylative properties of enterococci

Enterococci are often found as contaminants of milk and milk products (29, 30). Because these organisms are thermoresistant and because they can multiply rapidly during the first hours of cheesemaking, high densities can eventually be found. Nevertheless, they usually do not constitute the predominant microflora: 10^6 cfu/g can be regarded as very high, while 10^7 cfu/g is truly exceptional. Higher numbers are only found in cheese for which *S. faecalis* has been used as a starter (31). Dutch starter cultures do not contain enterococci.

In 1940 Gale demonstrated that *S. faecalis* can decarboxylate tyrosine to form tyramine (32). This capacity is shared by many other representatives of the enterococci (33). From the experiments of Dacre (26) and Dahlberg (34) it is clear that these organisms can also cause accumulation of tyramine in cheese, at least if they are added in large numbers to the cheese milk. However, it is not clear whether these microorganisms can also cause tyramine formation if at most 10^6 or 10^7 cfu/g are present. To study this, several cheeses were made from milk, inoculated after pasteurization with pure cultures of tyrosine decarboxylase-positive enterococci or with mixtures of enterococci. These mixtures had been isolated from cheese rich in tyramine. Microbiological and chemical analyses were performed after 1, 14, 30, 60, 90, 180 and 365 days of ripening. The results are shown in Table 4.

In the cheese contaminated with pure cultures of enterococci, tyramine formation was detected, but only in significant amounts if densities of 10^7 or higher were reached in the cheese. With $2 \cdot 10^9$ cfu/g, after 6 months of ripening, tyramine formation (9.5 mmol/kg) was accompanied by accumulation of phenylethylamine (12.8 mmol/kg). This was probably due to the presence of large amounts of the tyrosine-decarboxylating enzyme, which is known to possess activity towards phenylalanine as well (35). In the other experimental cheeses, no phenylethylamine was detected. The explanation for this probably lies in the fact that the enzyme has a much lower affinity for phenylalanine than for tyrosine (35).

Using a different species (*S. durans*) for inoculation of the milk, similar results were obtained: 10^7 cfu/g cheese yielded only tyramine at 0.75 mmol/kg after one year of ripening. Assuming that the decarboxylases of other strains are not more active (6), it is unlikely that enterococci are of practical importance for amine formation in (Dutch) cheese. The density needed for significant tyramine formation is hardly ever reached. This also explains why other investigators did not observe a correlation between tyramine content and number of enterococci (30, 36).

At first sight, the tyramine found in the cheese contaminated with mixtures of enterococci is in contradiction with this hypothesis; in one of these up to 4.2 mmol/kg was detected, while only $6 \cdot 10^6$ enterococci were present (Exp. 7, Table 4). However, lactobacilli and pediococci must also have been added to the cheese milk, since these bacteria reached even higher densities than did the enterococci. The presence in the cheese of pediococci and lactobacilli was an undesired effect of using homogenized KF streptococcal agar with colonies as inoculum. KF is a less selective medium (37) and many other bacteria can also grow on it. As it is known now that certain lactobacilli can also bring about the formation of tyramine (Section 3.2), it is likely that these organisms

Table 4. The formation of biogenic amines in cheese by enterococci[1].

Exp.	Strain(s)	Density in cheese milk (cfu/ml)	Maximum density[2] in cheese (cfu/g)	Age of cheese (days)	Amine contents (mmol/kg)[3]					
					TA	HA	PTR	CAD	TPA	PHEA
1	*S. faecalis* H1	3.6×10^4	1.1×10^6	30	–[4]	–	–	–	–	–
				60	0.10	–	–	–	–	–
				90	0.10	–	–	–	–	–
2	*S. faecalis* H1	3.5×10^5	1.0×10^7	30	0.30	–	–	–	–	–
				60	0.45	–	–	–	–	–
				90	0.85	–	–	–	–	–
3	*S. faecalis* H1	4×10^6	1.3×10^8	30	0.65	–	–	–	–	–
				60	1.10	–	–	–	–	–
				90	2.15	–	–	–	–	–
4	*S. faecalis* H1	1.1×10^7	2.0×10^9	30	1.80	–	–	–	–	1.80
				90	4.40	–	–	–	–	7.45
				180	9.55	–	–	–	–	12.80
				365	9.10	–	–	–	–	11.55
5	*S. durans* J1	n.d.[5]	1.0×10^7	30	n.d.[5]	–	–	–	–	–
				90	0.35	–	–	–	–	–
				180	n.d.	–	–	–	–	–
				365	0.75	–	–	–	–	–
6	Mixture[6]	7×10^3	9×10^6	30	–	–	–	–	–	–
				90	–	–	–	–	–	–
				180	–	–	–	–	–	–
				365	–	–	–	–	–	–
7	Mixture[7]	3×10^4	6×10^6	30	–	–	–	–	–	–
				90	0.80	–	–	–	–	–
				180	2.15	–	–	–	–	–
				365	4.15	–	–	–	–	–

8	Mixture[8]	1.5×10^4	3×10^6	30	–	–	–	–	–
				90	–	–	–	–	–
				180	–	–	–	–	–
				365	–	–	–	–	–

[1] The enterococci were added to the cheese milk after pasteurization.
[2] Maximum density of enterococci found in the cheese, usually after 1 to 3 days.
[3] See Table 2, note 2.
[4] See Table 2, note 3.
[5] not determined.
[6] In addition to the enterococci, also 5×10^7 lactobacilli and 3×10^7 pediococci/g were found after 3 months of ripening.
[7] In addition to the enterococci, also 3×10^6 lactobacilli and 1×10^6 pediococci/g were found after 3 months of ripening.
[8] In addition to the enterococci, also 2×10^7 lactobacilli and 2×10^7 pediococci/g were found after 3 months of ripening.

caused the decarboxylation of tyrosine. This suggestion is also supported by the fact that the rise of the tyramine concentration with increasing age shows more similarity to the pattern of the cheeses contaminated with *L. brevis*: the highest amounts were found after one year. In the cheese with *S. faecalis*, tyramine contents did not increase any more after six months of ripening.

Absence of biogenic amines in the other two cheeses (Exp. 6 and 8) made with mixtures of enterococci, indicates that even a fairly heavy density of enterococci, lactobacilli and pediococci does not necessarily lead to the formation of these substances.

3.4 Decarboxylative properties of Enterobacteriaceae

For the growth of coliform bacteria in Gouda and Edam cheese only a short time is available. Within 6 hours multiplication is restricted by the accumulation of lactic acid and the low pH (38). After 1 day, a rapid decline of the number of coliform bacteria is observed. Depending on characteristics of the species and on the initial count, complete disappearance will usually be observed within 1 to 3 months (39, 40).

Cheese made from pasteurized milk mostly does not contain *Enterobacteriaceae*. For example, examination by a Dutch control institute in 1976 showed that 99 % of the samples of 12-day-old cheese tested for the presence of these bacteria had counts lower than 100 cfu/g (41). However, in very exceptional cases, if contamination of the milk after pasteurization has been too heavy, or if cheese is made from raw milk, high densities can be reached (42).

Many strains of the *Enterobacteriaceae* are known to decarboxylate several amino acids, especially lysine and ornithine (20, 32, 43). Schormüller and Leichter detected lysine decarboxylase from these bacteria in Sauermilchkäse (44), but only little is known about the extent to which they can form biogenic amines in other types of cheese.

To investigate this, several experimental cheeses were made from milk deliberately contaminated with coliform bacteria. Pure cultures as well as mixtures of isolates were used for these experiments. The mixtures were obtained by incubating diluted samples from young cheese in VRBA agar. After 1 day of growth at 30 °C, the contents of the petri dishes were homogenized with a 0.85 % NaCl solution and the suspension was added to the cheese milk.

The results of the chemical and microbiological analyses are in Table 5. Except for one experiment carried out with strain RC1, all experimental cheeses showed an accumulation of cadaverine. Little cadaverine (0.50 mmol/kg) was detected when the milk was inoculated with LN2, even though this strain reached a density of $1 \cdot 10^8$ cfu/g in the cheese after one day. The cheeses made

with LN1 contained 2.5-3.0 mmol cadaverine per kg after 2-3 months. After 3 months all samples were almost free of coliform bacteria. An explanation for the stopping of the formation of cadaverine is that probably only viable microorganisms produce this amine.

Putrescine was also detected in some of the cheeses, with a highest concentration of 1.30 mmol/kg. Also in this case most of the amine was produced during the first months of ripening. Considerable variations existed in putrescine accumulation among cheeses contaminated with different strains, indicating differences in decarboxylase activities.

While an inoculum of $4 \cdot 10^3$ cfu *Hafnia alvei* LN1 per ml cheese milk did not lead to detectable putrescine formation in the cheese, $4 \cdot 10^5$ cfu/ml resulted in 1.3 mmol/kg. In this case the positive correlation between putrescine concentration and inoculum sizes may have been caused by the increasing decarboxylase content: with the small inoculum there is not enough enzyme to bring about detectable putrescine formation. This will be investigated in more detail in the future.

In the cheese made with mixtures of *Enterobacteriaceae*, cadaverine reached maximum concentrations of 1.0-5.9 mmol/kg, which suggests that strains with even stronger decarboxylative activities must exist. The presence of tyramine and histamine in one of the cheeses is presumably to be attributed to the simultaneous activity of lactobacilli (see also Section 3.3). Indeed some coliform bacteria can also decarboxylate histidine (45, 46), but the observed lasting increase of the histamine concentration makes it more likely that other bacteria caused this accumulation.

In practice, cadaverine formation may be expected in cheese contaminated with coliform bacteria. As an indicator for hygienic treatment, cadaverine formation has only a limited significance, since many false-negative results must be expected.

3.5 Decarboxylative properties of pediococci

While examining cheese rich in biogenic amines, fairly high densities of bacteria of the genus *Pediococcus* were sometimes observed. These organisms are known to occur as adventitious microflora in Cheddar cheese (47, 48), but little information is available about their presence in Gouda and Edam cheese. Nor is much known about the decarboxylative properties of these bacteria. For some single strains it was found that amine formation did occur when tested in selected growth media (49). *Pediococcus* starter cultures, used for the production of fermented sausages, were decarboxylase-negative (50). In 1974, Mayer reported the formation of histamine in sauerkraut coinciding with *Pediococcus* multiplication. He suggested that these organisms were the

Table 5. The formation of biogenic amines in cheese by coliform bacteria[1].

Exp.	Strain(s)	Density in cheese milk (cfu/ml)	Maximum density[2] in cheese (cfu/g)	Age of cheese (days)	Amine contents (mmol/kg)[3]					
					TA	HA	PTR	CAD	TPA	PHEA
1	*E. coli* RC1	2×10^5	2×10^8	30	–[4]	–	0.45	–	–	–
				90	–	–	0.80	–	–	–
				180	–	–	0.70	–	–	–
				365	–	–	0.70	–	–	–
2	*E. coli* LN2	3×10^5	1×10^8	90	–	–	–	0.35	–	–
				180	–	–	–	0.50	–	–
				365	–	–	–	0.45	–	–
3	*Hafnia alvei* LN1	3×10^5	3×10^7	30	–	–	0.20	1.55	–	–
				182	–	–	0.60	2.60	–	–
				365	–	–	0.40	2.45	–	–
4	*H. alvei* LN1	4×10^3	5×10^5	15	–	–	–	1.45	–	–
				30	–	–	–	1.95	–	–
				60	–	–	–	2.90	–	–
				90				3.80		
5	*H. alvei* LN1	3×10^4	9×10^6	15	–	–	0.25	1.55	–	–
				30	–	–	0.35	1.95	–	–
				60	–	–	0.45	2.60	–	–
				90	–	–	0.75	3.15	–	–
6	*H. alvei* LN1	4×10^5	5×10^7	15	–	–	0.85	1.50	–	–
				30	–	–	1.25	1.95	–	–
				60	–	–	1.30	2.34	–	–
				90	–	–	1.85	3.15	–	–
7	Mixture	1×10^5	2×10^8	90	–	–	–	4.40	–	–
				180	–	–	–	5.60	–	–
				365	–	–	–	5.90	–	–

8	Mixture	1×10^5	9×10^7	30	–	–	0.20	1.00	–	–
				60	lost[5]					
9	*Mixture*[6]	4×10^4	3×10^7	30	0.20	–	–	2.10	–	–
				90	1.05	0.40	–	2.75	–	–
				180	1.30	0.90	–	2.70	–	–
				365	3.50	1.40	–	2.70	–	–

[1] The coliform bacteria were added after pasteurization.
[2] Maximum density of coliform bacteria in the cheese. This was reached within 24 h, thereafter their numbers declined.
[3] See Table 2, note 2.
[4] See Table 2, note 3.
[5] Lost, due to 'late blowing'.
[6] Among which lactobacilli must have been present; after three months of ripening 4×10^7 cfu of these bacteria per g of cheese were found.

causative agents (51).

To examine the role of pediococci in the formation of biogenic amines in cheese, milk was inoculated with mixtures of strains isolated from cheese rich in biogenic amines. As there is no selective medium available for these micro-organisms, the inoculum of the cheese milk in the first experiment was prepared by mixing several pure cultures. In the second experiment the inoculum consisted of two *Pediococcus acidilactici* strains from a Parmesan cheese.

Even after one year of ripening the cheese of the first experiment did not contain biogenic amines. After 1-3 months the added organisms attained maximum densities of $3 \cdot 10^7$ to $9 \cdot 10^7$ cfu/g. In the second experiment a large inoculum of the *P. acidilactici* strains were added because this species is believed not to multiply in cheese. However, an increase up to $8 \cdot 10^7$ was observed. After one year no biogenic amines were detected in this cheese either.

No further attempts were made to isolate decarboxylase positive strains.

3.6 Decarboxylative properties of Bacterium proteolyticum

B. proteolyticum can cause serious off-flavours in cheese, but the bacterium occurs very rarely in cheese (52, 53, 54). Little is known about its taxonomical place and even less about its decarboxylative properties.

A portion of cheese milk was inoculated with $2.5 \cdot 10^3$ *B. proteolyticum* per ml. Its development in the cheese to about $5 \cdot 10^7$ cfu/g was accompanied by strong off-flavours. However, biogenic amines were not detected until after 12 months of ripening. At that time, when the cheese was already completely unpalatable, 1.30 mmol putrescine/kg was found. No further experiments were done with this species.

Conclusion

The aim of this work was to determine which non-starter bacteria can play a role in the formation of biogenic amines in cheese. The most important adventitious bacteria were investigated: mesophilic lactobacilli (non salt-tolerant), coliform bacteria, enterococci and pediococci, as well as some less frequently encountered bacteria such as salt-tolerant lactobacilli and *Bacterium proteolyticum*. No attention was paid to propionic acid bacteria, clostridia and thermophilic lactobacilli, because these bacteria do not develop in Gouda cheese made under good manufacturing practices. The propionic acid bacteria used for the production of Maasdam type of cheese were studied previously (1).

Any amine formation occurred only if the cheese milk was contaminated after pasteurization. The investigated pediococci were not able to cause amine formation in cheese. Contamination with *B. proteolyticum* gave slight putrescine formation (1.30 mmol/kg) after one year. This amount is not considered to be of practical importance. Enterococci caused tyramine formation and, if present in high densities, phenylethylamine was also found. However, these organisms probably do not play a significant role in amine build-up in Dutch cheese since it only rarely contains enough enterococci to form tyramine.

Contamination of the cheese milk with *Enterobacteriaceae* poses a serious risk because these microorganisms may give rise to biogenic amines at much lower densities. Especially the decarboxylase-positive strains that have a low death rate are of importance, because cadaverine and putrescine formation were observed in cheese contaminated with these bacteria.

Some lactobacilli can form biogenic amines. Tyramine, histamine and putrescine were detected in some cheeses, while cadaverine was also found in a cheese containing salt-tolerant lactobacilli. In one cheese, more than 29 mmol cadaverine/kg was found, together with an appreciable amount of putrescine (8.30 mmol/kg) after one year of ripening. However, the latter results have a limited practical significance, because in the Dutch cheese factories measures are taken to prevent contamination of the brine with this type of lactobacilli. The salt-sensitive lactobacilli did not produce more than 10 mmol total amines per kg cheese including a maximum of 6.0 mmol of histamine. Cheese associated with food poisoning contained 13.5 or more mmol histamine per kg (55, 56). Still, we believe that lactobacilli are the most important causative agents of the build-up of biogenic amines in cheese. In ensuing publications we will show that excessive proteolysis can greatly enhance amine formation in cheese containing decarboxylative lactobacilli.

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Samenvatting

H. M. L. J. Joosten en M. D. Northolt, *Omstandigheden waaronder biogene aminen in kaas kunnen worden gevormd. 2. Decarboxylerende eigenschappen van enige niet-zuurselbacteriën*[1]

In deze tweede publikatie over de vorming van biogene aminen in kaas wordt ingegaan op het decarboxylerend vermogen van bacteriën die regelmatig in zuivelprodukten worden aangetroffen maar doorgaans niet in zuursels gebruikt worden. Hiertoe werden kazen bereid uit melk welke opzettelijk besmet was met de te onderzoeken organismen. Na verloop van tijd werd het aminegehalte van de kazen bepaald.

Allereerst werd de invloed onderzocht van de aanwezigheid van grote aantallen Gram-negatieve organismen in de rauwe kaasmelk, zoals incidenteel in de praktijk worden gevonden, indien de melk gedurende langere tijd bij lage temperatuur wordt bewaard. Het bleek dat aminevorming in de kaas niet optrad, mits de melk werd gepasteuriseerd en er geen nabesmetting optrad.

De overige onderzochte bacteriegroepen werden na de kaasmelkpasteurisatie aan de kaasmelk toegevoegd.

Lactobacillen bleken verantwoordelijk voor het ontstaan van verschillende biogene aminen in kaas. Terwijl de meeste stammen geen decarboxylerend vermogen bezitten, konden mengsels van niet nader gekarakteriseerde stammen vorming van tyramine, histamine en putrescine in de kaas bewerkstelligen. Zouttolerante lactobacillen bleken zeer grote hoeveelheden cadaverine en putrescine in de kaas te vormen. Verschillende reinculturen van stammen die in staat waren om histidine of tyrosine te decarboxyleren, werden nader onderzocht in kaasbereidingsproeven.

Enterokokken worden doorgaans geacht belangrijke veroorzakers van tyraminevorming in kaas te zijn. Deze opvatting kon niet worden bevestigd. Slechts bij 10^8 en 10^9 cfu/g werd tyramine in grote hoeveelheden aangetroffen, terwijl bij 10^7 cfu/g slechts lage tyraminegehalten worden gevormd. Bij 10^6 cfu/g werd een zeer geringe tyraminevorming waargenomen. In alle gevallen was uitgegaan van een stam met een sterk decarboxylerend vermogen. Daar Nederlandse kaas slechts bij hoge uitzondering 10^6 of meer enterokokken per gram bevat zijn deze organismen voor de vorming van biogene aminen blijkbaar niet van belang.

Bacteriën van de coligroep veroorzaakten vorming van cadaverine en putrescine in de kaas. De hoogste gehalten werden gevonden indien de melk besmet werd met stammen die slechts langzaam afsterven in de kaas en welke tevens beschikken over een actief decarboxylase. De andere onderzochte organismen, pediokokken en *Bacterium proteolyticum*, bleken van geen belang voor aminevorming in Nederlandse kaas.

In het algemeen waren de gevonden gehalten niet bijzonder hoog. Met uitzondering van die gevallen waarin een buitengewoon zware besmetting was aangebracht, bleken alle kazen minder dan 5 à 6 mmol van een amine per kg te bevatten. Tryptamine werd in geen enkele kaas aangetroffen, phenylethylamine alleen in een kaas met $2 \cdot 10^9$ enterokokken.

[1] Dit artikel maakt deel uit van een serie artikelen over biogene aminen die ook wordt verspreid als Verslag V272 van NIZO te EDE (1988).

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Conditions allowing the formation of biogenic amines in cheese. 3. Factors influencing the amounts formed*

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Summary

The precursor concentration puts a limit to histamine formation in cheese infected with *Lactobacillus buchneri* St2A. Parameters influencing the production of free amino acids were investigated in cheese-making experiments.

Histamine formation was accelerated if the cheese was stored at higher temperatures (18 or 21 °C), while more histamine was also found in cheese with a higher pH or a lower salt content than normal Gouda. Pasteurization of the milk scarcely affected amine formation. Histamine concentrations in (infected) cheese produced with different mesophilic starters were much alike. When in addition to these mesophilic starter bacteria and *L. buchneri* St2A, thermophilic lactobacilli and propionic acid bacteria were also added to the cheese milk, as is usual for the production of Maasdam cheese, histidine liberation and hence histamine formation was strongly stimulated.

No such stimulatory effect on histamine formation was detected when other (non-decarboxylating) mesophilic lactobacilli or coliform bacteria were added to the cheese milk, while enterococci (*Streptococcus faecalis* var. *liquefaciens*) were capable of accelerating proteolysis only when present in a very high density (2×10^9 cfu/g).

In a Gouda cheese with about 1×10^8 cfu/g of a tyrosine decarboxylating *Lactobacillus brevis* strain, tyramine formation was also restricted by the precursor concentration. Tyramine formation was stimulated by addition of tyrosine to the milk and in cheese with faster protein degradation, but the addition of extra co-factor of tyrosine decarboxylase to the milk did not result in increased tyramine formation.

Ornithine is the precursor amino acid of putrescine. Its concentration in Gouda cheese shows much more variation than that of the other precursor amino acids, but in 12 months old cheese its concentration is usually lower than 6.0 mmol/kg. Accumulation of putrescine was observed when members of the *Enterobacteriaceae* were added to the milk, with 2.2 mmol/kg as the maximum concentration found.

Several cheeses were made with *Hafnia alvei* LN1, a lysine decarboxylating strain, added to the milk. When very high inocula (4×10^5 cfu/ml) were used it was found that these bacteria died off much more rapidly than if 4×10^3 cfu were added per ml.

Cadaverine formation during the first month of ripening went equally fast in each of the cheeses infected with LN1, but after six months the highest cadaverine contents were found in the cheese for which the smallest inoculum was used. This probably results from the accumula-

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tion of nitrite: addition of $NaNO_2$ to washed cell suspensions of LN1 accelerated the death rate and also caused a rapid decline of lysine decarboxylase activity.

Strains with decarboxylases specifically active towards phenylalanine were not found. Phenylethylamine was only formed when many tyrosine decarboxylating bacteria were present in the cheese, probably as a result of slight activity of tyrosine decarboxylase towards this structurally related amino acid. Tryptamine was not found in any cheese.

1 Introduction

In exceptional cases, cheese with a very high content of biogenic amines is encountered (1-4). Since consumption of cheese rich in biogenic amines may lead to food intoxication (1, 5-7), an investigation was started to reveal the factors that lead to accumulation of these amines. Biogenic amines are formed by action of bacterial decarboxylases. Therefore, we first examined starter and non-starter bacteria for their decarboxylative properties in cheese.

It was found that the starter bacteria commonly used for Dutch cheesemaking do not have the capacity to form biogenic amines in cheese (8). When Gouda cheese was made from milk contaminated with other bacteria, noticeable amine formation was sometimes found. Many strains among the lactobacilli and the coliform bacteria may cause accumulation of tyramine, histamine, putrescine or cadaverine (9).

Nevertheless, the amounts of biogenic amines found in the cheeses were generally rather low. Further studies revealed that cheese containing histidine decarboxylating bacteria had a very low free histidine concentration. There was not enough precursor present to allow massive formation of histamine (only the free amino acid can be decarboxylated). Obviously, proteolysis had not proceeded that far. Histidine decarboxylase activity was shown to be high enough to allow for far more histamine formation (10).

In this article we will first give a short review of the literature on protein degradation in cheese and also add the results of our own experiments on this subject. Thereafter we present the results of investigations on the influence of proteolysis on the formation of biogenic amines. From a toxicological point of view, histamine is the most interesting amine (1, 11, 12), which therefore received most attention.

2 The free amino acid content of Dutch-type cheese

2.1 Literature

Whereas milk has a very low free amino acid content, cheese, especially after

a prolonged ripening period, contains these substances in rather high proportions. This accumulation can be attributed mainly to the hydrolytic activities of several enzymes on paracasein, the principal protein found in cheese. These enzymes comprise the proteinases from milk, the milk clotting enzymes (rennet) and the proteolytic enzymes from the starter bacteria and possibly other, adventitious bacteria (13-16). Studies with aseptically made starter-free and/or rennet-free cheese showed that in a normal Gouda type of cheese, amino acid formation is mainly brought about by the action of the starter bacteria (15-17). It was found that the enzymes from the rennet also play an important role as they contribute to the breakdown of large casein molecules into smaller peptides. These subsequently serve as a substrate for proteinases and peptidases from the starter bacteria. Milk proteinases only have limited importance, as these account for a minor fraction of the amino acids found in Gouda cheese (18, 19).

The proteolytic activity of the bacteria involved is very variable and the action of the enzymes is influenced by environmental factors (20). Therefore, it is not surprising that considerable variation in the content of free amino acids among various types of cheese is found (21-23).

In this section, we will discuss the most important factors influencing the contents of free amino acids in Gouda, Edam and Maasdam type of cheese. It must be borne in mind that cheese ripening is a very complex process. Changing one of the parameters usually elicits changes of other parameters as well. Mostly it is also necessary to gather information on the history of the cheese: for example if two cheeses have the same pH at a certain age this does not imply that this was always so. When for instance the pH during the actual cheese making was not the same it is very likely that the amount of rennet retained in the two cheeses is different, and this in turn influences proteolysis.

2.1.1 Temperature. After brining, Gouda and Edam cheese is usually stored at 13-14 °C until marketed. Initially, Maasdam cheese is also kept at 14 °C, but after two weeks the cheese is transferred to a room with a temperature of 18 °C. Here propionic acid fermentation takes place. After two or three weeks, the cheese has usually developed enough gas and the storage temperature is then gradually decreased to 8 °C. At this temperature the cheese is kept for another three or four weeks. At the age of about 2 months Maasdam cheese can be marketed. Continued storage, although not recommended, should take place at 5-8 °C.

Several authors have investigated the effect of storage temperature on proteolysis during ripening (24, 25). It was shown that Edam cheese stored at 17 °C contained twice as much amino acid nitrogen as cheese stored at a tem-

perature of 9 °C (26). In Section 2.3.1 the results of some new experiments on this subject are discussed.

2.1.2 pH. During ripening, the pH of Gouda cheese usually increases from about 5.15 immediately after brining to about 5.4-5.5 after 6 months of ripening. The pH of Maasdam cheese immediately after brining is 5.35 and it also increases during ripening. Investigations on the influence of the pH on the liberation of amino acids (27) revealed that in cheese with a high pH the concentration of amino acid nitrogen increases more rapidly than in cheese with a low pH. Ali (28) performed amino acid analyses on Edam cheeses with a different pH, and found that this effect could be observed for most of the individual amino acids. Unfortunately, exact information on the influence of the pH on the liberation of the precursors of biogenic amines is lacking as rather high amounts of unidentified amines were found in many samples. However, it seems very likely that these amino acids were also liberated in larger quantities in the cheese with a high pH.

2.1.3 Salt and water content. The salt and water content influence the rate of proteolysis in cheese. Experiments with Edam and Gouda cheese showed that most amino acid nitrogen was found in cheese with a high water and a low salt content (29). In normal Gouda cheese, the salt-water ratio after two weeks is about 0.051. The water content is about 41.5 % at that time. Due to evaporation, the water content slowly decreases during ripening. After 13 weeks some 37 % of the cheese consists of water, and the salt/water ratio has then increased accordingly. Maasdam type cheese contains 41.5 % water after two weeks and its salt/water ratio at that time is 0.031 (average values). This low ratio contributes to accelerated liberation of amino acids in Maasdam cheese, although there are other factors involved.

2.1.4 The microflora of the cheese. Proteolytic enzymes of bacterial origin are paramount for the liberation of amino acids in cheese. In normal Gouda and Edam cheese the starter bacteria reach densities of more than 1×10^9 cfu/g and they are the principal source of bacterial proteinases and peptidases. The adventitious bacteria usually form a minority with counts of more than 1×10^7 or 1×10^8 only in exceptional cases being found. Studies by Stadhouders (13) and Kleter (30) clearly show that the presence of all kinds of non starter bacteria such as mesophilic lactobacilli, micrococci etc. do not influence the liberation of amino acids in cheese, at least if their numbers do not exceed 1×10^8 cfu per gram. Neither was proteolysis markedly affected by high psychrotrophic counts of the raw milk (31, 32). Still, the possibility that

an excessive contamination with undesired bacteria changes the amino acid pattern cannot be excluded.

The proteolytic capacity of the bacteria that are commonly used as starter organism, have been the subject of many studies (20, 22, 33). Kleter (17) investigated the formation of amino acid nitrogen in cheese made with different single-strain starters of *Streptococcus cremoris**. It was found that the amount of amino acids formed was highly dependent on the strain used. However, for Dutch cheese making, mixed strain starters are more commonly used. In Section 2.3.2 new investigations on the proteolytic activity of some of these cultures are described. The results show that all the investigated mixed starters yield nearly equal amounts of amino acids in the cheese. However, the possibility that other starters cause accumulation of specific amino acids cannot be ruled out and may be important for the formation of biogenic amines in cheese. In this respect, the behaviour of thermophilic lactobacilli deserves attention. Amino acid analysis of cheese made with these bacteria has revealed very high free histidine contents in some cases (23, 35). The amount of this compound relative to the total amino acid content was considerably higher than in paracasein. Reuter, investigating changes of the composition of growth media, observed accumulation of histidine after incubation of *Lactobacillus bulgaricus* (36). Although we cannot readily explain this accumulation, these results may be important regarding the formation of histamine in Maasdam cheese. Maasdam is usually made with the starter Bos, a mesophilic mixed strain starter containing streptococci, but the cheese milk is also inoculated with propionic acid bacteria and thermophilic lactobacilli. In Section 3.3.1.4 we will show that addition of these bacteria indeed affects histamine formation.

In recent years there has been a tendency to develop starters which can rapidly proliferate in milk and also produce lactic acid at greater speed (so called fast starters). Fast variants carry a plasmid coding for the production of a cell wall proteinase, while slow variants do not carry such a plasmid (37). However, cheese made with 100 % fast variants produced at our Institute did not contain more amino acid nitrogen than cheese made with a conventional starter, containing about 20 % fast variants. On the other hand, when a starter devoid of fast strains was used, amino acid liberation was retarded substantially. It was concluded that although this proteinase is very important for

* The nomenclature of the genus *Streptococcus* has recently been changed (34). *S. cremoris, S. lactis* and *S. lactis* subsp. *diacetylactis* are now referred to as *Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis* and *L. lactis* subsp. *diacetylactis* respectively. In this publication the old names are used.

cheese ripening, in normal Gouda cheese it is not a limiting factor for the liberation of amino acids. It seems more likely that the activity of the peptidases determines the amino acid concentration (38).

The liberation of free amino acids by other cultures (yeasts, moulds, enterococci) may be different from the above mentioned bacteria, but will not be discussed here because they are not used for the production of Dutch cheese.

2.1.5 Clotting enzymes. A mixture particularly of chymosin and some bovine pepsin is widely used as a clotting agent in the cheese making process. This mixture initiates the coagulating process by cleaving the Phe105-Met106 bond of kappa-casein. Thereafter, most of the enzyme escapes with the whey, but a part of it is retained in the curd, the amount of which depends on several factors such as pasteurization and cooking temperature, water content of the curd and the pH during manufacturing (39). These retained clotting enzymes contribute to further degradation of the protein fraction of cheese and their importance has been investigated in starter-free curd and cheese (16, 18, 40). It was found that the contents of soluble N in these cheeses were comparable to that found in normal cheese. However, free amino acids were scarcely detected. In Edam cheese made with an overdose of rennet, the amino acid content was not higher than in a comparable cheese for which a normal amount of rennet was used (13).

Outside the Netherlands other rennets are sometimes used for cheesemaking. Investigations by Zwaginga et al. on protein degradation in cheese made with a mixture of 50 % pig pepsin and 50 % calf rennet showed that amino acid nitrogen in this cheese was about the same as in cheese made with 100 % calf rennet (41). When a microbial type of rennet was used (from *Mucor pusillus* Lindt) for the production of Gouda the liberation of free amino acids was somewhat accelerated (about 5 to 15 %) (42). Because these enzymes have other cleaving sites (43), the abundance of the individual amino acids might have been different too.

2.1.6 Pasteurization. Pasteurization reduces the number of viable bacteria in milk. Therefore, cheese made from raw milk generally contains far more adventitious microorganisms than does cheese made from pasteurized milk. The role that these bacteria may play is discussed in Section 2.1.4.

Some authors have suggested that pasteurization may increase the activity of alkaline proteinase (plasmin) (44), while others found a decrease of activity after pasteurization (45). Visser investigated the contribution of milk proteinase to the accumulation of amino acids in aseptic rennet-free starter-free

cheese. After 6 months of ripening only very low levels of these compounds were found (18). The soluble N fraction was also very small. This means that plasmin only plays a minor role in the degradation of protein in normal Dutch cheese and, even if its activity is increased by pasteurization, no significant effect on the amino acid content is to be expected.

2.2 Materials and methods

2.2.1 Cheesemaking. Cheesemaking was performed as described in an earlier publication (9). Only bactofuged and pasteurized milk was used. To prevent clostridial blowing 0.015 % $NaNO_3$ was added to the milk.

2.2.2 Amino acid analysis. The concentration of individual free amino acids was determined with an LKB 4151 Alpha plus Amino acid analyzer. Sample clean up was performed in the same way as for determination of biogenic amines (46). Prior to injection the pH of the extract was adjusted to 2.2 with 4N NaOH and norleucine was added as internal standard.

2.3 Results and discussion

2.3.1 The influence of the ripening temperature on the liberation of amino acids in Gouda cheese. In Section 2.1.2 it was already stated that high storage temperatures strongly enhance liberation of amino acids in cheese. From the literature on this subject it was not clear whether this stimulatory effect was equally strong for each of the individual amino acids of paracasein. Therefore we set up an experiment in which 4 lots of Gouda cheese from one vat were stored at 9, 14, 18 and 21 °C respectively. After three and six months, the amino acid content was analyzed (Table 1). As expected, the highest content was found in the cheese stored at 21 °C. This was also true for the concentration of the amino acids that serve as precursor for the formation of biogenic amines. Only the ornithine contents of the cheeses were not always substantially higher when higher storage temperatures were employed. This is probably explained by the fact that ornithine is not formed by hydrolysis of paracasein like most of the other amino acids but is formed instead from arginine by certain bacteria (47, 48). Possibly the activity of these bacteria is negatively influenced by a high storage temperature. Biogenic amines were not detected in the samples.

2.3.2 The liberation of amino acids in Gouda cheese made with six different mesophilic starters. Kleter investigated proteolysis in cheeses made with sev-

Table 1. The free amino acid content (mmol/kg) of Gouda cheese stored three or six months at different ripening temperatures[1].

Amino acid	9 °C		14 °C		18 °C		21 °C	
	3	6	3	6	3	6	3	6
Glu	10.6	20.0	15.6	25.1	18.6	32.0	20.9	47.1
Pro	1.4	2.3	2.2	3.6	2.5	5.2	2.6	5.2
Gly	1.4	2.8	2.3	4.3	2.8	6.0	3.5	8.0
Ala	2.1	3.8	3.1	5.3	3.7	7.3	4.3	10.7
Val	4.3	8.0	6.4	11.4	8.0	14.8	9.0	19.7
Met	1.1	2.3	1.6	3.6	2.2	4.8	2.7	6.2
Ile	1.2	3.4	2.1	5.8	3.1	8.3	4.4	12.9
Leu	10.2	18.1	14.7	23.5	17.7	28.2	18.7	35.8
Tyr	1.7	3.1	2.3	4.4	2.9	5.6	3.6	8.1
Phe	5.0	7.6	6.4	9.4	7.3	11.2	9.0	14.0
His	0.6	1.5	1.1	2.4	1.3	3.3	2.6	4.4
Cit	0.3	0.7	0.4	0.9	0.5	1.4	0.7	2.5
Orn	3.3	4.8	4.2	5.1	4.2	4.9	4.8	6.0
Lys	4.2	9.0	6.6	14.0	8.4	19.8	15.0	28.9
Arg	1.4	2.5	2.0	4.1	3.0	5.7	4.0	6.8
Gln	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Asn	7.8	12.5	10.9	15.4	12.8	18.6	14.7	28.4
Asp	1.2	2.3	1.7	3.3	1.9	4.1	3.5	6.5
Thr	1.5	3.3	2.3	4.9	3.1	6.6	3.8	10.1
Ser	1.8	3.6	2.8	3.7	3.5	7.3	4.1	9.8

[1] The cheeses were all from one production vat. The starter Bos was used (0.6 %). After two weeks of ripening the pH was 5.25, while the salt/water ratio was 0.049 at that time.

eral mixed strain starters and found that the amino acid content was about the same in all cases (30). However, no information was given on the amounts of the individual amino acids, leaving open the possibility that the abundance of some of the amino acids shows greater variation. To investigate this we performed amino acid analyses on cheese produced with six different mixed strain starters (Table 2).

When the contents of the individual amino acids are compared, only minor differences between the cheeses are observed. An exception must be made for arginine, citrulline and ornithine, which show a large variation. This can probably be explained by the fact that the bacterial population of the cheese can degrade arginine to several compounds: besides ornithine, agmatine and citrulline can be formed (48, 49). The capacity to do so obviously depends upon the starter used.

The results also show that the relative amounts of most amino acids resemble those of paracasein. Obviously, the combined action of the proteolytic en-

Table 2. The free amino acid content of paracasein hydrolysate and of Gouda cheese made with various starters after one year of ripening.

Amino acid	Fr8		Fr18		Fr19		Bos		A		Ur		Paracasein[1]
	mmol/kg	mol %	mmol/kg	mol %	mmol/kg	mol %	mmol/kg	mol %	mmol/kg	mol %	mmol/kg	mol %	mol %
Glu	35.6	16.6	42.4	17.4	35.7	17.4	31.1	17.0	27.5	16.5	33.8	17.5	19.0
Pro	6.9	3.2	5.6	2.3	4.3	2.1	5.4	2.9	7.9	4.7	4.2	2.2	12.9
Gly	6.5	3.0	7.7	3.2	6.0	2.9	5.2	2.8	5.5	3.3	6.1	3.2	3.1
Ala	7.4	3.5	8.3	3.4	6.9	3.4	6.3	3.4	5.4	3.2	5.7	3.0	4.3
Val	16.2	7.6	17.9	7.3	15.0	7.3	13.5	7.4	12.7	7.6	13.5	7.0	6.9
Met	5.1	2.4	6.0	2.4	5.2	2.5	4.8	2.6	3.5	2.1	4.9	2.5	2.2
Ile	9.7	4.5	12.2	5.0	9.3	4.5	7.7	4.2	6.5	3.9	9.2	4.8	4.9
Leu	32.3	15.1	34.5	14.1	30.2	14.7	28.0	15.3	25.8	15.4	27.0	14.0	8.9
Tyr	7.5	3.5	8.1	3.3	7.0	3.4	6.2	3.4	5.1	3.0	6.2	3.2	3.8
Phe	12.0	5.6	12.8	5.2	11.5	5.6	10.8	5.9	9.9	5.9	9.1	4.7	3.8
His	4.1	1.9	4.9	2.0	3.9	1.9	3.1	1.7	3.3	2.0	3.7	1.9	2.0
Cit	5.2	2.4	5.4	2.2	2.0	1.0	5.0	2.7	2.4	1.4	1.3	6.7	0
Orn	5.5	2.6	5.9	2.4	2.2	1.1	4.0	2.2	2.3	1.4	3.2	1.6	0
Lys	23.3	10.9	28.2	11.6	22.8	11.1	18.8	10.3	17.1	10.2	20.9	10.8	7.2
Arg	0.6	0.3	1.1	0.4	7.4	3.6	0.6	0.3	5.0	3.0	8.9	4.6	3.1
Gln	<0.5	<0.3	<0.5	<0.3	<0.5	<0.3	<0.5	<0.3	<0.5	<0.3	<0.5	<0.3	–[2]
Asn	19.1	8.9	21.9	9.0	18.6	9.1	17.4	9.5	16.1	9.6	18.7	9.7	–[3]
Asp	4.2	2.0	5.6	2.3	5.0	2.4	4.2	2.3	2.7	1.6	4.4	2.3	6.5
Thr	6.1	2.8	7.6	3.1	5.6	2.7	5.2	2.8	3.6	2.2	5.9	3.0	4.4
Ser	6.5	3.0	8.0	3.3	6.2	3.0	5.8	3.2	4.4	2.6	6.3	3.3	7.0
Total	213.8	100	244.1	100	204.8	100	183.1	100	166.7	100	193.0	100	100
pH[4]	5.21		5.20		5.17		5.11		5.25		5.24		
salt/water[4]	0.061		0.073		0.067		0.069		0.069		0.063		

[1] P. van Rooijen, NIZO.
[2] Detected as glutamic acid, due to hydrolysis.
[3] Detected as aspartic acid, due to hydrolysis.
[4] The salt, pH and water content of the cheeses were determined after 6 weeks of ripening.

zymes of these mesophilic starter bacteria and renneting enzymes has no clear preference for certain amino acids.

3 The influence of precursor concentration and density of decarboxylating bacteria on the formation of biogenic amines in cheese

3.1 Introduction

In the following sections we will present results of experiments in which cheese was made from milk inoculated with various amounts of decarboxylating bacteria. It was investigated to what extent the formation of biogenic amines by these bacteria is influenced by the factors as described in Section 2. Most attention is paid to the formation of histamine, because of its toxicological significance.

3.2 Materials and methods

3.2.1 Cheesemaking. Cheesemaking was performed as described in Section 2.2.1. To achieve variation in pH, salt and water content, the method was adapted as described in the text. Decarboxylating bacteria were added after pasteurization of the milk. Unless stated otherwise, the starter Fr 19 was used (0.6 %).

3.2.2 Cultures. Most of the strains and cultivation methods have been described previously (9). For one experiment, a cocktail of 50 mesophilic *Lactobacillus* strains was added to the cheese milk, together with *Lactobacillus* sp. 4720-2. These 50 strains were obtained from various lots of Gouda cheese by means of a selective agar for lactobacilli, TGV 5.4 (50). Their decarboxylative properties were tested with the modified method of Möller (9).

3.2.3 Microbiological and chemical analysis of the cheese. The microflora of the cheese was investigated after 1, 14, 30, 90 and 182 days of ripening. Most of the methods used have been described previously (8, 9). To enumerate thermophilic lactobacilli, LB agar was employed (51). The plates were incubated for two days at 44 °C.

Other methods have been described in earlier publications (8, 9, 46).

3.2.4 Preparation of washed cell suspension and cell-free extract of H. alvei LN1. *H. alvei* LN1 was grown on 250 ml BHI (Brain heart infusion broth, Difco) for 24 h at 30 °C. Cells were harvested by centrifugation (10 min,

10 000 *g* at 4 °C). The pellet was resuspended with saline (0.85 %), centrifuged again and resuspended in 20 ml 0.2 M phosphate buffer, pH 5.2.

Five ml of this suspension was sonicated (10 × 10 s) in a W375 Cell Disruptor (Heat Systems, Ultrasonics Inc., Plainview, N.Y.) and centrifuged (30 min at 45 000 *g*). The supernatant was used as cell-free extract.

For the enzyme assay, both the washed cell suspension and the cell-free extract were diluted, 1:10, with 0.2 M phosphate buffer, pH 5.2.

3.2.5 Lysine decarboxylase assay. The activity of lysine decarboxylase was determined manometrically with a Gilson Differential Respirometer (Gilson Medical Electronics Inc., Middleton, Wis.). 2.3 ml of the diluted washed cell suspension or cell-free extract and 0.2 ml pyridoxal phosphate solution (2 mg/ml) were added to the main compartment of the Gilson flask. One side arm contained 0.5 ml 0.1 M lysine and the other side arm 0.1 ml water or $NaNO_2$ (4 mg/ml). The assay was carried out under anaerobic conditions (N_2 atmosphere). Reaction components were allowed to equilibrate for 10 min at 30 °C before the substrate was tipped in. Release of CO_2 was monitored for 25 min and results were expressed as microlitre CO_2 released/min.

3.3 Results and discussion

3.3.1 Histamine. Many different bacteria have been investigated for their decarboxylative properties in cheese (9). The formation of histamine was only observed when certain strains of lactobacilli contaminated the cheese. It was investigated under which circumstances histamine formation was stimulated.

3.3.1.1 Influence of the ripening temperature on histamine formation. Gouda cheese made from one vat of milk contaminated with *L. buchneri* St2A (about 2000 cfu/ml) was stored at 9, 14, 18 and 21 °C. The amine contents were determined after 3, 6 and 12 months of ripening (Table 3). The results clearly show that the formation of histamine is strongly influenced by the storage temperature. In cheese no. 6, stored at 21 °C, 6.8 mmol/kg was found after one year of ripening, while only 2.2 mmol/kg was found in cheese no. 1 stored at 9 °C. The observed differences probably reflect differences in precursor concentration; histidine decarboxylase is sufficiently active to form much more histamine (10).

3.3.1.2 Influence of the pH of the cheese on histamine formation. Gouda cheese was made from milk containing *L. buchneri* St2A. Extension of the stirring time combined with the use of more washing water, resulted in cheese

Table 3. The influence of pH and storage temperature on the formation of histamine in Gouda cheese, infected with *L. buchneri* St2A[1].

Cheese no.	Milk lot	Ripening temperature (°C)	pH[2]	Salt-water ratio[2]	Ripening time (months)	Histamine (mmol/kg)
1	1	9	5.19	0.046	3	0.5
					6	1.1
					12	2.2
2	1	14	5.19	0.046	3	0.7
					6	1.4
					12	3.4
3	2	14	5.39	0.043	3	1.3
					6	2.4
					12	6.5
4	1	18	5.19	0.046	3	1.0
					6	2.2
					12	5.6
5	2	18	5.39	0.043	3	ND[3]
					6	4.7
					12	8.6
6	1	21	5.19	0.046	3	1.6
					6	3.7
					12	6.8
7	2	21	5.39	0.043	3	2.5
					6	6.8
					12	9.4

[1] About 2000 cfu/ml were added to the milk. Outgrowth in the cheese took place to about 2×10^8 cfu/g.
[2] The pH and salt and water content were determined after two weeks of ripening.
[3] ND: not determined.

with a pH of 5.39 after two weeks of ripening. The formation of histamine in this cheese (no. 3, Table 3) was compared with that found in cheese with a pH of 5.19 (no. 2). In the former cheese almost twice as much histamine was found after one year as in the latter (6.5 and 3.4 mmol/kg respectively). Combination of higher pH and a storage temperature of 21 °C resulted in further increased histamine formation: 9.4 mmol/kg was found after one year of ripening, probably also because histidine liberation was enhanced.

3.3.1.3 Influence of the salt content of the cheese on histamine formation. Gouda cheese, containing *L. buchneri* St2A, was brined for 15, 24, 48 and 70 h, giving salt/water ratios of 0.026, 0.029, 0.041 and 0.048 respectively (after two weeks of ripening). The cheese was stored at 14 °C and the histamine content was measured after 1, 3 and 6 months (Table 4). The results indicate

Table 4. The influence of salt content on the formation of histamine in Gouda cheese infected with *L. buchneri* St2A[1].

Cheese no.	Salt-water ratio[2]	pH[2]	Ripening time (months)	Histamine (mmol/kg)
1	0.026	5.16	1	0.4
			3	1.4
			6	3.5
2	0.029	5.18	1	0.4
			3	1.2
			6	3.2
3	0.041	5.18	1	0.4
			3	1.2
			6	2.4
4	0.048	5.12	1	0.4
			3	1.0
			6	2.1

[1] See Table 3, note 1.
[2] See Table 3, note 2.

that low salt in water content results in a higher amine content: after 6 months 3.5 mmol/kg was found in the cheese with the low salt/water ratio, and 2.1 mmol/kg with the highest ratio. Although the activity of histidine decarboxylase is diminished by increasing salt concentration (10), the differences in histamine content can be best explained by the effect of the salt concentration on the proteolysis.

3.3.1.4 Influence of several starter cultures on histamine formation. The formation of histamine was investigated in Gouda cheese produced with 6 different starters. Pasteurized milk, containing *L. buchneri* St2A, was inoculated with 0.6 % of the starters Fr19, Bk2, Ur, A, Fr8 or Bos. These are all mesophilic, mixed strain starters, commonly used for the making of Dutch cheese. The results of the chemical analysis are presented in Table 5. Bearing in mind the influence of factors such as pH, salt and water content, it appears that the choice of starter culture has at most a minor effect on histamine formation, probably because precursor concentrations are much alike (see Table 2).

The formation of histamine was also investigated in a Maasdam cheese produced from pasteurized milk containing *L. buchneri* St2A. Although this type of cheese is usually marketed after two months of ripening, histamine formation was monitored during 12 months. Furthermore we employed a ripening temperature of 8-14 °C (see Table 6), in order to enhance proteolysis. (The recommended storage temperature is 5 °C). It was found that after six

Table 5. The influence of several mesophilic starters[1] on the formation of histamine in Gouda cheese, infected with *L. buchneri* St2A[2].

Cheese no.	pH[3]	Salt-water ratio[3]	Starter	Ripening time (months)	Histamine (mmol/kg)
1	5.28	0.040	BK2	3	1.2
				6	2.0
				12	5.0
2	5.26	0.037	Ur	3	1.3
				6	2.0
				12	6.0
3	5.24	0.041	A	3	1.2
				6	2.0
				12	5.6
4	5.23	0.039	Fr8	3	1.2
				6	1.8
				12	5.3
5	5.18	0.040	Fr19	3	0.9
				6	1.9
				12	4.5
6	5.33	0.050	Bos	3	0.8
				6	2.2

[1] Starter, 0.6 % added to the milk.
[2] See Table 3, note 1.
[3] See Table 3, note 2.

months of ripening, the histamine content of this infected Maasdam cheese was much higher than that of the infected Gouda cheese of the same age: 3.5 vs about 2.0 mmol/kg. Maasdam cheese has a higher pH and a lower salt content than Gouda, but these factors explain only a part of the difference in histamine contents. The addition of propionic acid bacteria together with the thermophilic lactobacilli probably accounts for the greater part of the increase of precursor concentration. The importance of the thermophilic lactobacilli is indicated by the remarkable histamine formation in no. 2, in which cheese the inoculum size of the thermophilic lactobacilli was increased. After six months 9.6 mmol/kg was found already. After 12 months, histamine contents had increased to 21.6 mmol/kg. These results show that very high histamine levels in cheese can indeed be found eventually. However, here we must again emphasize that these levels are only to be expected when a number of irregularities and abnormals handling coincide.

Other types of cheese, for which thermophilic lactobacilli are used as starter are probably also more susceptible to histamine formation.

Table 6. The influence of the number of thermophilic lactobacilli on the formation of histamine in Maasdam cheese[1], infected with *L. buchneri* St2A[2].

Cheese no.	pH[3]	Salt-water ratio[3]	Thermophilic[4] lactobacilli (cfu/g)	Ripening time (months)	Histamine (mmol/kg)
1	5.36	0.028	1.4×10^8	3	1.3
				6	3.4
				12	14.5
2	5.34	0.028	4.5×10^8	3	3.0
				6	9.6
				12	21.6

[1] Besides propionic acid bacteria and thermophilic lactobacilli, the starter Bos was added (0.8 %) to the milk. After brining the cheeses were first stored for two weeks at 14 °C, then 2½ weeks at 18 °C; one week at 14 °C; 13 weeks at 7 °C and thereafter at 14 °C.
[2] See Table 3, note 1.
[3] See Table 3, note 2.
[4] Maximum density in the cheese, reached within one week.

3.3.1.5 Influence of other non-starter bacteria on histamine formation. We investigated whether the formation of histamine is influenced by the presence of non-histidine-decarboxylating non-starter bacteria in the cheese. One vat of milk was inoculated with the regular starter for Gouda cheese, Fr19, and a skim milk culture of *Lactobacillus* sp. 4720-2, a strain that can also decarboxylate histidine (9). In another vat, we added Fr 19, 4720-2 and a mixture of 50 mesophilic *Lactobacillus* strains, each first tested for histidine decarboxylase activity and found to be negative in this respect. Microbiological examination of the cheese during ripening revealed that both 4720-2 and non-histidine decarboxylating strains developed well in the cheese. After three months the maximum density was found: 2×10^8 cfu/g, of these about 60 % were able to decarboxylate histidine.

In a third vat Fr 19 and *L. buchneri* St2A were added, while starter Fr 19, *L. buchneri* St2A, *H. alvei* LN1 and *S. faecalis* H1* were added to the fourth vat. These last two strains have been described previously (9). In the fifth cheese vat, a very large inoculum of *S. faecalis* H1 (2×10^7 ml^{-1}) was added together with Fr 19 and St2A. A maximum density of 1.6×10^9 enterococci per gram of cheese was found in this cheese.

The formation of histamine in the above mentioned cheeses was followed

* The use of *S. faecalis* as a starter organism is described for Cheddar (52) and Emmental (53) cheese, but it is not used for the production of Dutch cheese. Therefore we discuss this bacterium together with the other adventitious bacteria.

Table 7. The formation of histamine in Gouda cheese infected with *L. buchneri* St2A or 4720-2[1]. Influence of the concomitant growth of some other, adventitious bacteria.

Cheese no.	pH[2]	Salt-water ratio[2]	Addition	Ripening time (months)	Histamine (mmol/kg)
1	5.14	0.055	4720-2	3	0.4
				6	1.3
				12	3.3
2	5.21	0.060	4720-2 + 50 non-histidine decarboxylating *Lactobacillus* strains[3]	3	0.5
				6	ND[7]
				12	3.8
3	5.18	0.040	St2A	3	0.9
				6	ND
				12	4.5
4	5.37	0.040	St2A + *H. alvei* LN1[4] + *S. faecalis* H1[5]	3	1.0
				6	1.9
				12	5.2
5	5.31	0.043	St2A + *S. faecalis* H1[6]	3	3.0
				6	8.2
				12	16.2

[1] The histidine decarboxylating *Lactobacillus* strains were added at a density of about 2000 cfu/ml to the milk. Outgrowth took place to about 2×10^8 cfu/g after three months of ripening.
[2] See Table 3, note 2.
[3] See text, Section 3.3.1.5.
[4] *H. alvei* LN1 was added to the milk at a density of 2×10^5 cfu/ml and reached a maximum density of 5×10^7 cfu/g in the cheese.
[5] *S. faecalis* H1 was added to the milk at a density of 1×10^4 cfu/ml, yielding after one week 1×10^6 cfu/g cheese.
[6] *S. faecalis* H1 was added to the milk at a density of 2.5×10^7 cfu/ml, yielding after one week 1.6×10^9 cfu/g cheese.
[7] ND: not determined.

during 6-12 months (Table 7). When comparing the results, differences in pH, salt in water content should be taken into account as these also affect the production of free amino acids. The results clearly show that a combination of histidine decarboxylating lactobacilli and other, non-histidine decarboxylating lactobacilli does not lead to a greatly increased histamine content of the cheese. Neither did the concomitant growth of coliform bacteria (*H. alvei*) and enterococci affect histamine formation. Only if an extremely high number of enterococci was present in the cheese was an increased histamine formation observed, which is probably caused by an increased rate of proteolysis.

In the preceding paragraph it was shown that thermophilic lactobacilli could enhance the liberation of histidine when added as starter to the milk in high numbers. However, these bacteria show poor growth in Dutch cheese,

because of the relatively low temperatures employed and probably lactose depletion. Therefore they are never important constituents of the adventitious microflora.

3.3.1.6 Influence of pasteurization on histamine formation. Two portions of 60 litres standardized milk from one lot of aseptically drawn milk were used for the production of Gouda cheese: one portion was pasteurized (10 s, 74 °C), while the other portion was not. To both cheese vats *L. buchneri* St2A was added (2000 cfu/ml) together with the starter bacteria.

Microbiological examination of the ripening cheese revealed that growth of bacteria other than St2A and the starter organisms was negligible. The histamine content was determined after 3, 6 and 12 months of ripening (Table 8). In the cheese made from raw milk 7.0 mmol/kg was found after one year of ripening, while at that time the cheese made from pasteurized milk contained 5.6 mmol/kg. Unfortunately, the salt and water contents of the cheese are not known. The usual analysis after two weeks of ripening was not performed since only one loaf of cheese was available per vat, but the water content was probably above normal. Nevertheless, the results show that pasteurization of the milk as such does not greatly affect the formation of histamine.

This does not exclude the general experience that the risk of histamine formation in cheese from pasteurized milk is smaller than in cheese from raw milk. The reason is that raw milk cheese is more often contaminated with decarboxylating bacteria than is pasteurized milk.

3.3.2 Tyramine. The formation of tyramine was observed in cheese made from milk deliberately contaminated with *Lactobacillus brevis* strains and

Table 8. The influence of pasteurization of aseptically drawn milk on the formation of histamine in Gouda cheese, infected with *L. buchneri* St2A[1].

Cheese no.	pH[2]	Pasteurization	Ripening time (months)	Histamine (mmol/kg)
1	5.35	+	3	1.0
			6	2.4
			12	5.6
2	5.35	–	3	1.3
			6	2.8
			12	7.0

[1] *L. buchneri* was added to the milk at a density of about 2000 cfu/ml, if need be after pasteurization.
[2] The pH was determined after 6 months of ripening.

Table 9. The formation of tyramine by *L. brevis* Hem 3 and *S. faecalis* H1 in Gouda and Maasdam cheese, and the influence of adding tyrosine or pyridoxal phosphate.

Cheese no.	Type of cheese	pH[1]	Salt-water ratio[1]	Addition				Ripening time (months)	Tyramine (mmol/kg)
				L. brevis[2]	*S. faecalis*[3]	tyrosine[4]	p-5-p[5]		
1	Gouda	5.22	0.043	+	–	–	–	1	0.6
								3	1.4
								6	2.6
								12	4.8
2	Gouda	5.26	0.045	+	–	–	+	1	0.8
								3	1.4
								6	2.6
								12	5.1
3	Gouda	5.32	0.047	+	–	+	–	1	4.2
								3	5.2
								6	6.0
								12	8.8
4	Maasdam	5.38	0.025	+	–	–	–	1	1.3
								3	2.2
								6	5.5
								12	9.0
5	Gouda	5.37	0.041	–	+	–	–	1	<0.2
								3	<0.2
								6	<0.2
6	Gouda	5.33	0.041	–	+	–	+	1	<0.2
								3	<0.2
								6	<0.2
7	Gouda	5.35	0.049	–	+	+	–	1	<0.2

[1] See Table 3, note 2.
[2] *L. brevis* addition: after pasteurization ca. 1000 cfu/ml, outgrowth to about 10^8 cfu/g after three months of ripening.
[3] *S. faecalis* addition: after pasteurization ca. 10^4 cfu/ml, outgrowth to about 2×10^6 cfu/g.
[4] Tyrosine was added after removal of the first whey to give a concentration of 2.5-5.0 mmol/kg in the cheese.
[5] Pyridoxal-5-phosphate was added after removal of the first whey to give a final concentration of about 40 μmol/kg in the cheese.

also when large numbers of *S. faecalis* were added to the milk (9). However, the enterococci are not considered to be important for the formation of tyramine in Dutch cheese, as they are hardly ever found at the densities required for substantial enzyme action: if less than 1×10^7 cfu/g were present in the cheese, no more than 1.0 mmol tyramine/kg was found after six months of ripening (9).

We have not investigated the formation of tyramine by *L. brevis* Hem3 as thoroughly as the histamine formation by *L. buchneri* St2A, but it seems likely that also in this case amine formation is mainly restricted by the precursor concentration. However, a shortage of cofactor can also put a limit to enzyme action. Like most other decarboxylases, tyrosine decarboxylase requires pyridoxal-5-phosphate (vitamin B6) as a coenzyme (54, 55). Histidine decarboxylases from *L. buchneri* St2A, *Clostridium welchii, Lactobacillus* sp. 30A and probably many other Gram-positive bacteria are exceptional, as they do not need a coenzyme (56, 57). Instead, the function of this group is fulfilled by a pyruvate molecule, covalently linked to the enzyme. It actually arises by nonhydrolytic serinolysis of the proenzyme (58).

Milk contains about 2 μmol vitamin B6 per litre and this is hardly affected by pasteurization as this substance is relatively heat-stable. In Gouda, Edam and Maasdam cheese it is present at concentrations between 2.5 and 6 μmol/kg (59, 60). These amounts are well above the apparent Km of pyridoxal phosphate for tyrosine decarboxylase from *S. faecalis*, which is about 3×10^{-7} M (61). However, Schormüller (62) showed that the activity of lysine decarboxylase in suspensions of Sauermilchkäse increased when pyridoxal-5-phosphate was added.

To check whether tyramine formation is affected by an increased level of coenzyme, we added pyridoxal phosphate to curd, already containing tyrosine decarboxylating bacteria, to yield a concentration of at least 40 μmol/kg cheese. In other experiments, tyrosine was added to the curd, to study whether tyramine formation can be enhanced by raising the precursor concentration. A drawback of this experiment is that abnormal induction of the decarboxylating enzymes is possible (54, 63), so the results should be interpreted with caution. Tyramine formation was also studied in a Maasdam cheese, made from milk inoculated with *L. brevis* Hem3. The results of all these experiments are in Table 9.

Addition of tyrosine to the curd increased tyramine formation in the cheese with *L. brevis*: after one month already 4.2 mmol/kg was found but any further increase was about equal to that of the comparable cheese without added tyrosine.

Addition of tyrosine to cheese milk infected with *S. faecalis* did not stimu-

late tyramine formation (Table 9, no. 7), indicating that in this case not the presence of precursor but the activity of the decarboxylating enzyme was the limiting factor.

In both cases (contamination with *S. faecalis* and *L. brevis*), the addition of vitamin B6 did not enhance the formation of tyramine. Its normal concentration in cheese is probably sufficient for maximum enzyme action. In the Maasdam cheese containing *L. brevis* 9.0 mmol tyramine/kg was found after one year of ripening, which is more than is found in Gouda cheese containing these bacteria. Probably the higher precursor concentration in this type of cheese accounts for the observed difference.

3.3.3 Putrescine. The formation of putrescine was observed in cheese made from milk contaminated with coliform bacteria, salt-tolerant lactobacilli and mixtures of non-salt-tolerant lactobacilli (9). The highest amounts (8.3 mmol/kg after one year of ripening) were found in the cheese containing salt-tolerant lactobacilli, but as the presence of these bacteria is rare no further attention was paid to them. The formation of putrescine in cheese by coliform bacteria only occurred during the first months of ripening (Table 10): it seems likely that amine formation ceases because the decarboxylating enzymes are inactivated, as the contents of free ornithine should permit more putrescine to be formed (Tables 1 and 2).

The concentration of this precursor in Gouda cheese shows more variation that that of the other precursors. The aseptically produced cheese (Table 2) generally contained less of it, after 12 months of ripening, than did the 6 months old cheese ripened at 14 °C from Table 1. As the ornithine content is greatly influenced by the starter, we performed an experiment in which several lots of Gouda cheese were made with two different starters: Fr19 and Bos. *H. alvei* were added to the milk, resulting in maximum densities ranging from 5×10^5 to 5×10^7 cfu/g cheese (Table 10). In addition, a Maasdam cheese was made from milk containing the ornithine decarboxylating bacteria. The results show that the number of these coliform bacteria present influences the amount of putrescine formed: while 5×10^5 cfu/g did not lead to detectable putrescine formation, 9×10^6 cfu/g and 5×10^7 cfu/g effected 0.8 and 1.8 mmol/kg respectively after three months of ripening when Bos was used as starter. Gouda cheese, made with Fr19 and containing 3×10^7 cfu/g after one day, showed little putrescine formation: after three months only 0.3 mmol/kg was detected. However, this cheese had a much lower pH and a higher salt content than the other three Gouda cheeses infected with LN1. The highest amine contents were found in the Maasdam cheese infected with *H. alvei*, 2.2 mmol putrescine/kg after three months of ripening.

Table 10. The formation of putrescine in Gouda and Maasdam cheese by *H. alvei* LN1.

Cheese no.	pH[1]	Salt-water ratio[1]	Type of cheese	Maximum[2] density	Starter	Ripening time (months)	Putrescine (mmol/kg)
1	5.43	0.044	Gouda	5×10^5	Bos	1	<0.2
						3	<0.2
						6	<0.2
2	5.44	0.045	Gouda	9×10^6	Bos	1	0.3
						3	0.8
						6	0.4
3	5.40	0.042	Gouda	5×10^7	Bos	1	1.2
						3	1.8
						6	1.6
4	5.11	0.052	Gouda	3×10^7	Fr19	1	0.2
						3	0.3
						12	0.4
5	5.36	0.027	Maasdam	4.5×10^7	Bos[3]	1	2.0
						3	2.2

[1] See Table 3, note 2.
[2] Number of LN1 after 24 hours (cfu/g).
[3] Propionic acid bacteria and the thermophilic lactobacilli were also added, as is usual for this type of cheese.

Still, the maximum contents of putrescine in the latter cheeses were not very high. It seems unlikely that coliform bacteria can cause much greater putrescine formation, since the liberation of arginine and the subsequent formation of ornithine proceeds slowly, while in the meantime, coliform bacteria die off and ornithine decarboxylase is probably rendered inactive. Higher amounts can be found in cheese containing ornithine decarboxylating lactobacilli, which remain active much longer. *L. buchneri* St2A also showed some ornithine decarboxylating activity, but this capacity only became manifest in Gouda cheese with a high pH and a high storage temperature. After twelve months, 1.2 mmol/kg was detected. *L. buchneri* St2A is also capable of forming the precursor, ornithine, from arginine (64). We did not investigate whether *Lactobacillus* strains with stronger decarboxylases may produce more putrescine.

3.3.4 Cadaverine. Besides massive formation of cadaverine by salt-tolerant lactobacilli, this amine was only found in cheese contaminated with members of the *Enterobacteriaceae* (9). We further studied the formation of cadaverine by the coliform bacterium *H. alvei* LN1 in Gouda and Maasdam cheese. The influence of the size of the inoculum and of the starter type and the addition of

Table 11. The formation of cadaverine in Gouda and Maasdam cheese infected with *H. alvei* LN1. The influence of the addition of lysine and pyridoxal-5-phosphate.

Cheese no.	pH[1]	Salt-water ratio[1]	Type of cheese	Starter	Maximum[3] density LN1	Addition		Ripening time (months)	Cadaverine (mmol/kg)
						lysine[4]	p-5-p[5]		
1	5.43	0.044	Gouda	Bos	5×10^5	–	–	1	2.0
								3	3.8
								6	6.0
2	5.44	0.045	Gouda	Bos	9×10^6	–	–	1	2.0
								3	3.2
								6	3.4
3	5.40	0.042	Gouda	Bos	5×10^7	–	–	1	2.0
								3	3.2
								6	3.2
4	5.11	0.052	Gouda	Fr19	3×10^7	–	–	1	1.6
								3	2.0
								6	2.6
								12	2.4
5	5.48	0.047	Gouda	Fr19	3×10^8	+	–	1	6.9
6	5.37	0.041	Gouda	Fr19	5×10^7	–	–	1	1.7
								3	2.8
								6	3.2
								12	3.1
7	5.33	0.041	Gouda	Fr19	7×10^7	–	+	1	1.9
								3	4.0
								6	5.2
								12	5.4
8	5.36	0.027	Maasdam	Bos[2]	4.5×10^7	–	–	1	4.8
								3	5.8

[1] See Table 3, note 2.
[2] See Table 10, note 3.
[3] See Table 10, note 2.
[4] Lysine was added after removal of the first whey, to give a concentration of 3.5-7.0 mmol/kg in the cheese.
[5] See Table 9, note 5.

precursor and of cofactor were studied. The results are in Table 11. In three experiments, the size of the inoculum was varied from 4×10^3 to 4×10^5 cfu/ml, leading to maximum densities of 5×10^5 to 5×10^7 cfu/g. After one month of ripening the cadaverine content of each cheese was about 2.0 mmol/kg (Table 11). Later on, only in the cheese with the smallest inoculum of *H. alvei* was a substantial increase of the cadaverine content found, while in the other two cheeses cadaverine formation was greatly retarded: after six months the contents were 6.0, 3.4 and 3.2 mmol/kg, respectively.

Microbiological examination revealed that the coliform bacteria in the last two cheeses died off rapidly. After three months of ripening less than 100 cfu/g were detected, while at that time 5×10^3 cfu/g were still detected in the cheese for which the smallest inoculum was used. Galesloot described the possibility of auto-intoxication of coliform bacteria that can take place in cheese with very high densities of these bacteria and he suggested that this might be caused by the accumulation of nitrous acid (65). This toxic substance can be formed from nitrate, which is added to the cheese milk to prevent clostridial blowing.

To investigate this, LN1 was grown on BHI for 24 h at 30 °C, as described under Section 3.2.4. The lysine decarboxylase activity was determined (Table 12). The cell-free extract was more active than the washed cell suspension (31.9 and 10.2 μl CO_2 released/min, respectively). Addition of nitrite immediately before the assay resulted in an increase of the activity of the washed cell suspension (15.0 μl/min) while the activity of the cell free extract decreased somewhat (28.2 μl/min). The nitrite concentration of 1.8 mM was chosen because nitrate is also added in this concentration to the milk for Gouda production. Obviously, in this concentration nitrite has no remarkable immediate inhibitory effect on enzyme activity.

The remainder of the washed cell suspension was divided into three portions, A, B and C. $NaNO_2$, at a concentration of 1.8 mM was added to suspension B and $NaNO_2$, together with pyridoxal phosphate (1.8 and 0.6 mM, respectively) was added to suspension C. All three portions were incubated at 37 °C and after 1, 2, 3 and 6 days the lysine decarboxylase activity and the viable count were determined (Table 12). The bacteria treated with nitrite died rapidly: after 6 days only 400 cfu/ml were detected (the initial count was 1×10^9 cfu/ml). In suspension C to which both $NaNO_2$ and pyridoxal phosphate were added even contained less viable bacteria: 15 cfu/ml, while 4×10^6 cfu/ml were still present in suspension A. The activity of lysine decarboxylase also declined fast in the suspension with nitrite: 4.0, 2.2 and 1.3 μl CO_2 was released per minute after 1, 2 and 3 days incubation at 37 °C respectively. After 6 days lysine decarboxylase activity was not detectable any longer. In suspen-

Table 12. The influence of nitrite and pyridoxal-5-phosphate on death rate and lysine decarboxylase activity of *H. alvei* LN1[1].

Enzyme preparation	Days incubated at 37 °C	cfu/ml	Lysine decarboxylase activity[2]
cell free extract	0	–	31.9
cell free extract + nitrite	0	–	28.2
A. washed cell suspension	0	1×10^9	10.2
	1	4.5×10^7	6.8
	2	2×10^7	7.0
	3	1.5×10^7	7.3
	6	4×10^6	8.7
B. washed cell suspension + nitrite (1.8 mM)	0	1×10^9	15.0
	1	1×10^6	4.0
	2	7×10^3	2.2
	3	8×10^2	1.3
	6	4×10^2	<0.05
C. washed cell suspension + nitrite 1.8 mM pyridoxal phosphate 0.6 mM	0	1×10^9	15.0
	1	$<10^6$	4.4
	2	40	4.2
	3	20	2.5
	6	15	0.1

[1] *H. alvei* was grown on BHI. From this culture a sonicated extract and a washed cell suspension were prepared. To these $NaNO_2$ or $NaNO_2$ + pyridoxal-5-phosphate was added. After 0, 1, 2, 3 and 6 days incubation at 37 °C, lysine decarboxylase activity and the number of viable bacteria were determined.

[2] μl CO_2 released/minute.

sion C 4.4, 4.2, 2.5 and 0.1 μl CO_2 was released per minute after 1, 2, 3 and 6 days incubation. The untreated suspension showed a relatively stable lysine decarboxylase activity: 6.8, 7.0, 7.3 and 8.7 μl was released per minute after 1, 2, 3 and 6 days incubation. This clearly demonstrates that NO_2^- has a deleterious effect on both viability and lysine decarboxylase activity. Therefore cheese varieties for which nitrate is not used may contain much more cadaverine when the milk is heavily infected with coliform bacteria.

The presence of large quantities of coenzyme retards inactivation of the enzyme. This can probably be explained by the findings of Sabo et al. (66), who showed that binding of coenzyme to lysine decarboxylase apoenzyme resulted in a stabilization of the native conformation of the holoenzyme. This probably also explains why more cadaverine was eventually found in cheese no. 7 (Table 11), to which besides LN1 pyridoxal phosphate was also added. After one year of ripening 5.4 mmol/kg was found, while the control cheese

(no. 6) contained 3.0 mmol/kg at that time. In the former cheese lysine decarboxylase could remain active for a longer period.

However, as the concentration of vitamin B6 does not show great variation in Dutch cheese (60), differences in cadaverine content probably hardly ever reflect differences in cofactor concentration.

It seems likely that during the first weeks of ripening cadaverine formation is limited by the precursor concentration: the content of this amine of the one month old cheese correlates well with the estimated lysine content of uninfected cheeses. Precursor limitation during the first weeks of ripening is also indicated by the fact that addition of lysine to curd, already containing LN1, resulted in a strongly enhanced cadaverine formation: after one month of ripening 6.9 mmol/kg was already found (Table 11, no. 5).

Cheese made with Fr 19 as a starter, contained about the same amount of cadaverine as cheese made with the starter Bos, probably because the liberation of lysine in Gouda cheese is not greatly influenced by the starter used. In Maasdam cheese *H. alvei* caused a higher cadaverine content: 4.8 mmol/kg was found after one month. This is probably caused by the high precursor concentration in this type of cheese.

3.3.5 Tryptamine. Although tryptamine is often mentioned when the formation of biogenic amines is discussed (1, 67), we did not observe detectable amounts (i.e. more than 0.4 mmol/kg) either in cheese made at our laboratory, or in samples from the market (to be published). This can probably be explained by the low tryptophane content of casein, and hence the low precursor content in Gouda cheese. Also from a toxicological point of view tryptamine is of little concern, since (to our knowledge) it has never been associated with food poisoning.

3.3.6 Phenylethylamine. Phenylethylamine concentrations in cheese samples from the market were almost always below 0.4 mmol/kg (to be published). We never detected bacteria with a decarboxylase specific for phenylalanine nor does the literature on this subject mention the existence of such bacteria. It seems likely that phenylethylamine formation in cheese results from the action of tyrosine decarboxylase, since this enzyme also has some affinity for phenylalanine (68, 69). Likewise, this amine was only found in cheese containing much tyramine. Appreciable amounts of phenylethylamine (12.8 mmol/kg) was found in a cheese heavily infected with *S. faecalis* (Table 13). Small quantities (0.4 mmol/kg) were found in a one year old Maasdam cheese containing *L. brevis* Hem3, and in Gouda cheese to which tyrosine (and *L. brevis*) was added. The precursor content is not a limiting factor, as it can be found in large amounts in ripened cheese (Tables 1 and 2).

Table 13. The formation of phenylethylamine in Gouda and Maasdam cheese infected with *S. faecalis* H1 or *L. brevis* Hem 3.

Cheese no.	pH[1]	Salt-water ratio[1]	Type of cheese	Addition/maximum density			Ripening time (months)	Phenylethylamine (mmol/kg)
				S. faecalis[2]	*L. brevis*[3]	tyrosine[4]		
1	5.34	0.047	Gouda	1×10^7	–	–	1	< 0.4
							3	< 0.4
							6	< 0.4
2	5.34	0.041	Gouda	1.3×10^8	–	–	1	< 0.4
							3	< 0.4
							6	< 0.4
3	5.31	0.043	Gouda	1.6×10^9	–	–	1	0.6
							3	4.1
							12	6.8
4	5.23	0.035	Gouda	2.2×10^9	–	–	1	1.8
							3	7.4
							6	12.8
							12	11.6
5	5.22	0.043	Gouda	–	1×10^8	–	1	< 0.4
							3	< 0.4
							6	< 0.4
							12	< 0.4
6	5.32	0.047	Gouda	–	1×10^8	+	1	< 0.4
							3	< 0.4
							6	< 0.4
							12	0.5
7	5.38	0.025	Maasdam	–	1×10^8	–	1	< 0.4
							3	< 0.4
							6	< 0.4
							12	0.4

[1] See Table 3, note 2.
[2] *S. faecalis* H1 was added to the milk after pasteurization. The maximum density, given in cfu/g, was reached within one week.
[3] *L. brevis* was added to the milk after pasteurization (about 1000 cfu/ml). After about one month the maximum density was reached.
[4] see Table 9, note 4.

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Samenvatting

H. M. L. J. Joosten, *Omstandigheden waaronder biogene aminen in kaas kunnen worden gevormd. 4. Factoren die de gevormde hoeveelheden beïnvloeden**

De precursorconcentratie is de limiterende factor voor de vorming van histamine in kaas besmet met *Lactobacillus buchneri* St2A. Daarom werden er nieuwe kaasbereidingsproeven uitgevoerd waarbij parameters die mogelijk de vrije-aminozuurconcentratie beïnvloeden gevarieerd werden. Histaminevorming bleek sneller te verlopen in kaas die bij een hogere temperatuur bewaard werd (18 of 21 °C). Dit was ook het geval in kaas met een hogere pH of een lager zoutgehalte dan normaal is voor Goudse. Pasteurisatie van de melk beïnvloedde de aminevorming slechts in geringe mate, wat erop wijst dat deze hittebehandeling waarschijnlijk niet tot gevolg heeft dat de hoeveelheid vrij histidine verandert.

De mesofiele zuursels die voor de bereiding van Nederlandse kaas het meest worden gebruikt, bleken slechts in geringe mate te verschillen in hun vermogen om histidine vrij te maken en dientengevolge was de hoeveelheid histamine gevormd in besmette kazen bereid met verschillende zuursels praktisch gelijk. Als naast deze mesofiele zuurselbacteriën ook thermofiele lactobacillen en propionzuurbacteriën aan de kaasmelk werden toegevoegd, zoals gebruikelijk is voor de bereiding van Maasdammer, wordt de vorming van histidine in sterke mate gestimuleerd. Als dit type kaas besmet geraakt is met histidine-decarboxylerende lactobacillen hoeft een hoog histaminegehalte niet onmiddellijk verwacht te worden, omdat Maasdammer gewoonlijk al na twee maanden op de markt wordt gebracht. Hoge gehalten kunnen pas dan optreden als zo'n besmette kaas langere tijd bij te hoge temperatuur wordt opgeslagen en vooral dan als er teveel thermofiele lactobacillen bij de bereiding werden toegevoegd.

De gelijktijdige aanwezigheid van andere mesofiele lactobacillen en coliforme bacteriën bleek de vorming van histamine niet te beïnvloeden. Enterokokken (*Streptococcus faecalis* var. *liquefaciens*) versnelde de eiwitafbraak alleen wanneer er zeer grote aantallen van deze stam aanwezig waren (2×10^9 cfu/g). In een Goudse kaas met daarin ongeveer 1×10^8 cfu/g van een tyrosine-decarboxylerende stam van *Lactobacillus brevis* werd de vorming van tyramine ook beperkt door de precursorconcentratie. Tyraminevorming werd gestimuleerd door toevoeging van tyrosine aan de melk en ook in kaas met een snellere eiwitafbraak was het tyraminegehalte hoger. Toevoeging van pyridoxaalfosfaat aan de melk gaf geen versnelde tyraminevorming.

Ornithine is de precursor van putrescine. Het gehalte aan ornithine van Goudse kaas vertoont veel meer variatie dan dat van de meeste andere aminozuren, maar na twaalf maanden rijping is het gehalte meestal lager dan 6,0 mmol/kg. Putrescinevorming werd geconstateerd bij besmet-

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ting met bepaalde coliforme bacteriën, maar het maximale gehalte bedroeg dan slechts 2,2 mmol/kg.

Bij enkele kaasbereidingsproeven werd *Hafnia alvei* LN1, een lysine-decarboxylerende stam, aan de melk toegevoegd. Wanneer het inoculum erg groot was (4×10^5 cfu/ml) bleek dat de bacteriën veel sneller afstierven dan wanneer 4×10^3 cfu/ml werden toegevoegd aan de melk. De hoeveelheid cadaverine die aangetroffen werd na 1 maand rijping was in iedere kaas gelijk. Na 6 maanden rijping was het gehalte in de kaas met het kleinste inoculum beduidend hoger. Waarschijnlijk werd in de kazen met de zeer hoge aantallen van deze bacterie veel nitriet gevormd. Als deze stof werd toegevoegd aan gewassen celsuspensies van LN1, nam niet alleen het aantal kolonievormende eenheden snel af, maar ook verminderde de activiteit van het lysinedecarboxylase veel sneller dan in de suspensie zonder nitriettoevoeging.

Toevoeging van pyridoxaalfosfaat aan de met LN1 besmette kaasmelk stimuleerde de cadaverinevorming enigszins. De vrije-lysineconcentratie van kaas is relatief hoog en alleen in jonge kaas kan deze voor de aminevorming een beperkende factor zijn.

Ook phenylalanine komt in vrij hoge gehaltes voor in kaas. Phenylethylamine werd alleen gevormd als grote aantallen tyrosine-decarboxylerende bacteriën in de kaas aanwezig waren, waarschijnlijk als gevolg van een geringe activiteit van tyrosinedecarboxylase voor dit structureel verwante aminozuur.

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Neth. Milk Dairy J. 42 (1988) 3-24

Conditions allowing the formation of biogenic amines in cheese. 4. A study of the kinetics of histamine formation in an infected Gouda cheese*

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Summary

Gouda cheese was made from milk deliberately contaminated with a histidine decarboxylating *Lactobacillus* strain (St2A). During ripening, the density of the decarboxylating strain, its decarboxylase activity and the histidine and histamine concentrations were measured. Moreover, the influence of some environmental parameters such as pH, temperature, substrate and product concentration on enzyme activity were determined. Within one month St2A reached its maximum density of more than 10^8 cfu/g. Enzyme activity was found to reach its highest value after about 40 days of ripening, after which it gradually decreased. Free histidine was hardly detectable in the cheese, only in the rind a little (0.6 mmol/kg) was found. In the center of the cheese it was virtually absent (<0.03 mmol/kg). Histamine contents increased gradually during ripening. In the middle its concentration was higher than in the rind (5.8 against 3.8 mmol per kg after 13½ months of ripening).

A kinetic model is proposed for histamine formation in cheese and its validity is tested by comparing experimentally determined histamine and histidine concentrations with those calculated with the model. To do so, certain assumptions had to be made, for instance that the rate of histidine liberation in an uninfected cheese is the same as in the cheese infected with St2A. It was found that the results of the calculations corresponded well with experimentally determined values, at least for the center part of the cheese. The results of calculations with this model make it likely that histamine degradation does not occur in cheese and that the precursor concentration is rate-determining for histamine formation in the infected cheese (10^8 cfu/g). The situation in the rind is somewhat less clear: the observed histidine concentration was higher than expected according to the model. The explanation of this discrepancy probably lies in the very low water content of the rind, hence a very low diffusion rate, but the number of bacteria and enzyme activity were also reduced.

Furthermore it was calculated what histamine formation can be expected in cheese not so heavily contaminated with St2A. The results showed that for example in a regular Gouda cheese with a maximum density of 10^6 cfu/g only 0.5 mmol/kg will be formed after one year of ripening.

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1 Introduction

The starters commonly used in the Dutch cheese industry lack the capacity to form biogenic amines (1). Therefore, most of the cheese produced in this country does not contain these toxic compounds. However, if non-starter bacteria with decarboxylating properties gain access to the milk after the pasteurization process, they may eventually reach a high density in the cheese, thus leading to biogenic amine formation (2). Chemical analysis of Gouda cheese, deliberately contaminated with various strains of decarboxylating bacteria (e.g. some members of the *Lactobacillaceae* and the *Enterobacteriaceae*), revealed that even when large inocula were used, amine formation remained at a fairly low level. For example, Gouda cheese, made from milk to which approximately 2000 cfu of a histidine-decarboxylating strain of *Lactobacillus buchneri* was added per ml, and in which outgrowth took place to more than 10^8 cfu per gram, did not contain more than about 5 mmol histamine per kg after one year of ripening (2). On the other hand, there are reports of incidents of food poisoning, caused by consumption of cheese containing more than 15 mmol histamine per kg (3, 4). When another histidine decarboxylating strain was used instead (*Lactobacillus* 4720-2), almost the same histamine content (4 mmol/kg) was measured after one year of ripening (2).

It was postulated that histamine formation in the cheese heavily infected with these lactobacilli was not limited by the activity of histidine decarboxylase but by a shortage of precursor (free histidine). Indeed, higher histamine concentrations were found in infected Gouda cheese in which proteolysis was accelerated (5), while very high histamine levels were reached by adding histidine to curd already containing the earlier mentioned *Lactobacillus* strains (H. Joosten, unpublished results). Amine formation in cheese contaminated with other decarboxylating bacteria (e.g. tyrosine decarboxylating strains of *L. brevis*) was also increased by accelerated protein breakdown (5).

Nevertheless, the conditions that allowed faster proteolysis to occur and the increased precursor concentration itself may have influenced enzyme formation and enzyme activity. It is well known, for example, that the catabolic bacterial decarboxylases are inducible by high substrate concentrations (6-8). Therefore, on the basis of only these results, it cannot yet be ruled out that enzyme activity was actually a limiting factor.

To solve this problem we again made cheese from milk to which the histidine decarboxylating strain *L. buchneri* St2A was added. The enzyme activity of suspensions of the cheese, as well as histamine and histidine concentrations were measured. In addition, the influence of various parameters on decar-

boxylase activity was determined. The results were used to study the kinetics of histamine formation in this cheese and to predict the rate of histamine formation under other circumstances.

2 Materials and methods

2.1 Cheesemaking

Cheesemaking was performed as described in an earlier publication (2). Histidine decarboxylating lactobacilli were added after bactofugation and pasteurization of the milk. The starter Fr19 was used.

2.2 Cultures

L. buchneri St2A was kindly provided by Dr S. L. Taylor, Madison, Wis. The strain was propagated in sterilized litmus milk supplemented with 1 % yeast extract.

2.3 Preparation of a washed cell suspension of L. buchneri St2A

St2A was grown on TGV 5.4 medium (9) for 3 days at 30 °C. 2.5 ml of this culture was used to inoculate another 250 ml portion of TGV 5.4 medium, supplemented with 1 % L-histidine. After 3 days anaerobic incubation at 30 °C, the cells were harvested by centrifugation (10 min, 10 000 *g* at 4 °C). The cells were washed with saline (0.85 %), centrifuged again and suspended in 40 ml 0.2 M citrate buffer (pH 5.2). After addition of 7 % (w/v) lactose, portions of 5 ml were frozen at −40 °C. The suspension could be stored for several weeks at this temperature without detectable loss of activity.

2.4 Preparation of a cheese suspension for the enzyme assay

A borer sample of 10 g cheese was homogenized with 40 ml of a peptone salt solution and the pH adjusted to 5.2 with a few drops of 0.2 M HCl.

2.5 Enzyme assays

Histidine decarboxylase activity was measured by a manometric and an isotopic assay. The manometric assay was performed with a Gilson Differential Respirometer (Gilson Medical Electronics, Middleton, Wisc.). As an enzyme source, washed cell suspensions of strain St2A, diluted with 0.2 M citrate buffer, pH 5.2, were used. Unless stated otherwise, 2.3 ml of the suspension was added to the main compartment of the Gilson flask, and 0.5 ml 0.1 M L-histidine was added to the side arm. After 10 min equilibration at 30 °C, the reaction was started by tipping in the substrate and CO_2 release was measured during 30 minutes. Flasks with 0.5 ml H_2O instead of histidine

served as control. Some experiments were carried out at a pH of 5.5 or higher. In these cases the reaction was stopped after 30 minutes by addition of 0.3 ml 6 N H_2SO_4, which ensured complete liberation of all dissolved CO_2.

The sensitive isotopic assay was based on the method described by Levine & Watts (10). To determine histidine decarboxylase activity of cheese samples, 2 ml of the cheese suspension was mixed with 0.6 ml phosphate buffer (0.2 M, pH 5.2), 0.3 ml L-histidine (10 mM) and 0.1 ml (0.125 μCi) L-[carboxyl-^{14}C-]histidine (New England Nuclear, Boston, Mass) in a polyethylene scintillation vial. Immediately thereafter, the vial was closed with a rubber stopper in which a wire clip was inserted. To this wire clip a rolled filter paper was attached (1 × 3 cm, 3 MM Whatman), soaked with 75 μl phenylethylamine (Merck). The reaction was stopped after 1 hour incubation at 30 °C by injection of 2 ml 1 M $HClO_4$ into the vessel. 30 Minutes later, the filter paper was put in another scintillation vial together with 10 ml scintillation liquid (NE 265, Nuclear Enterprises, Edinburgh, UK). To stabilize light emission, the vials were stored for at least 24 hours in a dark place and thereafter the radioactivity was determined with a Beckman LS 7500 liquid scintillation counter (Beckman, Fullerton, CA). Counting efficiency was determined with ^{14}C-toluene (NEN, 4.0×10^5 dpm/ml), while the efficiency of the CO_2 trapping system was checked with $Na_2{}^{14}CO_3$ (NEN). Suspensions of uninfected Gouda cheese served as control for the assay (no histidine decarboxylase activity).

The isotopic assay was also used to determine the relationship between substrate concentration and initial reaction rate of washed cell suspensions of St2A: 1 ml of a properly diluted washed cell suspension of St2A in 0.2 M citrate buffer, pH 5.2, was added to a polyethylene scintillation vial and pre-incubated for 10 min at 30 °C. A substrate mixture containing both radioactive and non-radioactive L-histidine in 0.2 M citrate buffer, pH 5.2, was also pre-incubated for 10 min at 30 °C. To start the reaction, the substrate was added to the cells and the evolution of $^{14}CO_2$ was measured after 3, 6, 9 and 12 min incubation.

Enzyme activity was expressed as nmol histidine decarboxylated per h and per g of cheese (for cheese suspensions) and as nmol histidine decarboxylated per h and per 10^8 lactobacilli (for the washed cell suspensions).

Other microbiological and chemical methods were as published earlier (1, 2, 5).

3 Results and discussion

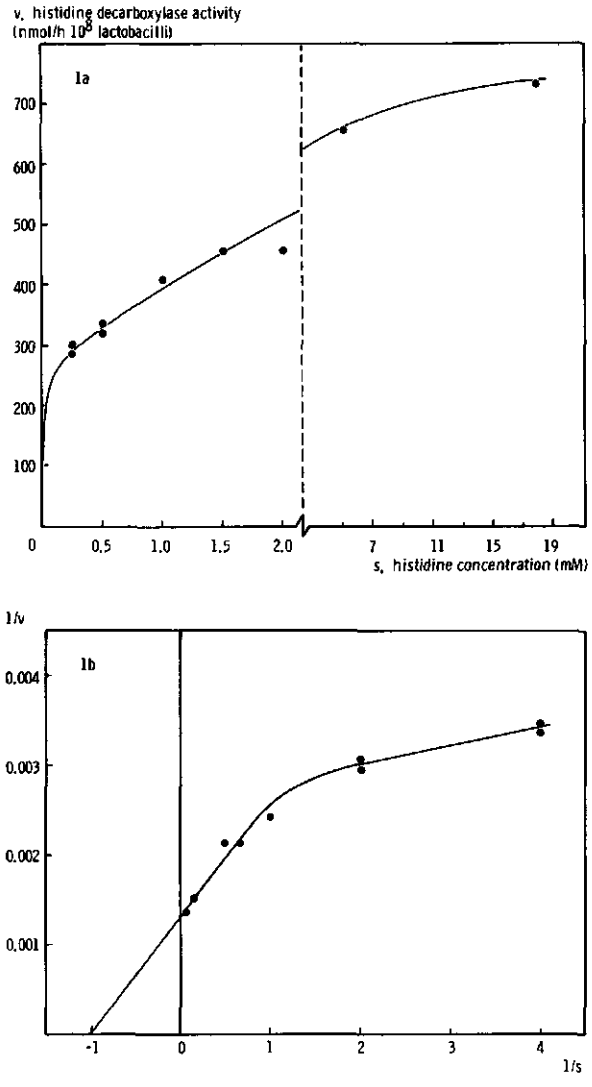
3.1 Influence of some environmental factors on histidine decarboxylase activity of strain St2A

3.1.1 Histidine

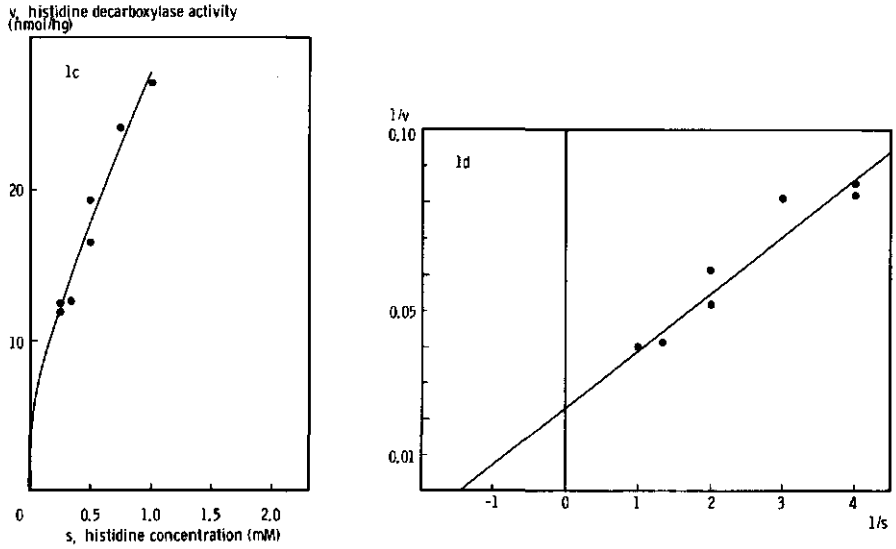
Using washed cell suspensions of strain St2A, the relation between enzyme activity and the substrate concentration was determined (Fig. 1a). Apparently there is no linear relationship between the reciprocals of these parameters (Fig. 1b). At first sight these observations are in contradiction with the results of Chang & Snell (11). Working with purified histidine decarboxylase from *Lactobacillus* 30A, an enzyme probably very similar to that of *L. buchneri* St2A (12), they found values of $1/v_0$ and corresponding $1/s$ that could be fitted to a straight line much better. However, our results were obtained with whole cells and because the enzyme is located within the cell, transport effects may easily disturb the expected linear relationship. It is well known that bacterial cells accumulate amino acids, and, especially at low concentrations of amino acids in the external medium, the accumulation factor (defined as the concentration within the cell divided by the external concentration) may reach high values (13). If the external amino acid concentration is relatively high, the accumulation factor would be lower. Assuming that at the highest substrate concentration employed, 17.8 mM, the accumulation factor is 1.0 and $Km \approx 1.0$ mM, then to fit the results to Michaelis-Menten kinetics at a histidine concentration of 0.25 mM an accumulation factor of 2.6 is calculated, which is not extraordinary if compared to accumulation factors reported in the literature (14-16).

Comparable experiments were performed using cheese suspensions as the enzyme source. The samples were taken from cheese contaminated with St2A, that had been stored for 6 months at 14 °C. The results are shown in Figs. 1c and 1d. After 6 months of ripening the number of lactobacilli was about 10^8 cfu per gram of cheese, thus permitting direct comparison with Figs. 1a and 1b. The most obvious difference between Figs. 1a and 1c is that the decarboxylase activity per bacterium in the washed cell suspension is much higher. This can probably be explained by the fact that the lactobacilli from which the washed cell suspension was prepared had been grown in a medium containing 1 % histidine. Under these circumstances enzyme formation is strongly induced. This will not occur in cheese because the histidine content in here is much lower.

Apart from this it can be noticed that in Figs. 1c and 1d the relative difference between enzyme activity at 0.25 mM and 1.00 mM is much greater (as



compared to Figs. 1a and 1b), and likewise the plot of $1/v_o$ against $1/s$ reasonably fits a straight line. Although these few results do not yet allow firm conclusions to be drawn, it is tempting to speculate that the latter difference of results obtained with the washed cell suspension and those obtained with the cheese suspension can be explained by assuming that the lactobacilli in the six months old cheese no longer accumulate histidine. This hypothesis is sup-



Figs. 1a-1d. Effect of substrate concentration on histidine decarboxylase activity of strain St2A. The experiments were performed with washed cell suspensions (a+b) and with suspensions of a 6 month old cheese infected with strain St2A (10^8 cfu/g) (c+d).

ported by the fact that for other lactic acid bacteria it has been shown that amino acid accumulation is an energy-dependent process (14-16). The growth of lactobacilli in Gouda cheese stops after 1 to 3 months of ripening and it seems likely that from then on the intracellular ATP concentration steadily decreases. When working with the cheese suspensions, care was taken not to use buffers containing a fermentable substrate, so as to avoid restoration of the proton motive force.

3.1.2 Temperature

Histidine decarboxylase activity was determined at 15, 20, 25 and 30 °C using an aliquot of the same cell suspension for each experiment. The results are shown in Fig. 2. Within this temperature range, the highest activity was measured at 30 °C (740 nmol histidine decarboxylated per hour and per 10^8 lactobacilli. Using the Arrhenius relation, we calculated an apparent activation energy of 57.2 kJ/mole ($Q_{10} = 2.2$).

3.1.3 pH

St2A was suspended in 0.2 M citrate buffers ranging from pH 2.7 to 5.9 and

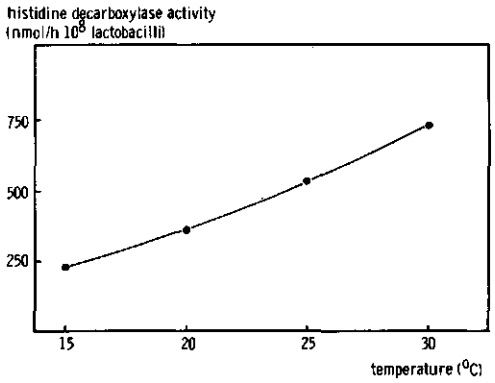


Fig. 2. Effect of temperature on histidine decarboxylase activity of washed cell suspensions of strain St2A.

enzyme activity was determined. Because the decarboxylation itself might cause a pH shift, the pH before and after the incubation period was measured. The difference between the two values was always smaller than 0.1 pH unit. The average values are plotted in Fig. 3, together with the corresponding enzyme activities. The optimum pH for CO_2 evolution was about 4.4.

In Section 3.1.1 it was postulated that the lactobacilli in the 6 months old cheese were no longer capable of accumulating amino acids, because they were in the starvation phase. In this phase, the pH gradient that exists be-

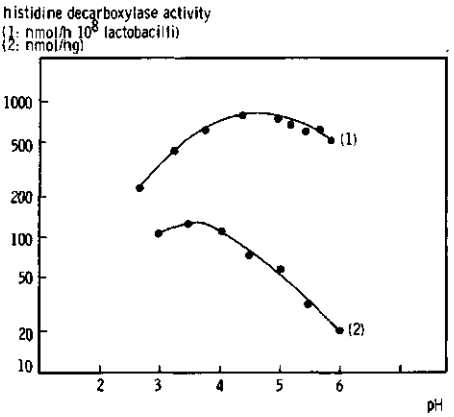


Fig. 3. Effect of pH on histidine decarboxylase activity of strain St2A. The experiments were performed with washed cell suspensions of strain St2A (1) and with suspensions of a 6 month old cheese infected with St2A (2).

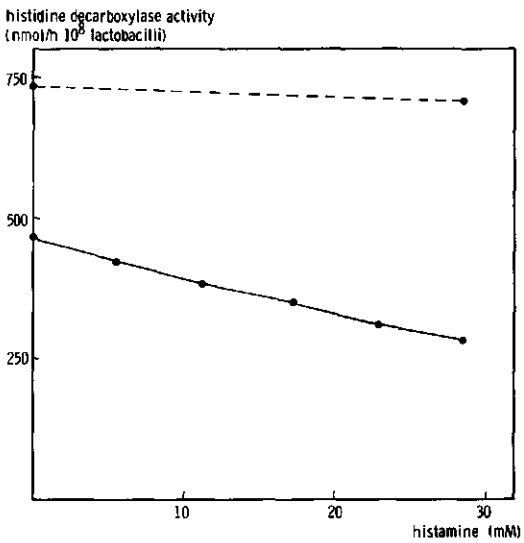


Fig. 4. Effect of histamine on histidine decarboxylase activity of washed cell suspensions of strain St2A. — (1) histidine concentration 2.1 mM; - - - (2) histidine concentration 17.8 mM.

tween the cytoplasm and the external medium decreases together with the proton motive force (14-17). It thus seems likely that the pH optimum determined with suspensions of old cheese should be more alkaline than the pH optimum determined with young cells. However, we were not able to confirm this (Fig. 3). In fact the pH optimum determined with the cheese suspension was about one pH unit lower. We do not have an explanation for this. Most other experiments were carried out at a pH of 5.2, because this closely resembles the situation in Gouda cheese.

3.1.4 Histamine

Addition of histamine (0-28.6 mM) to cell suspensions of St2A resulted in decreased enzyme activity (Fig. 4), if a low substrate concentration (2.1 mM) was employed. With a substrate concentration of 17.8 mM the inhibitory effect was no longer significant. This indicates a competitive inhibition mechanism, as is usually observed with product inhibition. Since the substrate concentration in the infected cheese remains low, we have to take product inhibition into account (see below).

3.1.5 Sodium chloride

To suspensions of St2A in 0.2 M citrate buffer, pH 5.2, NaCl was added (final

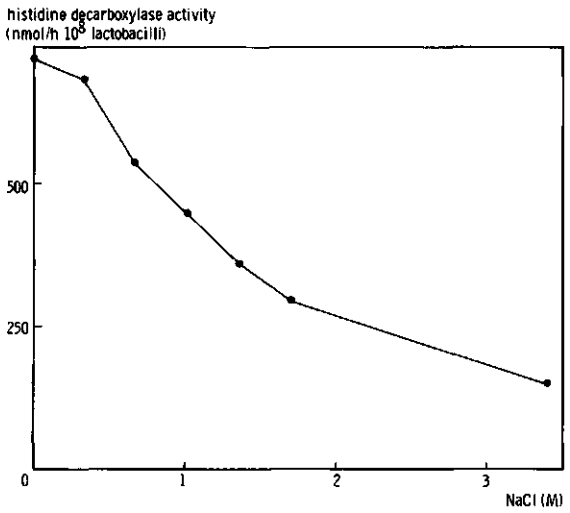


Fig. 5. Effect of NaCl on histidine decarboxylase activity of washed cell suspensions of strain St2A.

concentration 0-3.4 M). From Fig. 5 it can be seen that salt inhibits enzyme action: at 1.4 M NaCl enzyme activity is reduced to 50 % and at 3.4 M NaCl only 20 % of the activity is still found. Clearly this effect has to be taken into account when histamine formation in cheese is considered, where the salt concentration is about 1 M.

3.1.6 Lactate

The influence of lactate on histidine decarboxylase activity was determined (Fig. 6). The lactate concentrations employed (0-0.7 M) gave only a small reduction of activity. It must be noted that the lactate solution was adjusted to pH 5.2 with 10 M NaOH. Therefore the inhibitory effects may also have been due to sodium ions. Nevertheless, it can be concluded that the lactate concentration of Gouda cheese, which is never higher than 0.3 M, most probably does not affect histidine decarboxylase activity.

3.2 Growth of L. buchneri St2A in cheese and development of histidine decarboxylase activity

From 200 l of bactofuged and pasteurized milk, infected with approximately 2000 cfu of *L. buchneri* per ml, four Gouda cheeses were produced. During the ripening period, samples of 10 g were taken from the center part of the

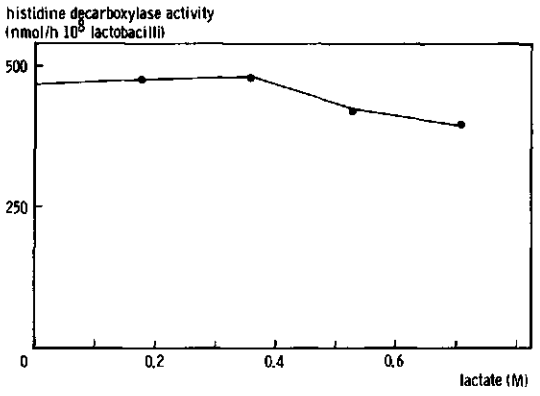


Fig. 6. Effect of lactate on histidine decarboxylase activity of washed cell suspensions of strain St2A.

cheese to determine outgrowth of St2A and to measure histidine decarboxylase activity. The results are shown in Fig. 7. Within one month St2A counts reached a maximum of 12×10^7 cfu/g and this slowly decreased to about 7×10^7 cfu/g after six months. Histidine decarboxylase activity also reached a

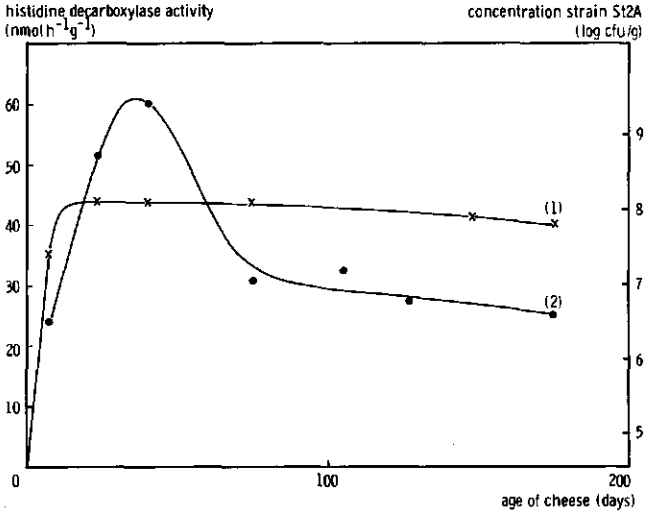


Fig. 7. Development of *L. buchneri* St2A (1) and histidine decarboxylase (2) in Gouda cheese, made from milk deliberately contaminated with this strain (10^3 cfu/ml).

maximum after about one month. Under the reaction conditions applied, a suspension of a 40 days old cheese decarboxylated 60.8 nmol histidine $g^{-1}h^{-1}$. In the older cheese samples lower activities were measured. After a decrease to 30.8 nmol $g^{-1}h^{-1}$ found in a three months old sample, enzyme activity remained relatively stable: in a six months old sample 25 nmol $g^{-1}h^{-1}$ could be decarboxylated.

Histidine decarboxylase activity was also measured in a 13.5 month old sample of Gouda cheese, infected with St2A but produced from another vat of milk. In this cheese histidine decarboxylase activity was still 22.5 nmol $g^{-1}h^{-1}$.

3.3 Kinetics of histamine formation in Gouda cheese infected with St2A

It should now be possible to calculate the rate of histamine formation in infected Gouda cheese from data on precursor (= histidine) formation and the decarboxylating activity of the bacteria involved. Before doing so, we must pay attention to the fact that the bacteria, and histidine (and other amino acids) and histamine (and other amines) are not evenly distributed throughout the cheese. In the first paper of this series (1), it was already apparent that the concentration of the amines was lower in the rind than in the middle of the cheese. Therefore, we also determined the distribution of some amino acids and histamine, the number of contaminating bacteria and decarboxylase activity in a 13.5 month old Gouda cheese, infected with *L. buchneri* St2A. The results are in Table 1, and they indicate that proteolysis is less in the rind than in the middle and that the number of lactobacilli is less by a factor 50 in the rind, whereas decarboxylase activity determined in suspensions of the rind was about 40 % of that in the middle. This may indicate that the density of St2A in the rind was initially much higher than later on and that after more than 13 months most of the bacteria are not able to reproduce in the counting medium anymore, although there still remains decarboxylase activity.

To estimate the rate of formation of histidine we used the data presented in Table 1 of a previous publication (5). At this stage it may be useful to distinguish between formation of histidine in the middle of the cheese and in the rind. The data in Table 1 of Reference 5 are results of sector samples, hence including the rind. From the results of the present Table 1 it can be deduced that, on average, the concentration in the middle is 1.16× the concentration in the sector sample; in this way we converted the data in Table 1 of Reference 5 to estimate the rate of histidine formation in the middle of the cheese. This rate could not be described by a first- or second-order rate equation; a zero-order equation gave the best approximation over the time scale con-

Table 1. Distribution of amino acids, histamine, lactobacilli and decarboxylase activity in a 13.5 month old Gouda cheese infected with *L. buchneri* St2A.

	Rind (8 mm)	Middle	Average in sector sample
amino acids (mmol/kg)			
asp	4.2	7.0	6.1
thr	3.9	8.7	6.9
ser	6.9	12.0	10.2
asn	16.5	29.7	26.4
glu	32.9	61.3	51.1
pro	8.8	15.6	12.9
gly	7.9	13.9	11.9
ala	6.4	11.2	9.4
val	15.5	33.7	25.9
cys	<0.2	<0.2	<0.2
met	5.6	8.4	7.3
ile	10.7	15.4	14.0
leu	27.8	41.5	36.4
tyr	6.5	8.0	7.4
phe	15.9	19.1	17.8
lys	20.7	33.5	29.7
his	0.61	<0.03	0.21
histamine (mmol/kg)	3.8	5.8	5.1
L. buchneri St2A (g^{-1})	1.5×10^6	8.2×10^7	ND
decarboxylase activity* (nmol his/g cheese/h)	9.0	22.5	ND

* As determined with suspensions of the cheese.

sidered (6 months). A zero-order rate equation also more or less applies when the formation of amino acid nitrogen in a normal Gouda cheese is considered over a period of 12 months (18). The rate constants thus determined are presented in Table 2 for four different ripening temperatures. An (apparent) activation energy, as determined from the Arrhenius equation, was found to be 64.3 kJ/mole. The Q_{10} is calculated as 2.5. It must be realized that these rate constants are crude approximations since we had only a few data for calculation. In passing, we note that the literature contains very few results on the kinetics of proteolysis in cheese; clearly this needs further research. Below we will use the results of Table 2 for the calculation of the formation of histamine, which implies the assumption that the formation of histidine is the same in uninfected and in infected cheese.

As far as the kinetics of decarboxylation are concerned we use the results described in Sections 3.1 and 3.2 of this paper. Most experiments were performed with cell suspensions; therefore we must convert some results in order to apply them to cheese. Although we found that the relation between rate of

Table 2. Calculated rate constants for the formation of histidine (k_1) and histamine (k_2)[1].

Temperature	k_1	k_2
9 °C	0.28	2.0
14 °C	0.38	3.1
18 °C	0.63	4.4
21 °C	0.85	5.5

[1] k_1 was derived from the results of amino acid analysis of uninfected Gouda cheese and is expressed as mmol kg^{-1} $month^{-1}$. k_2 was calculated from histamine formation in suspensions of Gouda cheese contaminated with St2A (10^8 cfu/g), and is expressed as $(mmol/kg)^{0.42}$ $month^{-1}$.

decarboxylation and substrate concentration could be described by Michaelis-Menten kinetics (at 30 °C at pH 5.2 in the absence of salt, using a suspension of a six month old cheese) we did not determine the parameters V_m and K_m as a function of temperature, salt content and pH. Therefore, we expressed the relation between enzyme activity and substrate concentration in a more general equation:

$$v = k_2 S^n \tag{1}$$

in which v is the formation of histamine per unit of time, k_2 the rate constant, and S the substrate concentration (= histidine). We determined n by power law regression of results shown in Fig. 1c: $n = 0.58$. It is realized that this is only a crude approximation of the relation between substrate concentration and enzyme activity, but at the moment it is the best approximation we can offer. We now assume that the temperature dependency of k_2 is the same for fresh cell suspensions (see Section 3.1.2) and (old) cells in cheese, namely with an apparent activation energy of 57.2 kJ/mole ($Q_{10} = 2.2$). We also assume that the effect of NaCl is the same as is described in Section 3.1.5. As an average value, we choose 6 % NaCl in moisture. In the beginning of ripening it will be lower, at the end it may be higher. With these assumptions the rate constants can be calculated for Gouda cheese containing 10^8 cfu of *L. buchneri* St2A per gram: see Table 2.

Then we have to take into account the phenomenon of product inhibition, as described in Section 3.1.4, but again we have to convert these data to make them applicable to cheese. We did this in the following way. It was assumed that product inhibition in cheese can be described according to the Lineweaver-Burke relation:

$$\frac{1}{v} = \frac{K_m}{v_m}\left(1 + \frac{p}{K_p}\right)\frac{1}{s} + \frac{1}{v_m} \tag{2}$$

in which p is product concentration (histamine) and Kp the association constant of the product-enzyme complex. From the data given in Fig. 4, we estimated K_p to be about 8 mmol/kg. Next the inhibition can be calculated for values of s and p. Since we deal with low histidine concentrations in the cheese when histamine is formed (see below) the decrease in enzyme activity can be considerable at high histamine concentrations, for instance a 50 % decrease of activity was calculated at $p = 10$ mmol/kg cheese and $s = 0.1$ mmol/kg cheese. However, since the concentration of histamine, if formed, gradually increases during the ripening of cheese, the inhibition also increases gradually. With the aid of equation (2), we calculated the decrease in v as a function of p at $s = 0.1$ mmol/kg. The relation found was fitted in an exponential function and expressed in the rate constant k_2:

$$k_2' = k_2 \times e^{-0.077p} \tag{3}$$

in which k_2' is the rate constant corrected for product inhibition at a substrate concentration of 0.1 mmol/kg cheese. We assume that temperature only has an effect on k_2, not on the correction for product inhibition.

Some uncertainties remain: is there a difference in inhibition for young and old cells, or in histamine concentration outside and inside the cells? We have no answers to these questions as yet, so we have to be content with the present derivation.

An unknown but conceivable phenomenon may be that histamine is further degraded into other products (for instance as a result of deamination). We do not have any indication that this actually happens, but we incorporated the possibility in the kinetic model: we simply assumed that histamine can be converted according to a first-order reaction. The model then can be described as follows:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D \tag{4}$$

in which A stands for histidine bound in protein, B for free histidine (liberated from the protein), C for histamine and D for a degradation product of histamine. The following differential equations can now be set up:

$$-\mathrm{d}[A]/\mathrm{d}t = k_1 \tag{5a}$$

$$\mathrm{d}[B]/\mathrm{d}t = k_1 - k_2'[B]^{0.58} \tag{5b}$$

$$\mathrm{d}[C]/\mathrm{d}t = k_2'[B]^{0.58} - k_3[C] \tag{5c}$$

$$\mathrm{d}[D]/\mathrm{d}t = k_3[C] \tag{5d}$$

and

$$k_2' = k_2 \times e^{-0.077[C]} \tag{6}$$

These equations were solved by numerical integration using a fourth-order Runge-Kutta method with boundary conditions $[A]_o$ = 47.5 mmol/kg, and $[B]_o = [C]_o = [D]_o = 0$. The values for k_1 and k_2 were taken from Table 2.

Before testing the validity of the model, we must consider the rate of diffusion. The production of histidine occurs at another place than does the production of histamine: histidine must diffuse to the cells before it is converted and this process could be rate determining. If we assume that the number of colonies N is the same as the number of inoculated bacteria, then $N = 10^4$ g^{-1}. Supposing that the number of bacteria is 10^8 g^{-1} and the volume of one bacterium is 1 μm^3, then the diameter d of one colony is about 20 μm. The average distance x between colonies can be estimated from the relation

$$x = N^{-1/3} - d \tag{7}$$

It then follows that $x \approx 440$ μm, which can be regarded as a typical distance over which histidine must diffuse before it meets a colony. The time necessary to halve the concentration difference is:

$$t_{1/2} = x^2/D \tag{8}$$

in which D is the effective diffusion coefficient, which may be taken as 10^{-10} m^2 s^{-1} for small molecules in cheese (19). Under these conditions, $t_{1/2}$ = 32 minutes, in other words it takes about half an hour to halve the concentration difference over 440 μm.

In fact, the above calculation is for a one-dimensional case. In a cheese, a three dimensional case, $t_{1/2}$ will be lower than 32 minutes. Considering the time scale of ripening (months) we may conclude that diffusion of precursor is not rate limiting for the production of histamine (or any other amine).

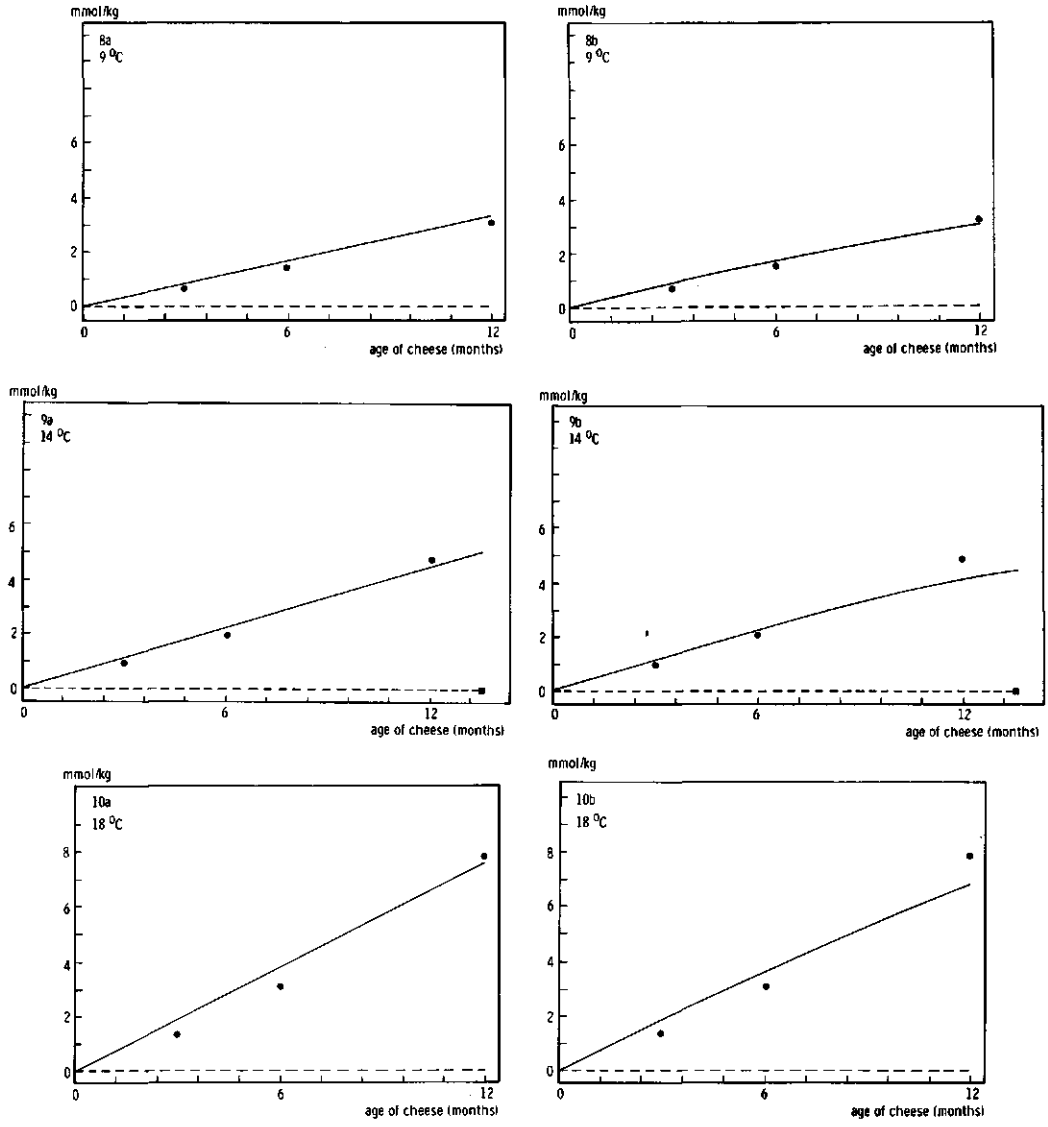
Then there is, of course, the effect of intracellular diffusion on the reaction rate; this effect is actually included in the reaction rate constant k_2. We assume this to be not rate determining inside the cells.

The results of the simulation are to be compared with the experimentally determined values. These were presented in a previous paper (Table 3 of Reference 5); these were again the results from analysis of sector samples including the rind. From the results given in Table 1 of Reference 1 it can be deduced that the values in the middle are about 1.4 times as high as the average

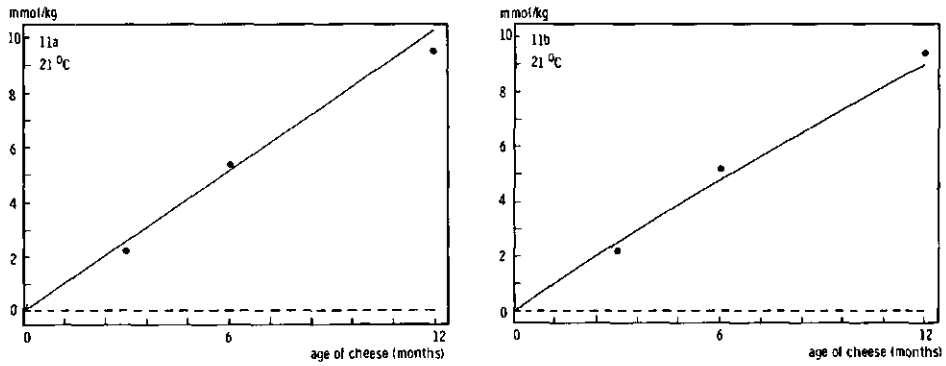
values of sector samples, hence we should multiply the values of Table 3 of Reference 5 by a factor 1.4.

Figures 8-11 compare the values calculated with the above model with the experimentally determined values for the center of the cheese. On the whole, the agreement is good. The results of the calculation show that the histidine values must remain very low, in agreement with the value found for histidine (<0.03 mmol/kg) in the center of an infected Gouda cheese (Table 1). In fact, the model shows that (in the center of the cheese) the production of histidine is rate determining: all histidine formed is converted to histamine. The agreement with experimental results does not improve, in most cases even becomes worse, if a finite value for k_3 is introduced; in other words it is not very likely that histamine is further degraded.

Let us now consider the formation of histamine in the rind. It can be deduced from the data in Table 1 that the concentrations of amino acids in the rind were, on average, 0.61 × those in the middle. However, since the concentration of amino acids is higher in the center than in the rind, a net migration will take place from inside the cheese to the rind because of diffusion. It takes about 100 days to halve the concentration difference over 3 cm (cf Eqn (8) with $D = 10^{-10}$ m^{2}s^{-1}). In the outer rind (with a thickness of, say, 5 mm), the water content may be as low as 16 % and, consequently, D is much lower, say 5×10^{-12} m^{2}s^{-1} (20). Hence, it takes another 60 days to halve the concentration difference over the outer 5 mm. Hence, because of diffusion, the proteolysis rate in the rind relative to the center is even lower than indicated by the factor 0.61 derived above. This diffusion effect makes it difficult to compare the experimentally determined values for histidine (0.61 mmol/kg) and histamine (3.8 mmol/kg) with the results of the model for the rind. Since the concentration of histidine in the center of the cheese is very low, its concentration in the rind would have been higher than 0.61 mmol/kg if diffusion did not occur. Therefore, if we apply the model (which neglects diffusion) to the rind we should find a lower value for histamine than 3.8 mmol/kg and a higher value for histidine than 0.61, but we cannot calculate how much higher or lower under the present circumstances. Calculations with the kinetical model showed for $0.5 \times k_1$ (which is less than $0.61 \times k_1$ as deduced above) and $0.4 \times k_2$ (see Table 1) almost no accumulation of histidine and about 2.5 mmol histamine per kg after 13.5 months (Fig. 12). In order to fulfil the requirement that the concentration of histidine be higher than 0.61 mmol/kg and that of histamine lower than 3.8 mmol/kg we had to adjust the value of k_1 to about $0.7 \times k_1$ and that of k_2 to about $0.1 \times k_2$ (Fig. 13). The model thus predicts that the rate of formation of histidine in the rind is higher than found for most of the other amino acids, and that the decarboxylase activity in the rind is

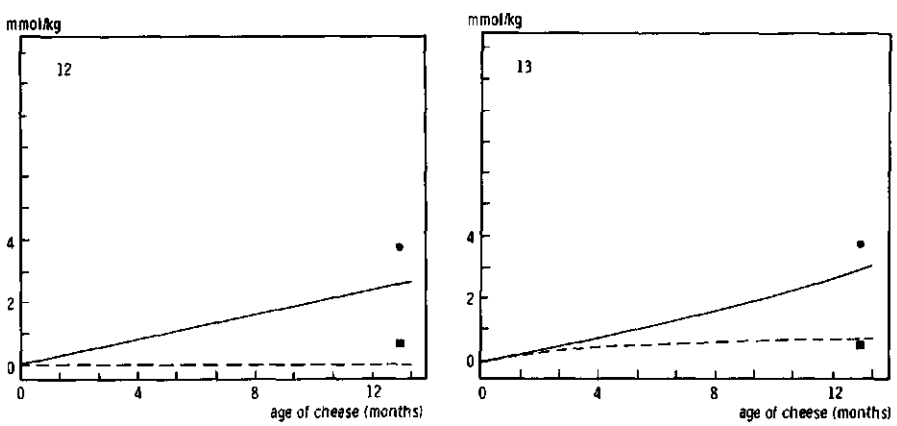


Figs. 8-11. Histidine and histamine concentrations in the center part of Gouda cheese, infected with *L. buchneri* St2A, stored at various ripening temperatures. —— values for histamine, obtained from calculations with the kinetic model; – – – values for histidine, obtained from calculations with the kinetic model; ● experimentally determined histamine concentration; ■ experimentally determined histidine concentration.
The values for k_1 and k_2 (histidine resp. histamine formation) are from Table 2. In Figs. 8a, 9a, 10a and 11a histamine degradation was assumed not to occur ($k_3 = 0$). In Figs. 8b, 9b, 10b and 11b a value of 0.02 for k_3 was introduced.



lower than found by experiment. The latter phenomenon is not very surprising because the circumstances in the rind differ substantially more from the circumstances in the decarboxylase assays, than do the circumstances in the center of the cheese. It may be that in the rind the effect of diffusion on the reaction rate itself comes into play, as a result of which k_2 must be lowered (remember that k_2 includes the effect of diffusion on the reaction rate).

We must admit that we do not have a full explanation for the differences in



Figs. 12-13. Histidine and histamine concentrations in the rind (8 mm) of Gouda cheese, infected with *L. buchneri* St2A, stored at 14 °C. — values for histamine, obtained from calculations with the kinetic model; – – – values for histidine, obtained from calculations with the kinetic model; ● experimentally determined histamine concentration; ■ experimentally determined histidine concentration.

In Fig. 12 the values for k_1, k_2 and k_3 were 0.19, 1.24 and 0 and in Fig. 13 k_1, k_2 and k_3 were 0.27, 0.31 and 0 respectively (see text).

histamine and histidine content as observed in the center and the rind of the cheese. Of the possible influences of difference in salt content, oxygen pressure, carbon dioxide content, water content and ionic strength, we consider the influence of the difference in water content to be the most important factor, because the magnitude of the diffusion coefficient so strongly depends on it. Nevertheless, the present model shows that histidine and histamine can both be present if the enzymatic activity (k_2) is not too high, and this apparently is the case in the rind of the cheese.

In conclusion we may state that the fairly simple model for histamine production, as presented in this paper, adequately describes the situation in cheese, if excluding the outermost layer.

Using the model it is possible to predict the effect of enzymatic activity, and hence that of the number of decarboxylating bacteria, on the level of histamine in cheese. Fig. 14 shows the result of such calculations. It appears that above a level of about 10^8 St2A/g, the enzymatic activity has no longer an effect on the histamine concentration, obviously because the formation of precursor, histidine, has then become rate determining. Fig. 14 thus clearly indicates that decreasing the number of decarboxylating bacteria and prevention

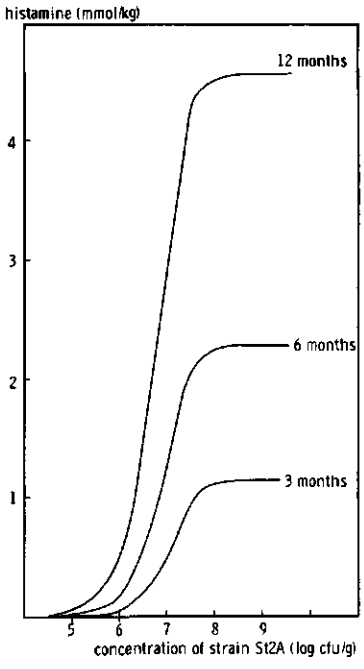


Fig. 14. Histamine formation in the center part of Gouda cheese, in relation to the density of *L. buchneri* St2A, as calculated with the kinetic model.

of excessive proteolysis are essential to reduce the risk of histamine formation.

Acknowledgement

This work was supported by the stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, the Netherlands. The authors thank Prof. Dr Ir P. Walstra (Wageningen Agricultural University) and Dr Ir J. Stadhouders (NIZO) for stimulating discussions on this subject. We also wish to thank P. J. van Rooijen for performing the amino acid analysis and M. v. Schaik for his assistance with the radiometric experiments.

Samenvatting

H. M. L. J. Joosten en M. A. J. S. van Boekel, *Omstandigheden waaronder biogene aminen in kaas kunnen worden gevormd. 4. Onderzoek aan de kinetiek van de vorming van histamine in een besmette Goudse kaas**

Kaas werd bereid uit melk die opzettelijk besmet was met histidine decarboxylerende lactobacillen (St2A). Tijdens de rijping van deze kaas werden regelmatig monsters genomen om na te gaan hoe de concentratie van deze bacterie zich ontwikkelde. Tevens werden de decarboxylase-activiteit en de histidine- en histamineconcentratie bepaald. Nagegaan werd in hoeverre de enzymactiviteit beïnvloed wordt door omgevingsfactoren als pH, temperatuur en substraat- en productconcentratie.

Uit de proeven bleek dat de decarboxylase-activiteit na ongeveer 40 dagen haar hoogste waarde bereikte. Nadien daalde de activiteit. Histidine was als vrij aminozuur nauwelijks aanwezig in de kaas. Het werd alleen in het randgedeelte aangetroffen (0.6 mmol per kg). In het midden van de kaas lag het gehalte onder de detectiegrens (0.03 mmol/kg). De histamineconcentratie steeg tijdens de rijping. In het midden van de kaas was het gehalte juist hoger dan in de rand (5.8 en 3.8 mmol per kg na 13½ maand rijping).

Van een kinetisch model dat werd opgesteld voor de vorming van histamine werd nagegaan of het de situatie in deze kaas adequaat beschrijft. Dit geschiedde door de experimenteel gevonden waarden voor histamine- en histidineconcentraties te vergelijken met de waarden die op basis van berekeningen met het model verwacht mochten worden. Hiertoe moesten wel enige veronderstellingen worden gemaakt. Zo werd bijvoorbeeld gesteld dat de mate waarin histidine wordt vrijgemaakt in een met St2A besmette kaas, even groot is als in normale, onbesmette kaas. Er kon worden geconstateerd dat voor wat betreft het middengedeelte van de kaas het kinetische model goed voldeed. Uit de berekeningen volgde tevens dat het volgens dit model onwaarschijnlijk is dat histamine-afbraak optreedt. Ook bleek dat de histidineconcentratie de beperkende factor is voor de vorming van histamine in deze besmette kaas.

De histidineconcentratie in de rand van de kaas was hoger dan verwacht mocht worden op basis van de berekeningen met het model. Mogelijkerwijs houdt dit verband met het lage vochtgehalte aldaar, omdat dit ondermeer zeer sterk de diffusie beïnvloedt. Daarnaast was in de rand

[1] Dit artikel maakt deel uit van een serie artikelen over biogene aminen die ook wordt verspreid als Verslag V272 van NIZO te Ede (1988).

echter ook het aantal lactobacillen en de decarboxylase-activiteit lager.

Met behulp van het model werd tevens nagegaan hoeveel minder histaminevorming verwacht mag worden in kaas met een geringere besmetting. Uit deze berekeningen bleek dat bijvoorbeeld bij een maximale concentratie van 10^6 cfu/g een gehalte van hooguit 0.5 mmol/kg gevonden kan worden na 1 jaar rijping.

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The biogenic amine contents of Dutch cheese and their toxicological significance*

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Summary

A survey is given of the literature about the toxicological actions of biogenic amines, with special reference to their presence in cheese. In most cases consumption of food containing biogenic amines does not lead to intoxication because amine destroying enzymes in the digestive tract prevent the uptake of these potentially hazardous compounds in the blood stream. As much as 1 mmol histamine and 3 mmol tyramine can be consumed without noticeable effects. However, when amine degradation is impaired, smaller quantities may cause food poisoning. Histamine destroying enzymes for example can be inhibited by concomitant ingestion of putrescine and cadaverine. It seems likely that such potentiation of histamine toxicity is involved in many cases of food poisoning. Tyramine toxicity is greatly increased by the (unselective) MAO inhibitors, but these drugs are hardly used any more because of their serious side effects. The toxic threshold doses of putrescine, cadaverine and tryptamine are not known, nor at which levels they can potentiate the actions of other amines. Phenylethylamine may be an important dietary factor in migraine, as little as 0.03 mmol can precipitate attacks in susceptible persons.

Results are presented of an investigation of the biogenic amine content of 63 samples of Dutch cheese. Innocuous quantities were found in three month old samples made from pasteurized milk.

Rather often biogenic amines were detected in cheese made from raw milk. Especially in mature cheese made from raw milk the quantities detected were relatively high. Cheese with a good bacteriological quality generally contained smaller amounts than cheese infected with many non-starter bacteria.

1 Introduction

In previous reports it was described under which circumstances the formation of biogenic amines in Dutch cheese can take place (1-3). It was found that amines are formed only by a contaminating microflora and that high amounts particularly could be found in heavily contaminated cheese with excessive

* This article is part of a series on biogenic amines, which is also distributed as NIZO Research Report V272 (1988).

proteolysis (3). To investigate the concentration of biogenic amines actually present in Dutch cheese, various lots of cheese were analyzed by the HPLC method described earlier (4).

The ingestion of amine containing foods may cause food poisoning (5-8), but the literature dealing with the toxicity of these amines is rather complex and sometimes presents conflicting results. Therefore the most important toxicological and pharmacological actions of various amines are reviewed here, with special reference to their presence in cheese.

2 Pharmacological and toxicological aspects of the presence of biogenic amines in cheese

2.1 Introduction

Tyramine, histamine, putrescine, cadaverine, tryptamine and phenylethylamine are generally considered to be the most important biogenic amines occurring in cheese, and therefore this review will cover only these six compounds. Recently, Zee et al. (9) reported the presence of large amounts (up to 4 mmol/kg) of adrenaline and noradrenaline in Cheddar cheese. Endogenous noradrenaline plays an important role in the 'cheese reaction' (see section 2.3), but food poisoning caused by the consumption of cheese has never been associated with the presence of these catecholamines. For this reason adrenaline and noradrenaline will not be discussed here.

Before discussing the toxicity of the individual amines in detail, it is important to emphasize at this point that harmful effects resulting from the consumption of food rich in biogenic amines can be expected only when these amines gain access to the bloodstream. This latter, however, is normally prevented by the action of several defence mechanisms of the body, of which mono- and diamino-oxidases situated in the digestive tract are the most important.

2.2 Histamine

2.2.1. Pharmacological actions of histamine. Histamine is a normal constituent of the body where it mediates several important functions (10, 11). It is formed from histidine by a pyridoxal phosphate dependent decarboxylase. Mast cells and blood basophils contain large amounts of histamine in which it is stored in special granules. For example, with an allergic reaction this histamine is released into the bloodstream. The principal pharmacological activities of histamine were already described in the period 1910-1919 in a series of

papers by Dale & Laidlaw, but even nowadays much research is still being carried out on the elucidation of its functions and mode of action in the body.

Histamine exerts its effects by binding to receptors on cellular membranes. At least two types of receptors are known to exist, which are distinguishable because of their different sensitivity for antihistaminica. Histamine receptors are found particularly in the cardiovascular system. Interaction of histamine with these receptors results in arteriolar dilatation in man and related species, whereas in some other species arteriolar constriction is observed. Furthermore, capillary permeability is increased, leading to leakage of plasma into the tissues and to haemoconcentration. Increase of the rate and strength of the heart-beat also belongs to the actions caused by histamine.

Histamine receptors are also found in various secretory glands. Gastric acid secretion is stimulated by histamine. To a lesser extent, the secretion of the pancreas, the intestine and the bronchii respond to histamine. Histamine liberates adrenaline and noradrenaline from the suprarenal gland. It also excites the smooth muscles of the uterus, the intestine and the respiratory tract.

In addition, histamine is probably a neurotransmitter in the central nervous system. It stimulates sensory and motor neurons, which explains the pain and itching felt by patients suffering from a histamine food poisoning (12).

Apparently, histamine is a very important and potent metabolite, and it is not surprising that its concentration is strictly regulated.

2.2.2 Toxicological actions of histamine. Orally ingested histamine normally does not influence the histamine levels in the various body tissues. Its uptake into the bloodstream is prevented by several defence mechanisms (see 2.2.3). Only when these systems fail to counteract all the ingested histamine, for example because a very high dose is ingested or because histamine metabolism is impaired by other toxic substances, will orally administered histamine cause poisoning (13, 14). The disease, sometimes also referred to as scombroïd poisoning, as consumption of fish from the *Scombroïdae* family is often implied (15), will become manifest several minutes to three hours after ingestion of the histamine containing food. At first, a flushing of the face and neck is usually observed, accompanied by a feeling of great heat and general discomfort. Often this is followed by an intense throbbing headache. Other symptoms may be: cardiac palpitations, dizziness, faintness, itching, rapid and weak pulse and gastrointestinal complaints (abdominal cramps, nausea, diarrhoea). In severe cases shock, bronchospasms, suffocation and severe respiratory distress are reported (12). However, the illness usually has a mild character and symptoms do not last long. In fact, in many individual cases the illness probably is not recognized as such (13).

It is also possible that the symptoms are falsely attributed to food allergy, because this affection has several characteristics in common with histamine poisoning (allergic reactions are mediated by histamine). However, in the case of food poisoning the attack rate can be as high as 100 %, while with food allergy the most of the consumers are not affected. So if the morbidity is high and if also large amounts of histamine in the incriminated food are detected, histamine food poisoning is diagnosed. In addition, negative skin prick tests and low IgE levels may help to distinguish it from allergy (16-18).

2.2.3 Histamine metabolism and potentiation of histamine toxicity. Histamine can be degraded by several enzymes (Fig. 1). The formation of N-methylhistamine, catalysed by a specific methyl transferase, and the formation of imidazole acetic acid by diamine oxidase (DAO, also referred to as histaminase) are the most important pathways (5, 12, 13, 19).

When administered orally, doses of up to 1 mmol histamine do not cause symptoms of food poisoning in normal healthy persons (20). On the other hand, only 0.07 μmol, injected intravenously, results in vasodilatation and increased heart rate. This demonstrates the importance of the histamine-metabolizing enzymes, which are particularly active in the digestive tract. In the literature many cases of food poisoning are reported that were attributed to the presence of histamine. However, the histamine contents of most of these foods do not readily explain its apparent toxic effects, as the doses ingested often remained well under 1 mmol. This controversy is now explained by the finding that the foods associated with food poisoning may also contain other substances that potentiate the action of histamine (21, 22).

Jung & Bjeldanes (23) postulated that the main potentiating effect was an increased uptake of histamine from the digestive tract. This was believed to

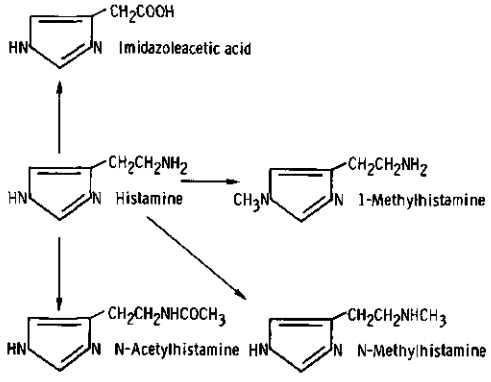

Fig. 1. Histamine metabolism.

be the result of inhibition of the histamine-binding capacity of mucin (24). There are several considerations that render this theory less plausible. Firstly, the mucin content of the intestinal tract is so high that significant inhibition is hardly imaginable. Secondly, this theory does not explain the difference in the ratio of undigested histamine to its metabolites in the urine between test animals receiving only histamine and those receiving both histamine and potentiating compounds (19). The latter observation fits better with the hypothesis developed by Taylor et al. (12) explaining the potentiating effect by inhibition of the histamine metabolizing enzymes. Inhibition could indeed be demonstrated by experiments in vitro. Amongst the inhibitors of methyl transferase or DAO were tyramine, putrescine, cadaverine and phenylethylamine (25, 26). Mono-amine oxidase inhibitors (see section 2.3.3) were also inhibitory to DAO.

2.2.4 Toxic threshold dose. Motil & Scrimshaw (20) performed experiments in which grapefruit juice and fresh tuna were spiked with histamine and given to volunteers. At a dose of 1.5 mmol mild symptoms, characteristic of scombroïd poisoning, were observed. However, as potentiating factors are probably common in cases of histamine food poisoning, it is impossible to determine which is the minimal histamine level in food to be considered as toxic. It is clear that more research has to be done to elucidate the quantitative effects of the concomitant ingestion of compounds such as putrescine and cadaverine. Constructing an overall biogenic amine index may be a valuable tool for the establishment of regulatory limits. At this moment in several countries only the histamine levels of some fish and fishery products are regulated by the authorities. The U.S. Food and Drug Administration for example has established a hazard action level of 4.5 mmol/kg for histamine in tuna. No such limits have been established for dairy products.

2.2.5 Epidemiology. Many cases of histamine poisoning are probably not recognized as such and in most countries scombroïd poisoning is not a notifiable illness. Therefore it is difficult to estimate how frequently this type of food poisoning really occurs. Nevertheless, it is certain that in most cases fish products are implicated (13, 15, 27). It is striking how many people were involved in some of the episodes. In 1973, for example, 2656 people in Japan suffered from histamine poisoning after consumption of dried horse mackerel.

There are only a few reports of histamine food poisoning caused by the consumption of cheese. In 1967, Gouda cheese containing 8 mmol histamine per kg was implicated in a case of food poisoning in the Netherlands. Only one

person was involved (28). In 1978, 38 people in the USA were reported ill after consumption of Swiss cheese, containing more than 9 mmol histamine per kg (29). A similar case was reported two years later in the USA: this time 6 people were involved and the incriminated (Swiss) cheese contained 16.8 mmol histamine per kg (30).

Besides fish and cheese some other food products such as sauerkraut, chicken, dry sausage and ham may also contain histamine (5). Often wine is mentioned as possibly containing histamine, but in comparison to the other foods, the histamine content is negligible (5, 31, 32). However, alcohol can potentiate the effects of histamine as it facilitates diffusion of amines through the gut wall, and it may also interfere with the degradation of histamine (33). Consequently, a combination of wine and histamine containing cheese may possibly give rise to food poisoning, whereas these foods would not cause any harm when ingested separately.

In addition, alcohol and its degradation product, acetaldehyde, may directly degranulate mast cells and likewise elicit symptoms that are also seen with histamine food poisoning. These effects, however, are beyond the scope of this study.

2.3 Tyramine

2.3.1 Pharmacological actions of tyramine. Unlike histamine, tyramine is not an important metabolite in the body and it is generally present at a very low concentration. When tyramine is injected intravenously it acts mainly indirectly by releasing noradrenaline from the sympathetic nervous system. Noradrenaline is a neurotransmitter in this system. It is stored in vesicles in the neurons. On excitation, noradrenaline is released in the synaptic cleft where it can interact with receptors on the postsynaptic membrane. Since many body functions are controlled by the sympathetic nervous system, tyramine ingestion may lead to a variety of physiological reactions. Increase of the blood pressure, by peripheral vasoconstriction and by increasing the cardiac output, is the most prominent effect of tyramine-induced noradrenaline release.

Tyramine also dilates the pupils, dilates the palpebral tissue, causes lacrimation and salivation and increased respiration. At high doses the blood sugar level is increased (5, 12).

2.3.2 Tyramine metabolism. In man oxidative deamination to p-hydroxyphenylacetic acid is probably the main degradative pathway (Fig. 2). It is catalyzed by monoamine oxidase (MAO), which is present in almost every organ.

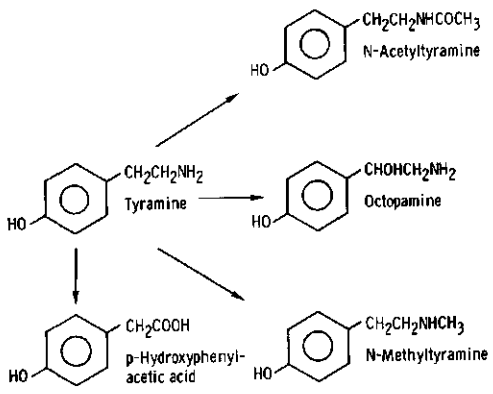

Fig. 2. Tyramine metabolism.

Type A and type B monoamine oxidases can be distinguished. Either type can degrade tyramine.

Other degradative pathways are oxidation to octopamine by dopamine-β-hydroxylase and methylation to N-methyltyramine by N-methyltransferase. Conjugation with either sulphate or acetate groups is also possible (34). Because these degrading enzymes are particularly active in the digestive tract, the toxicity of orally administered tyramine is low (35).

2.3.3 MAO inhibitors and the cheese reaction. In 1957 Crane (36) reported that the MAO inhibitor ipronazide, a drug at that time in use as tuberculostaticum, also had antidepressive properties. Thereafter many other MAO inhibitors were developed and prescribed mainly as antidepressives or as antihypertensives.

Because these drugs inhibit MAO in the mitochondria of sympathetic neurons, patients receiving MAO inhibitors accumulated large amounts of noradrenaline in these cells (MAO also regulates the noradrenaline content of these neurons). Therefore, the patients became very susceptible to the toxic actions of tyramine. Because MAO in the digestive tract is inhibited as well, ingestion of food rich in tyramine resulted in a dangerous intoxication, known as the 'cheese reaction': a hypertensive crisis, usually accompanied by a severe headache. This sometimes led to intracranial haemorrhage, neuronal sequelae, cardiac failure and pulmonary edema. Fatal incidents were also reported (37).

However, not only cheese but also other types of food such as marmite, pickled herring and dry sausage could bring about these side effects of the use of MAO inhibitors (38). As other and safer alternatives of these drugs became available, the use of MAO inhibitors was abandoned. Recently howev-

er, a new class of MAO inhibitors has been developed. Unlike the conventional type, these new drugs show selective inhibition towards type A or type B monoamine oxidases (39). Tyramine is degraded by MAO A as well as by MAO B. Treatment with the selective inhibitors does not result in completely impaired tyramine metabolism. In fact there are even reports of an antagonistic effect of L-deprenyl (a MAO B inhibitor) on the noradrenaline-releasing activity of tyramine, but the biochemical background of this unexpected phenomenon has not yet been resolved (40).

2.3.4 Tyramine and migraine. Soon after it was discovered that the presence of tyramine in food could cause severe complications in patients receiving MAO inhibitors, it was also observed that many of the foods eliciting the cheese reaction were also mentioned by migraine patients as precipitants of their attacks. Hanington et al. investigated the etiological role of tyramine in migraine by giving capsules with 100 mg (0.7 mmol) tyramine and capsules with 100 mg lactose on separate occasions to migraine sufferers claiming a dietary history. They observed a high attack rate after tyramine ingestion, but not so after ingestion of the placebo. It was also found that migraine sufferers had a decreased capacity to degrade tyramine. This led to the hypothesis that this low activity of the tyramine-metabolizing enzymes might be an important factor in the etiology of migraine (41). However, other investigators disputed some of the arguments (42). In the first place, there is no clinical similarity between the headache precipitated by tyramine in patients receiving MAO inhibitors and migraine headaches. Furthermore it was argued that chocolate, the type of food most often mentioned by migraine sufferers to cause their disease, did not contain any tyramine. In 1974, Ryan (43) investigated the role of tyramine in another and larger group of migraine patients. His results show that tyramine probably does not play a role as etiological factor in migraine (43).

2.3.5 Other disorders attributed to tyramine. In 1984 Schultz (44) reported on a patient whose regular consumption of rather large quantities of mature cheese was associated with polyneuropathy. Recently, Jacob & Carron (45) related attacks of palpitations and dyspnoea with the ingestion of food rich in tyramine. These are two examples of, probably several, reports in which tyramine-containing food is incriminated. However, in these two articles no substantial proof of the etiological role of tyramine (excluding the other amines) was given, neither were the actual amine contents of the foods implicated determined. The reason why tyramine is mentioned is probably due to tyramine causing such serious side effects at times when MAO inhibitors were still in

use. The toxic threshold level for this amine is probably so high that it is unlikely that it can do any harm in persons not using these drugs. However, the possibility that some individuals are more susceptible than others cannot be ignored.

Recently, tyramine was also investigated as a possible mutagenic precursor (46, 47). A reaction product of tyramine and nitrite, 3-diazotyramine, induced oral cavity cancer in rats. It was postulated that this mutagenic compound may be formed in the stomach: incubation of 5 mM tyramine and 50 mM nitrite at 37 °C and a pH of 1 to 2 for 60 minutes led to significant amounts of 3-diazotyramine. However, the nitrite concentration employed was extremely high and the reaction product has never been demonstrated in the stomach.

2.3.6 Toxic threshold dose and epidemiology. Grind et al. (35) examined the pressor response of orally administered tyramine. Various doses were given to 12 healthy persons not receiving any other drugs. No significant increase in systolic blood pressure occurred until a dose of 3 mmol was ingested (35). Although it was also noted that susceptibility to tyramine varied even within the individual, these results make clear that very large amounts of food rich in tyramine must be ingested before toxic effects can be expected. However, the possibility exists that in this case also food related factors potentiate its toxicological actions. Nevertheless, apart from the episode during which the conventional MAO inhibitors were widely used, food poisoning unambiguously caused by the consumption of tyramine rich food has not been reported.

2.4 Putrescine and cadaverine

Both putrescine and cadaverine have less pharmacological activity than the aromatic amines. Putrescine is associated with the synthesis of nucleic acids and protein. High concentrations can be found in actively growing tissues such as germinating plant seeds (7).

Only after ingestion of very large amounts of putrescine or cadaverine are toxic effects observed. Intoxication symptoms reported are hypotension, bradycardia, dyspnoea, lockjaw and paresis of the extremities (12).

The most important consequence of the presence of these compounds in food is probably the potentiation of the toxicity of other amines (13). Hui (19) demonstrated the potentiating effect by in vivo experiments in rats. At doses of 2.0 mmol cadaverine and 0.5 mmol histamine per kg body mass, more unmetabolized histamine was recovered from the urine than when only histamine was administered. It is not known at which levels the potentiating ef-

fect in man can be expected (19).

Putrescine and cadaverine can be degraded by diamine oxidase and possibly also by monoamine oxidase (12). There are no reports of cases of food poisoning caused by the sole presence of putrescine or cadaverine in the ingested food.

2.5 Phenylethylamine

Pharmacologically, phenylethylamine resembles tyramine as it is also a neurosympathomimetic amine. This means that its effects are mainly exerted indirectly by releasing noradrenaline from sympathetic neurons (12). Oxidative deamination by type B MAO is the most important degradative pathway (48).

During the time that MAO inhibitors were still widely used, hypertensive crises occurring as a side effect were in some instances also attributed to the ingestion of foods containing phenylethylamine. However, this only concerned a few cases, since the presence of phenylethylamine in food is not so frequently observed as that of tyramine.

In addition to its sympathomimetic action, phenylethylamine has also been associated with migraine. Doses as low as 0.03 mmol were reported to precipitate a headache in persons not receiving any other drugs (31, 48). It is well known that chocolate may contain phenylethylamine and this may explain why this type of food is so often mentioned by migraine sufferers as a dietary trigger of their attacks. It may be noted that cheese rich in tyramine quite often also contains some phenylethylamine (3). This results from the fact that tyrosine decarboxylase also shows a weak decarboxylating activity towards phenylalanine. Perhaps this explains why tyramine-rich cheese has been associated with migraine.

Urinary phenylethylamine excretion is increased in periods of stress (49). High concentrations were also found in urine samples of paranoïd chronic schizophrenes, but not so with non-paranoïd chronic schizophrenes (50). Observations like these make clear that phenylethylamine may well be an important endogenous metabolite. However, it remains as yet unresolved whether dietary factors influence its effective concentration in the brain.

2.6 Tryptamine

Tryptamine is a neurosympathomimetic amine, but it can also exert direct effect on smooth muscles and thereby increase the blood pressure. It is destroyed by MAO (as well by type A as by type B (51)). There are no reports of

tryptamine intoxication or of hypertensive crises due to tryptamine, probably because foods never contain large quantities.

2.7 Conclusion

Ingestion of biogenic amines may result in food poisoning. When these compounds are separately administered in their pure form, toxic effects have to be reckoned with after ingestion of more than 100 mg (approx. 1 mmol) histamine or more than 400 mg (approx. 3 mmol) tyramine. The threshold doses for putrescine, cadaverine and tryptamine are not known. As little as 3 to 4 mg (approx. 0.03 mmol) phenylethylamine may precipitate migraine attacks in susceptible persons. The toxicity of these substances may be altered by potentiating effects of other food constituents.

3 Some observations on the biogenic amine content of Dutch cheese

3.1 Introduction

The aim of this study was to find out whether Dutch cheese may contain high amine contents. Most cheeses produced in the Netherlands are made from pasteurized milk, and amine formation in these is unlikely (1). Therefore a selection was made: only cheese made from raw milk and cheese made from pasteurized milk but still containing more than 10 million lactobacilli per gram was investigated.

The chemical and bacteriological quality of cheese produced in the Netherlands is regulated by the authorities. Moreover, many of the producers of cheese made from raw milk have 12 days old cheese samples subjected to an additional examination which is effected by the Netherlands controlling authority for milk and milk products in Leusden. To obtain the predicate 'good quality', the following requirements have to be met: less than 5×10^4 cfu *Enterobacteriaceae* and less than 5×10^6 cfu lactobacilli per gram of cheese, the pH should lie between 5.2 and 5.4, the nitrate content must not be lower than 25 mg/kg, nor should it be higher than 50 mg/kg. The water content must be lower than 39 % and the fat content higher than 48 %. The bacteriological quality is called 'doubtful' when 5×10^4 to 5×10^5 cfu *Enterobacteriaceae* or when 5×10^6 to 5×10^7 cfu lactobacilli are present per gram of cheese and 'inferior' when higher densities of these bacteria are found. If producers have problems in meeting these requirements, they can get advice on how to improve the quality of their cheese.

We investigated whether this control system could also contribute to re-

duce the risk of amine formation. Therefore 30 cheeses from 30 different producers were analyzed after 12 days by the Netherlands controlling authority for milk and milk products in Leusden. After 3 months of ripening, the biogenic amine contents were determined.

In addition 9 cheeses with excessive gas production and 15 more mature cheeses, also made from raw milk, were analyzed.

3.2 Materials and methods

Samples. A cheese factory provided 9 samples of Gouda and Edam cheese, made from pasteurized milk and containing more than 10^7 lactobacilli per gram. The latter was confirmed at our Institute with TGV 5.4 agar (52).

The cheeses made from raw milk were obtained from various suppliers.

Chemical analysis. Biogenic amine contents were determined as described earlier (4).

3.3 Results and discussion

Relatively low biogenic amine contents were found in the cheeses made from pasteurized milk even though they contained large numbers of lactobacilli (Table 1, nos. 1-9). Tyramine was detected in one sample at a level of 1.4 mmol/kg and in another cheese 0.5 mmol/kg was found. The results show that contamination of the cheese with lactobacilli led to the formation of biogenic amines only in a few cases. In cheese made from raw milk (Table 1, nr. 10-63), biogenic amines were more often detected than in cheese made from pasteurized milk. Thirty 12 day-old samples (Table 1, nr. 10-39) were investigated by the Netherlands controlling authority for milk and milk products (Leusden). Of these, 17 samples had a good quality, 12 samples had a doubtful bacteriological quality and one sample had an inferior quality because it contained more than 5×10^7 cfu lactobacilli per gram cheese. To obtain the predicate 'good quality', the control institute also puts limits to the nitrate and moisture content and the pH, but these aspects were not considered in this investigation.

After 3 months of ripening, the biogenic amine contents were determined in these cheeses. Of the 17 cheeses with a good quality, 9 did not contain detectable amounts of biogenic amines. The maximum amounts detected in the other 8 samples were 1.2 mmol/kg for tyramine, 0.4 mmol/kg for histamine and 1.1 mmol/kg for cadaverine. Tryptamine and phenylethylamine were not found. The 12 cheeses (nos. 27-38) with a doubtful quality contained up to 2.8

mmol tyramine per kg, 1.5 mmol histamine, 0.5 mmol putrescine and 0.7 mmol cadaverine per kg. Only two of these did not contain detectable amounts of biogenic amines. The sample with the inferior quality contained 3.2 mmol tyramine, 1.2 mmol histamine and 1.0 mmol putrescine per kg. These results make clear that the estimation of the bacteriological quality may be a valuable tool to reduce the risk of amine formation.

In addition to these 30 cheeses, 9 other three months old samples of cheese made from raw milk were analyzed (nos. 40-48). They were selected because of their excessive gas production, which probably resulted from massive growth of undesired non-starter organisms. Up to 2.4 mmol tyramine, 2.2 mmol histamine, 4.4 mmol putrescine and 1.8 mmol cadaverine per kg were detected. In one cheese phenylethylamine was found at 0.4 mmol/kg.

The older cheeses made from raw milk sometimes contained relatively high amounts of biogenic amines. In the 9 month old cheese up to 6.6 mmol tyramine, 6.4 mmol histamine, 6.7 mmol putrescine and 3.6 mmol cadaverine per kg were detected. One sample contained phenylethylamine (0.4 mmol/kg). The highest contents were found in the 23 months old cheeses: one sample contained both tyramine (8.0 mmol/kg), histamine (10.2 mmol/kg) and phenylethylamine (1.4 mmol/kg). The reason the old cheese contained more biogenic amines must be more extensive proteolysis, causing a higher concentration of free amino acids.

It is clear that tyramine is the most frequently encountered amine, but histamine, putrescine and cadaverine were all found fairly often as well in cheese made from raw milk. Tryptamine was not detected and phenylethylamine only at low concentrations.

Because our main interest was to find cheeses with a high amine content, this survey particularly deals with cheese made from raw milk. It does not give a representative picture of the amine content of Dutch cheese, as most of this is made from pasteurized milk.

Other investigations have shown that some cheese varieties may contain higher amine concentrations (53, 54). In recently analyzed samples of blue veined cheeses for example, de Boer & Kuik (55) detected up to 18.6 mmol tyramine, 17.2 mmol histamine, 14.1 mmol putrescine and 41.9 mmol cadaverine per kg. We were able to confirm these data by analyzing the same samples with the HPLC method employed at our Institute.

At this moment it cannot be said to what extent the presence of these quantities of biogenic amines is a public health hazard. Clearly, more toxicological research has to be done first. However, susceptible persons may be advised to consume cheese made from pasteurized milk, and which is not very mature.

Table 1. Biogenic amines in various cheeses.

Sample	Cheese variety	Age (months)	Biogenic amine contents (mmol/kg)[1]						Remarks	
			TA	HA	PTR	CAD	TPA	PHEA		
1	Gouda	3	–	–	0.3	–	–	–	PM[2],	1×10^7 Lb./g[3]
2	Gouda	3	–	–	–	0.4	–	–	PM,	4×10^7 Lb./g
3	Edam	3	1.4	–	0.2	–	–	–	PM,	3×10^7 Lb./g
4	Edam	3	–	–	–	–	–	–	PM,	4×10^7 Lb./g
5	Gouda	3	–	–	–	–	–	–	PM,	2×10^7 Lb./g
6	Gouda	3	–	–	–	–	–	–	PM,	2×10^7 Lb./g
7	Gouda	3	0.5	–	–	–	–	–	PM,	2×10^7 Lb./g
8	Gouda	3	–	–	–	–	–	–	PM,	2×10^7 Lb./g
9	Gouda	3	–	–	–	–	–	–	PM,	2×10^7 Lb./g
10-18	Gouda	3	–	–	–	–	–	–	RM[2],	good quality[4]
19	Gouda	3	1.2	–	–	0.6	–	–	RM,	good quality
20	Gouda	3	0.3	–	–	–	–	–	RM,	good quality
21	Gouda	3	0.3	–	–	–	–	–	RM,	good quality
22	Gouda	3	0.3	0.4	–	–	–	–	RM,	good quality
23	Gouda	3	0.3	–	–	–	–	–	RM,	good quality
24	Gouda	3	–	–	–	1.1	–	–	RM,	good quality
25	Gouda	3	0.3	0.2	–	0.6	–	–	RM,	good quality
26	Gouda	3	0.3	0.2	–	0.5	–	–	RM,	good quality
27	Gouda	3	–	–	–	–	–	–	RM,	doubtful quality[4]
28	Gouda	3	2.2	0.4	–	0.3	–	–	RM,	doubtful quality
29	Gouda	3	0.4	0.2	–	–	–	–	RM,	doubtful quality
30	Gouda	3	0.6	0.9	–	–	–	–	RM,	doubtful quality
31	Gouda	3	0.2	–	–	0.3	–	–	RM,	doubtful quality
32	Gouda	3	1.8	–	–	–	–	–	RM,	doubtful quality
33	Gouda	3	0.7	0.2	–	0.7	–	–	RM,	doubtful quality
34	Gouda	3	–	0.2	–	–	–	–	RM,	doubtful quality
35	Gouda	3	2.8	1.5	0.5	–	–	–	RM,	doubtful quality
36	Gouda	3	0.3	0.3	–	0.3	–	–	RM,	doubtful quality
37	Gouda	3	–	–	–	–	–	–	RM,	doubtful quality
38	Gouda	3	1.6	–	0.3	0.4	–	–	RM,	doubtful quality
39	Gouda	3	3.2	1.2	1.0	–	–	–	RM,	inferior quality

Table 1. (Continued)

Sample	Cheese variety	Age (months)	Biogenic amine contents (mmol/kg)[1]						Remarks	
			TA	HA	PTR	CAD	TPA	PHEA		
40	Gouda	3	2.2	0.3	0.6	–	–	–	RM,	excessive gas production
41	Gouda	3	1.9	0.2	4.4	1.2	–	0.4	RM,	excessive gas production
42	Gouda	3	0.3	0.7	0.2	0.9	–	–	RM,	excessive gas production
43	Gouda	3	2.3	–	1.1	1.8	–	–	RM,	excessive gas production
44	Gouda	3	2.4	2.2	1.1	0.4	–	–	RM,	excessive gas production
45	Gouda	3	2.2	–	0.2	0.2	–	–	RM,	excessive gas production
46	Gouda	3	1.0	–	1.4	1.7	–	–	RM,	excessive gas production
47	Gouda	3	0.4	–	0.5	1.6	–	–	RM,	excessive gas production
48	Gouda	3	1.7	–	–	–	–	–	RM,	excessive gas production
49	Gouda	9	6.6	–	–	–	–	–	RM,	–[5]
50	Gouda	9	0.8	–	3.0	0.7	–	–	RM,	–
51	Gouda	9	6.4	0.2	6.7	3.7	–	–	RM,	–
52	Gouda	9	6.0	6.4	1.4	1.8	–	0.4	RM,	–
53	Gouda	9	2.0	–	0.6	0.4	–	–	RM,	–
54	Gouda	9	1.2	–	0.3	–	–	–	RM,	–
55	Gouda	9	6.2	–	0.3	5.6	–	–	RM,	–
56	Gouda	9	3.9	3.7	0.4	2.4	–	–	RM,	–
57	Gouda	9	0.3	–	–	–	–	–	RM,	–
58	Gouda	9	0.2	1.0	–	1.1	–	–	RM,	–
59	Gouda	23	1.0	–	–	–	–	–	RM[2],	–
60	Gouda	23	2.7	0.3	0.3	0.3	–	–	RM,	–
61	Gouda	23	2.5	–	–	–	–	–	RM,	–
62	Gouda	23	8.0	10.2	0.3	0.4	–	1.4	RM,	–
63	Gouda	23	–	–	–	–	–	–	RM,	–

[1] TA = tyramine, HA = histamine, PTR = putrescine, CAD = cadaverine, TPA = tryptamine, PHEA = phenylethylamine. – = <0.2 mmol/kg for ta, ha, ptr and cad and <0.4 mmol/kg for tpa and phea.

[2] PM = made from pasteurized milk; RM = made from raw milk

[3] The number of lactobacilli was determined after 3 months of ripening.

[4] Bacteriological quality of the 12-day old cheese. Good quality means less than 5×10^4 cfu *Enterobacteriaceae* and less than 5×10^6 cfu lactobacilli per gram of cheese. Doubtful quality means 5×10^4 to 5×10^5 cfu *Enterobacteriaceae* or 5×10^6 to 5×10^7 cfu lactobacilli. Inferior quality: more than 5×10^5 *Enterobacteriaceae* or more than 5×10^7 cfu lactobacilli per gram of cheese.

[5] –: no apparent defects. The samples nos. 49 to 63 were not microbiologically examined.

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Samenvatting

H. M. L. J. Joosten, *Het gehalte aan biogene aminen van Nederlandse kaas en de toxicologische betekenis daarvan**

Dit artikel begint met een overzicht van de literatuur betreffende de toxicologie van biogene aminen. Met name wordt aandacht geschonken aan de vraag of voedselvergiftiging op kan treden ten gevolge van consumptie van kaas welke biogene aminen bevat.

In het algemeen leidt orale opname van deze stoffen niet tot intoxicatieverschijnselen, omdat enzymen in het spijsverteringsstelsel aminen afbreken en aldus de opname van deze potentieel gevaarlijke stoffen in de bloedbaan verhinderen. Gezonde proefpersonen verdragen doses van 1 mmol histamine en 3 mmol tyramine zonder problemen. Als echter de amine afbrekende enzymen niet goed functioneren kunnen kleinere doses al tot voedselvergiftiging leiden. Remming van histamine afbrekende enzymen treedt bijvoorbeeld op bij gelijktijdige consumptie van histamine met putrescine of cadaverine. Waarschijnlijk is er in veel gevallen, waarbij voedselvergiftiging door histamine wordt vermoed, sprake van dergelijke synergistische effecten. De toxiciteit van tyramine kan in belangrijke mate worden vergroot door de zogenaamde MAO remmers, maar deze geneesmiddelen worden bijna niet meer gebruikt vanwege hun ernstige bijwerkingen. Sinds kort zijn er selectieve MAO remmers verkrijgbaar die deze bijwerkingen niet hebben. Het is niet bekend bij welke doses putrescine, cadaverine en tryptamine zelf vergiftigingsverschijnselen teweeg kunnen brengen, evenmin is het bekend hoeveel van deze stoffen nodig is om de toxiciteit van bijvoorbeeld histamine duidelijk te beïnvloeden. Kleine hoeveelheden (0.03 mmol) phenylethylamine kunnen bij daarvoor gevoelige personen migraine-aanvallen opwekken.

Het tweede deel van dit artikel geeft de resultaten van een onderzoek naar het voorkomen van biogene aminen in Nederlandse kaas. Hiertoe werden 63 monsters onderzocht. Volkomen onschadelijke hoeveelheden werden aangetroffen in kaas bereid uit gepasteuriseerde melk. In kaas bereid uit rauwe melk werden biogene aminen vaker aangetroffen. De hoogste gehalten werden gevonden in de extra belegen en oude kazen. Voorts bleek dat indien de besmetting met niet-zuurselbacteriën beneden een aanvaardbaar niveau bleef ook de aminevorming in het algemeen lager was dan in kazen met een slechte bacteriologische kwaliteit.

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Summary

During the ripening of cheese, paracasein is degraded into smaller peptides and amino acids. Decarboxylation of the amino acids by bacterial enzymes gives rise to the formation of biogenic amines. While in most cheeses the content of biogenic amines is low, these compounds sometimes may be found at concentrations which must be regarded as toxicologically hazardous. The main purpose of the underlying investigations was to reveal the conditions that allow the formation of biogenic amines in cheese.

Firstly, a new HPLC method was developed. The analysis is performed by reversed phase ion-pair chromatography. Ninhydrin is used to detect the amines. It is dissolved in the eluent, so an extra reagent pump is not necessary. After passage through the column, the eluent is heated in a special reaction coil and the purple reaction product is detected at 546 nm. The method allows the detection of tyramine, histamine, putrescine, cadaverine, tryptamine and phenylethylamine, with a detection limit of 0.02 mmol per kg cheese. The amino acid histidine can be determined in a separate run, when use is made of a slightly modified eluent composition. Besides cheese, other foods can also be analyzed with this method.

In chapter two, it is described how Gouda-type cheese was made under very hygienic conditions, using aseptically drawn, bactofuged and pasteurized milk. Six different mixed strain starters were employed, covering the majority of the mesophilic starters used in the Netherlands. Non-starter bacteria were virtually absent, both in the milk after pasteurization and in the ripening cheese. Biogenic amines were not found, indicating that the starter bacteria most commonly used for the production of Dutch-type cheese lack the capacity to decarboxylate amino acids. It was also shown that the starters used for the production of Maasdam type cheese do not form amines.

In chapter three attention is paid to the decarboxylative properties of several non-starter bacteria. It was found that outgrowth of psychrotrophic bacteria in milk, which was thereafter pasteurized and used for the production of cheese, did not give rise to amine formation: no evidence was found for the existence of heat stable decarboxylases produced by pschychrotrophic bacteria. Amines were only formed as a result of the presence of viable bacteria with decarboxylating properties in the cheese. The formation of tyramine, histamine, putrescine, and in some cases also cadaverine was observed if mixtures of mesophilic Lactobacillus strains had been added to the milk after pasteurization.

Even though enterococci are well known for their capacity to decarboxylate tyrosine, tyramine formation was not observed in cheese contaminated with these bacteria. Only when present at extremely high densities (10^8 and 10^9 cfu per gram), tyramine formation was observed and then also phenylethylamine was formed. Since these microorganisms hardly ever are found in such high numbers in Dutch type cheese, they are not considered to be

important for the formation of biogenic amines.

Contamination of the cheese with members of the Enterobacteriaceae often led to the formation of putrescine and cadaverine. The other bacteria investigated, pediococci and Bacterium proteolyticum, showed at most only weak decarboxylating properties. Except for the cheeses containing salt tolerant lactobacilli and those containing extremely high numbers of enterococci, amine formation was restricted to about 5 - 6 mmol per kg after one year of ripening.

In chapter four it is investigated which other factors influence the formation of biogenic amines. It was found that in cheese, infected with a histidine decarboxylating Lactobacillus strain (St2A), acceleration of proteolysis also led to increased histamine formation. Factors that clearly promoted precursor liberation were a high pH and a low salt-in-moisture content of the cheese, and a high storage temperature. Histamine formation was also strongly enhanced by the presence of large numbers of thermophilic lactobacilli and propionic acid bacteria in the cheese, probably because they specifically contribute to the formation of histidine. Up to 22 mmol per kg was found in a one year old cheese containing these bacteria (and strain St2A).

Tyramine formation by strains of L. brevis was also increased by the factors that accelerate proteolysis. The cofactor of tyrosine decarboxylase, pyridoxal phosphate, is probably present in sufficient quantities in the cheese.

The kinetics of cadaverine formation in cheese contaminated with a lysine decarboxylating strain of Hafnia alvei is more complex: only during the first weeks of ripening, the precursor concentration is a limiting factor, thereafter enzyme activity is rate determining. However, addition of more of these coliform bacteria to the milk does not always lead to higher cadaverine contents. On the contrary, at very high densities nitrite formation will occur, at least in Gouda cheese for which nitrate has been used, and this leads to auto-intoxication of the bacteria and concomitant loss of enzyme activity. Pyridoxal phosphate addition reduced the loss of enzyme activity.

In chapter five the formation of histamine by a strain of L. buchneri (St2A) is studied in more detail. Gouda cheese was made from milk contaminated with this strain resulting in a maximum density of more than 10^8 cfu/g. Histidine decarboxylase activity was followed during six months of ripening. In model experiments the influence of environmental factors such as pH, temperature, salt and substrate concentration on enzyme activity was determined. The data were used for a calculation of the rate of histamine formation. The results of this calculation correlated well with the experimentally determined concentrations of histamine and histidine in the cheese. The results showed that the precursor concentration (free histidine) is the limiting factor for the production of histamine. Only in the rind of the cheese, where enzyme activity is somewhat lower, free histidine is still present in detectable amounts, but also there its concentration is rate determining. From the kinetic analysis it became clear that histamine breakdown probably does not occur in Gouda cheese. Furthermore it was calculated what histamine formation can be expected in cheese not so heavily contaminated

with St2A. The results showed that for example at a density of 10^6 cfu/g only 0.5 mmol per kg will be formed after one year of ripening.

Chapter six contains a survey of the literature on the toxicology of biogenic amines. In their pure form, most amines have only a relatively low oral toxicity, because degradative enzymes in the digestive tract effectively decompose ingested amines. Food poisoning may occur when the ingested dose is very large or when these body defense mechanisms do not function properly. The latter may be the case when certain drugs (MAO inhibitors) are used but also when several different biogenic amines are consumed at the same time. In most cases of food poisoning by biogenic amines, such synergistic effects probably play an important part. Therefore the establishment of regulatory limits for the individual amines is not very relevant; it is, however, recommendable to establish a limit that comprises all of the suspected amines. However, at present only little is known about the quantitative aspects of synergism and this clearly needs further research.

Chapter six also gives the results of an investigation of the biogenic amine content of 63 samples of Dutch cheese, obtained from various commercial suppliers. In cheese made from pasteurized milk only small amounts were found, whereas in cheese made from raw milk, especially in the more mature samples, sometimes relatively high quantities were detected. It was shown that the measures that are now being taken in the Netherlands to improve the bacteriological quality of cheese made from raw milk also reduce the risk of amine formation.

Table 1 and Fig. 1 briefly summarize some of the most important results presented in this thesis.

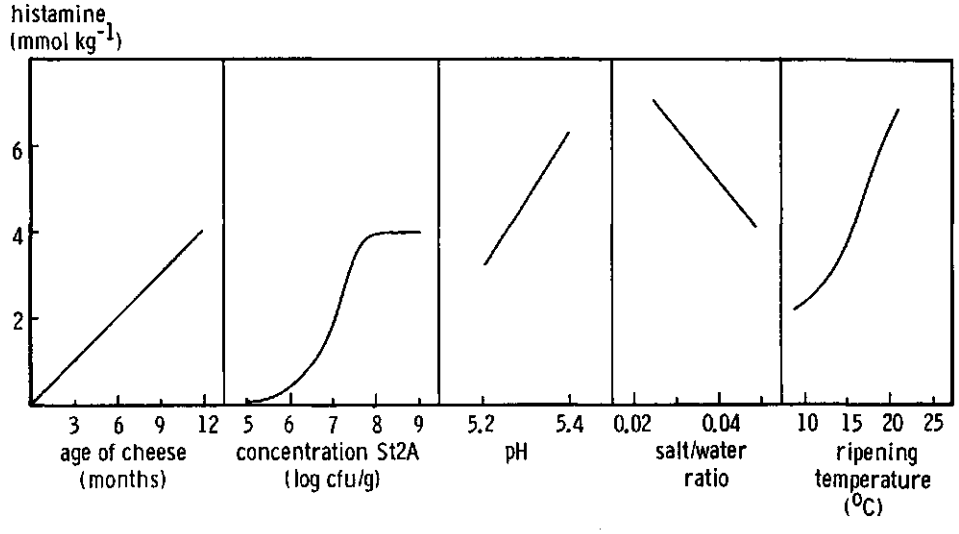
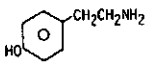
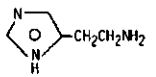
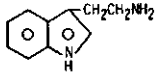
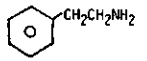


Fig. 1. The effect of various parameters on the formation of histamine in Gouda cheese, contaminated with a histidine decarboxylating L. buchneri strain. Designed to illustrate trends.

Table 1. Biogenic amines in cheese.

Name	Formula	Maximum concentration in cheese (mmol kg^{-1})	Toxic threshold dose[1] (mmol)	Precursor	Concentration of precursor in casein (mmol kg^{-1})	Responsible bacteria[2]
Tyramine		19	3	tyrosine	340	L. brevis and other mesophilic lactobacilli, enterococci[3]
Histamine		18	1	histidine	190	L. buchneri and other mesophilic lactobacilli
Putrescine	$H_2N(CH_2)_4NH_2$	14	high	arginine[4]	220	Hafnia alvei and other species of the Enterobacteriaceae, salt-tolerant lactobacilli
Cadaverine	$H_2N(CH_2)_5NH_2$	42	high	lysine	560	
Tryptamine		0.3	ND	tryptophan	60	ND[5]
Phenylethylamine		12	0.03[6]	phenylalanine	330	L. brevis and other mesophilic lactobacilli, enterococci[3]

[1] Quantity eliciting toxic response after oral uptake. In practice this value is probably not very relevant, because food poisoning is mostly the result of synergistic action of several amines and possible other food constituents.

[2] The indicated bacteria showed their capacity to form biogenic amines in Gouda cheese. However, mostly only specific strains possess decarboxylases.
The mesophilic, mixed-strain starters that are most commonly used in the Netherlands do not form amines.

[3] Only when present at very high concentrations.

[4] Arginine is first converted to ornithine.

[5] Not determined.

[6] Only for susceptible individuals.

Samenvatting

Tijdens de rijping van kaas wordt paracaseïne door eiwitsplitsende enzymen langzaam afgebroken, waarbij kleinere peptiden en aminozuren vrij komen. Enkele aminozuren kunnen daarna door bacteriële decarboxylasen omgezet worden in biogene aminen. In kaas is het gehalte aan biogene aminen meestal laag, maar een enkele keer worden grote hoeveelheden aangetroffen, die toxicologisch gezien gevaarlijk kunnen zijn. Nagegaan werd onder welke omstandigheden biogene aminen in kaas worden gevormd.

Allereerst werd een nieuwe HPLC-methode ontwikkeld waarmee de aminegehaltes van kaas bepaald konden worden. De methode berust op een scheiding in een reversed-phase kolom met behulp van ion-pair-chromatografie. Ninhydrine wordt gebruikt om de aminen spektrofotometrisch te kunnen detecteren. Dit kleuringsmiddel is reeds in de mobiele fase opgelost zodat met één pomp kan worden volstaan. De reactie met ninhydrine vindt plaats na passage door de kolom in een speciale reactiespiraal, welke in een oliebad met een temperatuur van 145 °C is gedompeld. Met deze methode kunnen histamine, tyramine, putrescine, cadaverine, tryptamine en phenylethylamine worden bepaald, met een detectielimiet van 0,02 mmol per kg kaas. Door de samenstellling van de loopvloeistof aan te passen kan met dezelfde opstelling het aminozuur histidine ook worden bepaald.

In hoofdstuk twee wordt beschreven hoe Goudse kaas bereid werd onder zeer hygienische omstandigheden. Aseptisch gewonnen melk werd gebruikt, welke ook nog gebactofugeerd en gepasteuriseerd werd. Als zuursel werden mengcultures gebruikt. Het betrof zes van de in Nederland meest gebruikte zuursels: Bos, Ur, A, Fr8, Fr18 en Fr19. Het aantal niet-zuurselbacteriën in de melk na pasteurisatie was zeer laag. Dit was ook het geval in de kaas gedurende de hele rijpingsperiode (1 jaar). Biogene aminen werden niet aangetroffen, wat er op wijst dat de betreffende zuurselorganismen waarschijnlijk niet het vermogen bezitten om aminozuren in aminen om te zetten. Tevens werd aangetoond dat dit ook geldt voor de cultures die gebruikt worden voor de bereiding van Maasdammer kaas.

In hoofdstuk drie wordt aandacht geschonken aan de decarboxylerende eigenschappen van enige niet-zuurselbacteriën. Tijdens de opslag van melk bij lage temperatuur kunnen enkele Gram-negatieve bacteriën zich nog vrij goed ontwikkelen, maar zij worden door het pasteurisatieproces weer gedood. Kaas bereid uit melk die voor het pasteuriseren flink besmet was met dergelijke organismen bevatte ook na langere tijd geen aminen. Aangenomen mag worden dat deze pschychrotrofe bacteriën onder deze groeiomstandigheden geen hitteresistente decarboxylasen vormen. Aminen werden slechts dan gevormd wanneer er bacteriën met decarboxylerende eigenschappen in de kaas aanwezig waren. De vorming van histamine, tyramine, putrescine en in enkele gevallen ook cadaverine werd waargenomen in kaas welke bereid was uit melk waaraan na pasteurisatie mengsels van mesofiele lactobacillen waren toegevoegd.

Enterokokken staan bekend om hun vermogen om tyrosine te de-

carboxyleren. Tyraminevorming werd evenwel niet geconstateerd in kaas welke met deze bacteriën was besmet. Alleen indien zeer hoge concentraties enterokokken aanwezig waren (10^8 - 10^9 cfu/g) trad vorming van tyramine en ook phenylethylamine op. Daar een dergelijk hoge besmettingsgraad met enterokokken in Nederlandse kaas niet voorkomt, is de rol die dit soort organismen spelen bij de aminevorming te verwaarlozen.

Besmetting van de kaas met bacteriën uit de familie der Enterobacteriaceae leidde vaak tot vorming van putrescine en cadaverine. De aanwezigheid van pediokokken en van Bacterium proteolyticum resulteerde niet in noemenswaardige aminevorming. Behalve in de kaas besmet met zoutresistente lactobacillen en de kazen welke extreem hoge aantallen enterokokken bevatten, waren de gehaltes aan biogene aminen van de proefkazen niet hoger dan 5 á 6 mmol per kilo na 1 jaar rijping.

In hoofdstuk vier wordt ingegaan op andere factoren die van invloed zijn op de vorming van biogene aminen. Het bleek dat in kaas besmet met een histidine decarboxylerende Lactobacillus stam (St2A), versnelling van de eiwitsplitsing ook tot een hoger aminegehalte leidde. Factoren die het vrijmaken van histidine uit eiwit sterk stimuleerden waren ondermeer een hoge pH, een laag zout-in-vocht-gehalte en een hoge rijpingstemperatuur. De vorming van histamine nam ook sterk toe door de aanwezigheid van grote aantallen thermofiele lactobacillen en propionzuurbacteriën in de met St2A besmette kaas. Hoeveelheden van 22 mmol histamine/kg werden in dergelijke kazen na 1 jaar rijping aangetroffen. Tyraminevorming door L. brevis stammen werd ook gestimuleerd door factoren welke de eiwitsplitsing versnellen. De cofactor van tyrosine-decarboxylase, pyridoxaalfosfaat, is in kaas waarschijnlijk in voldoende hoge concentraties aanwezig om maximale enzymwerking mogelijk te maken.

De kinetiek van de vorming van cadaverine door een lysine decarboxylerende Hafnia alvei stam is iets ingewikkelder. Alleen tijdens de eerste weken van de rijping is de precursorconcentratie de beperkende factor. Nadien wordt de enzymactiviteit limiterend. Bij erg hoge besmettingen van de kaasmelk werd er minder cadaverine gevormd. De verklaring hiervoor ligt waarschijnlijk in het feit dat deze bacteriën de bij de bereiding toegevoegde nitraat in nitriet omzetten, indien zij in zeer hoge aantallen in de kaas voorkomen. Dit leidt tot versneld afsterven van de bacterie en daarmee gaat waarschijnlijk ook de decarboxylase-activiteit verloren. Door toevoegen van pyridoxaalfosfaat, wat ook de cofactor is van lysinedecarboxylase, werd de afname van de decarboxylase-activiteit enigszins vertraagd.

In hoofdstuk vijf wordt de histaminevorming door L. buchneri St2A aan een nader onderzoek onderworpen. Goudse kaas werd bereid uit melk besmet met deze bacterie en de histidinedecarboxylase-activiteit van de kaas werd gedurende zes maanden gevolgd. De invloed van omgevingsfactoren zoals pH, temperatuur en zout- en substraatconcentratie op de enzymwerking werd ook onderzocht. De gegevens werden gebruikt voor een berekening van de te verwachten hoeveelheden histamine en histidine in de kaas. De resultaten hiervan kwamen goed overeen met de gehaltes die experimenteel waren bepaald. Tevens werd waarschijnlijk gemaakt dat histamine-

afbraak in kaas niet optreedt en dat de histidineconcentratie de beperkende factor is voor de vorming van histamine. Tenslotte werd berekend hoeveel histaminevorming verwacht mag worden bij minder grote besmettingen.

In hoofdstuk zes wordt een literatuuroverzicht gegeven van de toxicologische aspecten van de aanwezigheid van biogene aminen in het voedsel. Bij orale opname van de zuivere stof blijken de betreffende aminen slechts een geringe giftige werking te vertonen. Dit is te danken aan het feit dat er vooral in het spijsverteringsstelsel enzymen werkzaam zijn die de aminen snel onwerkzaam maken. Voedselvergiftiging zal daarom alleen optreden bij consumptie van zeer hoge doses, maar ook indien de amine afbrekende enzymen in hun werking belemmerd worden. Dit laatste treedt bijvoorbeeld op bij gebruik van bepaalde medicijnen (MAO-remmers) en bij gelijktijdige opname van verschillende aminen. In de praktijk spelen dergelijke synergistische effecten waarschijnlijk vaak een rol. Daarom is het zinvoller om een maximaal toelaatbaar niveau aan te geven voor het totale aminegehalte, dan dit te doen voor ieder van de aminen afzonderlijk. Op dit moment is er echter nog onvoldoende bekend over de mate waarin de verschillende aminen kunnen bijdragen aan voedselvergiftiging.

In hoofstuk zes worden ook de resultaten beschreven van een onderzoek naar het biogene aminegehalte van 63 Nederlandse kaasmonsters, welke van verschillende leveranciers waren betrokken. Kaas die uit gepasteuriseerde melk was bereid bevatte slechts weinig aminen. In kaas bereid uit rauwe melk, vooral in de wat meer belegen monsters, werden af en toe vrij hoge concentraties gevonden. Tegenwoordig worden er maatregelen genomen om de microbiologische kwaliteit van de uit rauwe melk bereide kaas te verbeteren. Uit de analyses bleek dat deze maatregelen ook het risico verkleinen dat er in dit soort kaas aminen worden gevormd.

In tabel 1 en figuur 1 worden in het kort enkele van de belangrijkste bevindigen uit dit proefschrift samengevat.

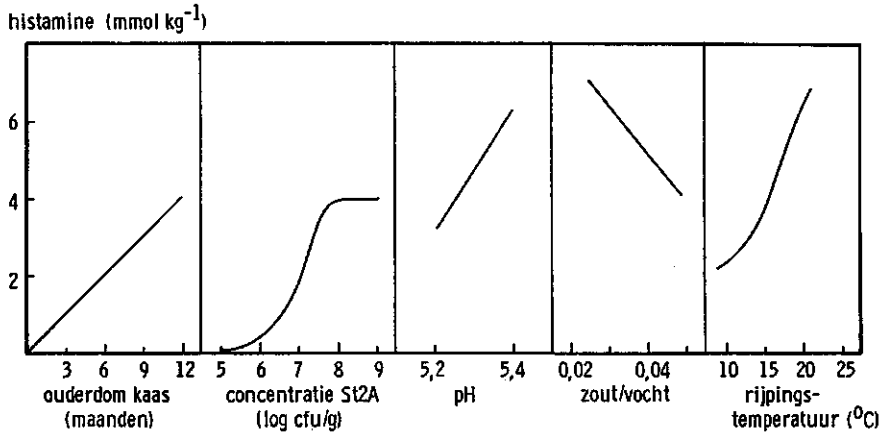
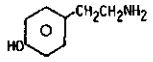
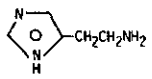
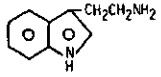
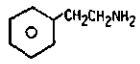


Fig. 1. De invloed van verschillende parameters op de vorming van histamine in Goudse kaas, besmet met een histidine decarboxylerende L. buchneri stam.

Tabel 1. Biogene aminen in kaas.

Naam	Structuurformule	Hoogste gehalte in kaas (mmol kg^{-1})	Schadelijke dosis[1] (mmol)	Precursor	Gehalte aan precursor in caseine (mmol kg^{-1})	Verantwoordelijke bacteriën[2]
Tyramine		19	3	tyrosine	340	L. brevis en andere mesofiele lactobacillen, enterokokken[3]
Histamine		18	1	histidine	190	L. buchneri en andere mesofiele lactobacillen
Putrescine	$H_2N(CH_2)_4NH_2$	14	hoog	arginine[4]	220	Hafnia alvei en andere soorten van de Enterobacteriaceae, zout-tolerante lactobacillen
Cadaverine	$H_2N(CH_2)_5NH_2$	42	hoog	lysine	560	
Tryptamine		0,3	NB	tryptofaan	60	NB[5]
Phenylethylamine		12	0.03[6]	fenylalanine	330	L. brevis en andere mesofiele lactobacillen, enterokokken[3]

[1] Hoeveelheid van de zuivere stof die bij orale opname tot vergiftigingsverschijnselen leidt. Voor de praktijk is deze waarde echter van weinig belang daar er bij gevallen van voedselvergiftiging meestal synergistische effecten een grote rol spelen.
[2] De aangeduide bacteriën waren in staat om aminevorming in Goudse kaas te bewerkstelligen. In het algemeen geldt dat slechts enkele stammen van de betreffende bacteriesoorten de beschikking hebben over decarboxylases.
[3] Alleen indien aanwezig in zeer hoge concentraties.
[4] Arginine wordt eerst omgezet in ornithine.
[5] Niet bepaald.
[6] Alleen bij daarvoor gevoelige personen.

Curriculum vitae

Han Joosten werd op 3 oktober 1956 in Goirle geboren. Hij behaalde in 1974 het diploma atheneum B aan het Elzendaalcollege te Boxmeer. In 1976 begon hij aan de Katholieke Universiteit van Nijmegen aan de studie biologie. In 1979 werd het kandidaatsexamen afgelegd en in 1983 het doctoraalexamen, met als hoofdvak microbiologie (Prof. Dr. Ir. G.D. Vogels) en als bijvakken medische microbiologie (Prof. Dr. J.van der Veen) en exobiologie (Prof. Dr. A.W. Schwartz). Sinds 1984 is de auteur verbonden aan het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede, waar het in dit proefschrift beschreven onderzoek werd verricht.