BACTERIOLOGICAL STUDIES ON LIMBURGER CHEESE

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BACTERIOLOGICAL STUDIES ON LIMBURGER CHEESE

(with a summary in Dutch)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN
OP GEZAG VAN DE RECTOR MAGNIFICUS, DR. IR. F. HELLINGA,
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THEOREMS

I

Organisms of the *Brevibacterium linens* type can not be considered as the main ripening agent in Limburger cheese. It may be stated that other cheese coryneforms, mainly arthrobacters, are responsible for the ripening process in this particular type of cheese.

This thesis

II

The colour of ripe Limburger cheese depends on the type of micro-organisms present on the cheese surface as well as on the presence or absence of light during the ripening period.

This thesis

III

In developing countries, knowledge of engineers and research workers could be easily transferred to the farmers in rural areas if those workers practise the same religion and follow the same way of living and thinking as the people in such areas.

IV

In developing countries bread and salt should be supplemented with different minerals and vitamins to avoid different cases of malnutrition.

V

In aiding developing countries, priority should be given to sending experts and to allowing as many students as possible from these countries to study abroad, and not to giving direct financial aid.

Thesis of A.F.M.El-Erian
Wageningen, 4th July, 1969

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1. GENERAL OUTLINE

1.1. INTRODUCTION

1.1.1. *Limburger cheese*

Limburger cheese is a semi-soft cheese on which a surface smear or slime, largely consisting of micro-organisms, is the main cause of the ripening process. The microbial flora of this surface layer includes many different micro-organisms. Therefore it is not surprising that various investigators have failed to agree in respect of the micro-organisms assumed to be of greatest importance for the ripening of this particular type of cheese.

WEIGMANN (1898) thought that spore-forming bacteria were responsible for the ripening of Limburger cheese. He reported that an anaerobic bacterium with polar spores was responsible for the typical aroma and flavour of this cheese. WEIGMANN considered this organism, which he identified as *Paraplectrum foetidum*, was associated in the smear with an aerobic spore-former, *Clostridium licheniforme (Bacillus licheniformis)*. He thought that the latter organism was preparing the way for *Paraplectrum foetidum* by breaking down the lactic acid and making the medium more alkaline. He also thought that the red smear, which covered the cheese surface, produced anaerobic conditions permitting growth of *Paraplectrum foetidum*.

In 1899 LAXA studied the flora of Harrack and Knoppist cheeses, two Bohemian varieties of Backstein cheese resembling Limburger. He stated that the organism *Oospora lactis* consumed a portion of the free acid and thus prepared the cheese for the bacteria which, growing together in symbiosis, produced the typical cheese aroma.

ORLA-JENSEN (1904) occasionally found *Oospora lactis* in young Limburger cheeses but did not consider it to contribute to the ripening. ORLA-JENSEN claimed that *Bacterium casei limburgensis* FREUDENREICH was of great importance in the cheese ripening process. He found that the most characteristic property of this bacterium was its ability to carry further the decomposition of the products of protein hydrolysis produced by other micro-organisms. When grown in milk in the presence of *Micrococcus casei liquefaciens*, protein decomposition was carried much further than when the latter was grown alone.

MAZÉ (1905) studied the non-spore-forming bacteria growing in the reddish smear or 'rouge' of Camembert cheese. He believed that the presence of these bacteria was desirable since they might help to digest the casein and render the medium alkaline.

In 1906 THOM maintained that Camembert cheese may have the typical Camembert flavour without development of any specific surface growth of bacteria.

The work of WOLFF (1909) is of special interest. He isolated nine different pigment-forming bacteria from smears on Tilsiter, Romadour and Woringer cheeses.

Organism I was a reddish-yellow short rod that liquefied gelatin and pro-

duced a faint alkaline reaction when grown in milk for three weeks. This organism originally was thought to be an important agent in cheese ripening, making 15 to 20 percent of the total flora, but eventually proved to be of lesser importance.

Organism II was a lemon-yellow short rod closely related to, but not identical with, *Bacterium erythrogenes* LEHMAN and NEUMANN.

Organism III was similar to II except for its more irregular colonies.

Organisms IV, V, VI, VII and VIII were found by WOLFF to be micrococci, related to, or identifiable with, *Sarcina aurantiaca* FLUGGE, *Micrococcus sulfureus* ZIMMERMAN, *Micrococcus bicolor* ZIMMERMAN and *Sarcina lutea* SCHROETER. Growth of these organisms on cheese after five weeks was insignificant in comparison with that of organisms II and IX.

Organism IX grew rapidly on a special milk medium with an orange-yellow, wet, shining growth and yielded an intensive typical odour like the cheese smear. Experimental Romadour (Limburger type) cheeses inoculated with cultures of this micro-organism were superior in every respect to control cheeses. A mixture of cultures II and IX when added to the cheese seemed to be more active than only one culture.

WOLFF continued his studies on organism IX and in 1910 he published a description, naming the organism *Bacterium linens*, according to a proposal made by WEIGMANN.

Other workers who have described the bacteria found on the surface of various types of surface-ripened cheeses were MAZÉ (1910), GRATZ and VAS (1914), FILIPOVIC (1923), PETER (1924) and HENNEBERG (1926).

STEINFATT (1930) gave a more detailed description of *Bacterium linens* WEIGMANN. It is a Gram-positive, aerobic, non-spore-forming, non-motile rod. Gelatin is liquefied. Little or no change in pH is produced in milk after 10 days, but afterwards the reaction gradually becomes alkaline. Growth on potato is mouse-grey in colour. No acid is formed from carbohydrates. The optimum growth temperature is 25 °C. A shiny orange growth is produced on agar. STEINFATT found that certain organisms, when growing together with *Bacterium linens*, increased protein degradation, while other organisms checked it, indicating that certain organisms present in the cheese smear may be undesirable.

WEIGMANN (1933) stated that the red smear was built up by red bacteria which covered the surface of the cheese and produced anaerobic conditions permitting the growth of *Paraplectrum* or *Plectridium foetidum*.

The nature of the protein decomposition products formed from casein by *Bacterium linens* was determined by GRIMMER and SCHMID (1936). Leucine, isoleucine and tyrosine were obtained in large amounts. Indol and skatol were never found.

KELLY (1937) made microscopic examinations of slides pressed against the surface of Limburger cheeses at 14 New York State factories and found a definite sequence in the microbiological changes from day to day. Budding yeasts appeared within 2 to 3 days and were present in large masses within 4 to 5 days. About the sixth or seventh day, short slender rods resembling *Bacterium linens* appeared and increased to large numbers at about the eighth day. From

10 to 18 days, the yeast cells tended to decrease in size, became destroyed, and disappeared entirely.

In 1937 MACY and EREKSON studied the microflora of the slime on Roquefort, Port de Salut, Tilsit and Limburger types of cheese. They found much the same sequence of changes as KELLY did for Limburger, the development of yeasts being followed by a predominance of rod-shaped bacteria.

KELLY and MARQUARDT (1939) stated that *Bacterium linens* does not grow below pH 5.85 and grows best at pH 6.5. Inasmuch as the pH of Limburger cheese is usually below 5.0 at the age of one day, *Bacterium linens* is not able to grow at this stage. The yeasts acting on both the protein and lactic acid, raise the pH above 5.85 at which point *Bacterium linens* becomes established and overgoes the yeasts.

LANGHUS and PRICE (1941) showed that the micro-organisms growing on the surface of Brick cheese were necessary for the development of the typical flavour. After salting, the surface flora of Brick cheese at first consisted largely of yeast-like organisms. About 4 days after salting, the latter began to be replaced by micrococci, many of them being capsulated. Small rod-shaped micro-organisms also appeared but more slowly than the micrococci.

YALE (1943) found that the surface smear of Limburger cheeses ranging from 2 to 14 days of age at two factories yielded a maximum yeast count of 6.3×10^9 per gram of smear on the fourth day, a maximum count of salt-tolerant bacteria of 1.29×10^{11} per gram of smear on the fourteenth day, and a maximum total bacterial count of 3.60×10^{11} per gram of smear also on the fourteenth day.

YALE made a study of 243 cultures of bacteria isolated from the surface of various types of surface-ripened cheeses. He showed that the predominant organism was a Gram-positive, non-chromogenic, non-spore-forming rod, producing a marked alkaline reaction in litmus milk and not liquefying gelatin. The next most common micro-organism was *Bacterium linens*, a strictly aerobic, Gram-positive, non-spore-forming rod, producing a neutral or slightly alkaline reaction in milk and liquefying gelatin. The colour of the growth of *Bacterium linens* on an agar medium ranged from light cream to deep orange.

Surface inoculation of Limburger cheese, made of raw milk, with strains of *Bacterium linens* did not improve the quality when compared with cheese held under good factory conditions. The addition of pure cultures of *Bacterium linens*, grown in milk, to pasteurized milk improved the flavour of the majority of the lots of experimental Limburger cheeses made in four trials at two factories, indicating that such a procedure may be of value.

TUCKEY and SAHASRABUDHE (1957) studied the ripening of Limburger and Brick cheeses by determining the quantity of individual amino acids liberated throughout the ripening period. They found that the ripening proceeds more rapidly in the rind portion than in the interior of the cheeses, but not exclusively in this area. The difference in amino acids in the rind and in the centre represented a quantitative rather than a qualitative difference, for the same amino acids were present in both areas at essentially the same time, but in higher concentration in the rind.

Bacterium linens was able to hydrolyse milk proteins more rapidly than other agents, including milk and rennet enzymes and other bacteria involved in the manufacture and ripening of Limburger cheese. The surface of the cheese was heavily inoculated with a culture of these organisms and, therefore, *Bacterium linens* was probably responsible for the rapid ripening of the exterior.

TUCKEY and SAHASRABUDHE (1957) also found a quantitative and a qualitative difference in the amino acid patterns at the end of ten weeks of ageing in the lots of the studied Brick and Limburger cheeses. After Limburger cheese was wrapped, the free amino acids continued to increase in concentration, whereas, after the Brick cheese was waxed, the rate of liberation of amino acids was reduced as compared with Limburger. Hydroxyproline was found to be markedly higher in concentration in the Brick cheese than in the Limburger.

These authors stated that there was no correlation between the presence of any single amino acid and the characteristic flavour development in Limburger or Brick cheese. This was established during the ageing period by organoleptic judging of the samples.

MULDER et al. (1966) found that more than 90% of the total surface flora of Limburger cheese were coryneforms. The majority of these coryneforms were grey-white coloured strains, followed by a smaller group of orange-coloured ones (9 to 24% of the total). They found that 2.0 to 3.0% of the total flora were yellow coryneforms.

1.1.2. *Cheese coryneforms*

Many reports in the literature are dealing with the importance of the Gram-positive rods in the ripening of soft cheeses. There has always been some confusion concerning the classification of these Gram-positive rods.

Coryneforms are easily recognized with the aid of a microscope, by their angular and palisade arrangement of cells. Many workers have reported the presence of coryneforms in aseptically drawn, raw and pasteurized milk (ABD-EL-MALEK and GIBSON, 1952; EDGELL and BIRD, 1949/50; and GALESLOOT 1951, 1952, and 1953).

STADHOUDERS and MULDER (1958), not agreeing with the classification of the Gram-positive rods mentioned in BERGEY's Manual (7th ed., 1957), used the following criteria in characterizing such bacteria which they isolated from milk and from the interior and surface of cheeses: liquefaction of gelatin, acid production from glucose, anaerobic growth at pH 7.0, colour, reduction of nitrate, growth in a protein-deficient medium, acid-fast staining and fat hydrolysis.

They divided their strains into three groups. Bacteria of group I were able to develop slowly under anaerobic conditions at pH 7.0; they werd white, did not liquefy gelatin and were related to *Corynebacterium bovis* types. Group II contained obligately aerobic strains which formed orange-coloured colonies, did not produce acid from glucose and liquefied gelatin. They classified this group as related to *Br. linens*. Bacteria of the third group belonged to the genus *Microbacterium*.

STADHOUDERS and MULDER continued their investigations and in 1959 they

published a study of the cheese surface organisms associated with fat hydrolysis in cheese. They isolated 53 strains from the surface of Gouda and Edam cheeses and arranged them into three groups. First the types related to *Corynebacterium bovis*, followed by intermediates between this group and the third group, which included the types related to *Br. linens*. They did not succeed in isolating any microbacteria from the cheese surface. They found that very few of the isolated strains were able to hydrolyse cheese fat to any extent.

MULDER and ANTHEUNISSE (1963) and MULDER (1964), in their studies about the bacteria belonging to the genus *Arthrobacter*, isolated a group of strains from the surface of different types of cheese and from milk. Most of the isolated cheese strains were found to be *Arthrobacter*-like bacteria. In 1966 MULDER et al., in their study about the relationship between *Brevibacterium linens* and bacteria of the genus *Arthrobacter*, found grey-white arthrobacters to be the main group on the surface of Limburger cheese.

Preliminary studies of the author confirmed the phenomenon found by MULDER et al. (1966) (Chapter III).

1.1.2.1. *Arthrobacter*

Arthrobacter is an aerobic, pleomorphic micro-organism belonging to the *Corynebacteriaceae*. In young cultures the cells appear as rods which may vary in size and shape, from straight to bent, swollen or club-shaped forms; sometimes developing filaments and true branching. Snapping division may give rise to angular cell arrangement. Upon ageing, the rod-shaped cells turn into coccoid forms. The coccoid cells are persistent as the predominant form in older cultures. It can be generally said that bacteria of this genus are characterized mainly by their cell morphology.

Bacteria of the *Arthrobacter* type, under the name of *Bacterium globiformis*, were described by CONN (1928) as typical soil bacteria. Long before the name *Arthrobacter* was introduced, JENSEN (1934) had described this group in detail as soil corynebacteria. The most commonly occurring species in his experience was *Corynebacterium helvolum* which is apparently identical with, or very closely related to *Arthrobacter globiformis*. Other cultures studied by JENSEN were found to be identical with other *Arthrobacter* spp. Throughout, JENSEN stressed the close relationship of the soil corynebacteria and certain species of *Nocardia*, and it seems highly probable that KRASSILNIKOW's mycobacteria and micrococci of the soil include also the arthrobacters (KRASSILNIKOW, 1934; BERGEY's Manual, 1957).

It was not until 1947 that bacteria of this type were described by CONN and DIMMICK under the name of *Arthrobacter globiformis*.

Extensive investigations concerning the morphology and physiology of bacteria of the genus *Arthrobacter* have been carried out by several workers. Morphological studies on these micro-organisms have been reported by TOPPING (1937), TAYLOR (1938), SACKS (1954), SGUROS (1955, 1957), CHAPLIN (1957), SUNDMAN (1958), BLANKENSHIP and DOETSCH (1961), STEVENSON (1961, 1962, 1963), STARR and KUHN (1962), ENSIGN and WOLFE (1964), SIEBURTH

(1964), MULDER and ANTHEUNISSE (1963), MULDER (1964) and MULDER et al. (1966). Nutritonal studies have been made by CAMPBELL and WILLIAMS (1951), MÜLLER (1957), MORRIS (1960), LOCHHEAD and his collaborators (1953, 1955, 1957, 1958) as well as by MULDER et al. (1962, 1966).

In studies of the arthrobacters from soil, activated sludge and dairy products, particularly the surface of cheeses, MULDER and ANTHEUNISSE (1963) and MULDER (1964) suggested that those from activated sludge would have a closer physiological relationship to the soil arthrobacters than those from cheese.

Of special interest is the work of MULDER et al. (1966) in which an extensive study was carried out about the relationship between *Brevibacterium linens* and bacteria of the genus *Arthrobacter*. The data collected by those authors, only partially agreed with the description given in BERGEY's Manual (1957). The majority of the soil arthrobacters were found to be Gram-negative or Gram-variable in the rod stage with only a slight tendency to become Gram-positive with age. MULDER et al. (1966) suggested that the cheese coryneforms should not be placed in the genus *Arthrobacter* because of the several existing differences, mainly physiological ones, between these organisms and the arthrobacters from soil. They found two distinct types of coryneform bacteria occurring on the surface of Edam, Gouda, Hervse, Hohenheim, Kernhemmer, Leidse kanter, Limburger, Mamirolle, Marville, Meshanger, Munster, Pénitent, Romadour, St. Paulin and Vacherin Mont d'Or cheeses, one forming grey-white colonies, sometimes with a light yellow or pink shade, and the other giving orange colonies. They stated that although both types had a number of characters in common, in other respects they were clearly distinct.

MULDER et al. (1966) found the grey-white type occurring as relatively short rods which tended to transform into cocci more readily than did the orange cheese strains and the soil arthrobacters. The orange type was found to be identical with *Brevibacterium linens*. They also showed that the formation of the orange pigment was light-dependent in more than half of the tested strains.

As to the nutritional requirements, expecially those of nitrogen compounds and vitamins, all but one of the orange strains tested required organic nitrogen. A number of strains gave good growth with glutamic acid, but more often other amino acids were needed. As for the grey-white strains, they were found to be more or less intermediate between the orange ones and the soil strains. Ammonium nitrogen was assimilated by approximately 85% of the tested grey-white strains partly requiring amino acids (methionine or glutamic acid) and/or vitamins.

MULDER et al. (1966) also stressed the ability of both types of cheese corynebacteria to tolerate large amounts of salt and, under certain conditions, even requiring relatively high concentrations of it. They suggested this to be an important character in both taxonomical and ecological respects.

1.1.2.2. *Brevibacterium linens*

In the literature on cheese ripening the significance of *Br. linens* is well documented. It was found to be usually present in large numbers in the slimy

orange or orange-brown growth developing on the surface of many soft cheeses, and was thought to contribute to the ripening process of such cheeses (WOLFF, 1909, 1910; WEIGMANN, 1911; STEINFATT, 1930; GRIMMER and SCHMID, 1936; KELLEY, 1937; KELLY and MARQUARDT, 1939 and ALBERT, LONG and HAMMER, 1944).

BREED (1953) gave this organism its present name. According to BREED, *Brevibacterium linens*, which forms orange-coloured colonies, should be a short, unbranched rod. LOCHHEAD (1955) considered the whole genus to exist of simple rod forms without any of the morphological implications of the soil coryneform bacteria.

This organism has been described in BERGEY's Manual (1957) as a rod, aerobic, catalase-positive, gelatin-liquefying and salt-tolerant. It belongs to the family of *Brevibacteriaceae*.

SCHEFFERLE (1957) observed that *Brevibacterium linens* resembles the corynebacteria and she showed it to be closely related to *Arthrobacter globiformis*.

STADHOUDERS and MULDER (1958) observed that *Br. linens* resembles the corynebacteria in their angular and palisade arrangement of cells.

MULDER et al. (1966) stated the close relationship between orange cheese coryneforms of the *Br. linens* type, and the grey-white cheese arthrobacters. They also stated that in spite of the presence of a number of characters in common, these two types, in some respects, were clearly distinct (see 1.1.2.1.).

1.2. SCOPE OF THE PRESENT STUDY

The purpose of the present investigation was to gain more information concerning the microbial flora of ripening Limburger cheese, particularly as to the effect of various types of organisms on the ripening process. An additional aim was to contribute to the classification of the Gram-positive coryneforms occurring on the surface of Limburger cheese. A study of the general amino acid pattern of Limburger cheese and of the effect of organisms of different types on this pattern was also made.

The following steps were included in the investigation:

a. A survey of the micro-organisms found on the surface of Limburger cheese throughout the different stages of ripening with special respect to the chemical composition of cheese during these ripening stages.
b. A taxonomical study of the micro-organisms growing on the surface of this particular type of cheese.
c. An investigation of strains isolated from ripening Limburger cheeses and thought to be responsible for the ripening process. These strains were introduced to sterilized fresh cheese slices and their growth was studied.
d. A comparison of the amino acid pattern of Limburger cheese, at different stages of ripening, with that of sterilized fresh cheese slices inoculated with different strains of coryneform bacteria, and that of Casamino acids-containing media inoculated with the same strains.
e. A study of the effect of a number of these micro-organisms on individual amino acids.

2. EXPERIMENTAL MATERIAL AND METHODS

2.1. BACTERIOLOGICAL METHODS

2.1.1. *Total viable count*

Plate counts were performed on tryptone soya agar and tryptone glucose extract agar. One ml of alcohol was placed in a sterile mortar and ignited. The pestle was stirred around the inner surface of the mortar to be touched by the burning alcohol. This procedure served to sterilize the mortar as well as to warm it and thus facilitating the homogenization of the scraped surface material of the cheese. After about one minute, 1 ml of a sterile solution of 20% sodium citrate and one gram of the prepared sample were placed in the mortar and homogenized. Then 8 ml of sterile water were gradually added to the suspension while stirring, thus obtaining a 1:10 dilution. From this suspension a dilution series was made, which was used for the inoculation of the plates. These plates had been dried by storage at 30°C for 24 hrs prior to use. Colonies were counted after 5 days incubation at 25°C.

2.1.2. *Replica method*

Strains isolated were tested for proteolytic activity, catalase reaction, utilization of carbon compounds and for *Arthrobacter* characteristics with the replica method of MALING (1960).

2.1.3. *Gram's stain*

The Gram's stain was carried out according to HUCKER's modification described in the 'Manual of Microbiological Methods' (1957).

2.1.4. *Media*

2.1.4.1. Oxoid tryptone glucose extract agar (T.G.E.A.)

This medium contains: beef extract, 3 g; tryptone, 5 g; glucose, 1 g; agar, 12 g; tap water, 1000 ml; pH: 7.0.

2.1.4.2. Oxoid tryptone soya agar (T.S.A.)

This medium consists of: tryptone, 17 g; soya peptone, 3 g; glucose, 2.5 g; NaCl, 5 g; K_2HPO_4, 2.5 g; agar, 12 g; tap water, 1000 ml; pH: 7.0.

2.1.4.3. Casein agar

This medium is composed of: casein, 1 g; yeast extract, 0.7 g; glucose, 1 g; K_2HPO_4, 1 g; $Ca(H_2PO_4)_2$, 0.25 g; $MgSO_4.7H_2O$, 0.25 g; $(NH_4)_2SO_4$, 0.25 g; agar, 10 g; tap water, 1000 ml; pH: 7.0.

2.1.4.4. Yeast extract glucose agar

This medium had the following composition: yeast extract, 7 g; glucose, 10 g; agar, 12 g; tap water, 1000 ml; pH: 7.0.

2.1.4.5. The mineral nutrient medium

The mineral nutrient medium employed for certain nutritional tests had the following composition: K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.05; $FeCl_3.6H_2O$, 0.01 g per litre; $CuSO_4.5H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1; $MnSO_4.7H_2O$, 1.0; Na_2MoO_4, 0.01 and H_3BO_3, 0.01 mg per litre of medium. The pH of this medium was 7.0.

2.1.4.6. The vitamin mixture

The vitamin mixture used, consisted of: biotin, 2; folic acid, 20; riboflavin, 100; thiamin, 100; pyridoxine, 100; nicotinic acid, 100; pantothenic acid, 100; p-amino-benzoic acid, 100 and vitamin B_{12}, 1 μg per litre of medium.

2.1.5. *Nutritional requirements*

2.1.5.1. Utilization of carbon compounds

The effect of different carbon compounds was tested on agar media containing 0.15 percent yeast extract and 0.5 percent of the compound to be tested.

2.1.5.2. Nitrogen and vitamin requirements

In this investigation, use was made of the mineral nutrient medium, supplied with 0.25 gram nitrogen in the form of ammonium sulphate or Casamino acids, and 5 g glucose per litre of medium; pH: 7.0. The vitamin mixture was added in this experiment.

2.2. Chemical methods

2.2.1. *Moisture content*

About 5 g of cheese were weighed and thoroughly mixed with previously washed and heated sand, then dried until constant weight at 100 °C.

2.2.2. *Total nitrogen*

About 1 g of cheese was weighed in a Kjeldahl flask, 10 ml of distilled water were added and the mixutre was digested with 10 ml concentrated H_2SO_4, 3 g K_2SO_4 and 0.3 g $CuSO_4.5H_2O$.

After the addition of NaOH to the mixture, the ammonia was steam-distilled into N/50 H_2SO_4. The excess of acid was titrated with N/50 NaOH.

2.2.3. *Soluble nitrogen*

A whole block of cheese was ground in a sterilized mortar. About 20 g of it were weighed into a 300 ml Erlenmeyer flask. Then 150 ml of distilled water and 0.5 ml of a 50% alcoholic phenol solution, to stop further biological changes, were added. The mixture was well stirred and then shaken according to the method of Sirks (1943). The flask was warmed to 40 °C and its contents were quantitatively transferred to another Erlenmeyer flask and brought up to 250 ml. It was then cooled to solidify the fat and filtered. Ten ml of this extract were used in the digestion followed by steam distillation.

2.2.4. *Amino acid nitrogen*

As the addition of 95% alcohol to a cheese extract in the proportion of 5 to 1 precipitates proteins and peptides, but no amino acids, it was used in preparing the samples for the amino acid estimations. A full obtaining of the amino acids in the cheese extract could be achieved in this way.

To each 50 ml of the filtrated cheese extract 250 ml of 95% alcohol were added. The mixture was shaken, allowed to settle for about 15 minutes for the precipitation of any remaining protein, and then centrifuged. Of the clear solution, 150 ml were evaporated at 60 °C using a rotary evaporator. The residue was quantitatively transferred into a volumetric flask. A quantity of 10 ml, equivalent to 2 g of cheese, or 20 ml, equivalent to 4 g of cheese were used in the determination.

2.2.5. *Salt*

Salt was determined by the method of DAVIES (1932). 2 g of cheese sample were digested using 25 ml HNO_3 + 10 ml saturated potassium permanganate in the presence of silver nitrate. Excess $AgNO_3$ was titrated back with potassium thiocyanate using iron alum as an indicator.

2.2.6. *The pH*

2 g of cheese were weighed and 30 ml of neutralized boiled distilled water were added and mixed with the cheese by means of a glass rod. The mixture was heated to 50 °C while stirring until a milky mixture of cheese in water resulted. After cooling to 20 °C, the pH was determined by using a Radiometer glass electrode pH meter.

2.3. QUALITATIVE PAPER CHROMATOGRAPHY OF AMINO ACIDS

The sample was applied to a sheet of filter paper at a point 8 cm from the top and the side of the paper and was confined to a circle 1–1.5 cm in diameter (drops of 0.01 ml were used). After drying, the paper was transferred to a cabinet in which the chromatography (descending method) was carried out.

A 2-dimensional procedure was used, first with phenol-water and then with collidine-lutidine-water as the moving phases. The cabinet used contained three troughs; in each trough two sheets of filter paper might be placed. The papers were held in the troughs by a heavy glass rod in such a way that the sample lay approximately 3.5 cm beyond the edge of the trough. Dishes with phenol-water (2:3) were placed at the bottom of the cabinet to allow the papers and the atmosphere to equilibrate with the solvent before adding the latter to the troughs

Then 80 ml of phenol-water (3:1, pH 5–5.5) were added to the troughs through a small hole in the top of the cabinet which was closed by a stopper. The phenol solution travelled through the paper, downwards, carrying the different amino acids at different rates. When the solvent had moved for approximately 45 cm, i.e. when it had reached a distance of approximately 1–2 cm from the lower edge (36 hours at 20 °C) the paper was removed from the cabinet and

allowed to dry at room temperature in an air stream for about 20 hours. A margin of about 2 cm, containing the phenol front was cut out from the paper. The paper was then transferred to a second cabinet in which a similar prodecure was carried out with collidine-lutidine-water (1:3:3, pH 8.0) in a direction at right angles to the first. As with the phenol treatment, the second cabinet contained dishes with the solvent to ensure equilibrium between paper and atmosphere. After equilibrium had been reached (4–6 hours), 80 ml of the collidine-lutidine-water mixture were added to the troughs and the second phase of the chromatography began. After 20–24 hours at 20 °C when the solvent had moved for approximately 35 cm, the procedure was stopped and the paper was again dried in an air stream (about 16 hours).

The chromatogram was now ready for spraying with ninhydrin. A solution of 1 % ninhydrin in 95 % ethanol containing 2 % of a collidine-lutidine mixure (1:3) was used for that purpose. A hand sprayer activated by compressed CO_2 was used.

The sheets were then transferred to the colour development cabinet which is a modification of the apparatus of THOMPSON et al. (1951). It consisted of a tank 54 by 34 by 60 cm in which the chromatograms were heated under ethanol-saturated anaerobic conditions. The bottom of the tank contained a layer of ethanol. Three perforated pipes connected to a metal cylinder containing CO_2 were immersed in this layer so that a flow of ethanol-saturated CO_2 passed across the paper sheets. The tank had accomodation for three sheets. A perforated shield above the surface of the ethanol prevented splashing of the papers. The chromatograms were hung vertically in the tank with the initial spot at the lower end. In order to keep the atmosphere saturated with ethanol, two filter papers were hung down along the walls of the tank from shallow troughs at the top. These filters papers were dipped into the ethanol at the bottom of the tank; the troughs also contained alcohol. The tank was contained in an outer cabinet with a constant-temperature control.

Under these conditions an optimum colour development was found to take place within 20–25 minutes at 60 °C. The sheets were then dried and photographed.

2.4. MATERIALS

2.4.1. *Samples used for the survey analysis*

These samples were brought from three different factories, two in The Netherlands and one in Belgium, specialized in manufacturing Limburger cheese. The three factories are owned by: P. GEELEN, Berg en Terblijt, The Netherlands, J. PINCKERS, Epen, The Netherlands, and P. PINCKERS, Neu-Moresnet, Belgium.

The samples taken from those factories included cheeses of different ages, brine and samples of scraped material from the shelves, the tables and the walls of the curing rooms.

2.4.2. *Cheese manufacture*

2.4.2.1. Cheese made under commercial conditions

The Limburger cheese used in this investigation was made in the factory of P. GEELEN, Berg en Terblijt, South Limburg, The Netherlands. This factory is specialized in manufacturing Limburger cheese which is its only product. Samples were taken from the running production in the factory. This was done to be sure of having samples of cheese made, handled and ripened in the same way as usual for the commercial process of manufacturing Limburger cheese.

The cheese was made from pasteurized cow's milk. Starter was added and rennet used for forming a curd. After cutting and dewatering, the curd was placed in wooden forms. When the curd was firm enough, it was cut to blocks weighing about 250 grams each. Brine salting was applied by dipping the cheese blocks into brine for overnight.

The cheese was now ready for the ripening process. This was carried out in a curing room maintained at about 15 °C and kept always at a high relative humidity. While being in this room, the cheese was rubbed a few times to close the surface openings and to help in distributing the ripening organisms all over the surface. After about 3 to 4 weeks of ripening, the cheeses were ready for consumption.

2.4.2.2. Manufacture of cheese for the preparation of cheese plates

Pasteurized cow's skim milk was used for making cheese for the cheese plates. The milk was warmed to 30 °C and the following substances were added for every 100 litre of milk: $CaCl_2$, 10 ml of a 35 % solution, and standard rennet, 30 ml.

After about 45 minutes of rennetting, when the curd became firm enough, it was cut to small cubes. These cubes were then turned, by hand, in the vat until the curd particles reached the desired firmness. This normally took about ten minutes after which the whey was drained. While draining, the curd was stirred by hand, to prevent the cheese particles from matting together. The curd was then placed into forms; it was not pressed but the hoops were turned several times during the first few hours after manufacturing. The cheeses were removed from the hoops 18 hours after they had been placed into them.

The cheeses were now ready for preparing the cheese plates. These were made by cutting the cheeses to round slices weighing about 40 grams each. The slices were placed into Petri dishes and every group of 4 plates wrapped in cellophane paper. The plates were sterilized twice in two following days at 105 °C for 20 minutes, after which they were ready for inoculation with the pure strains.

3. SAMPLING AND CHOICE OF THE PROPER MEDIA FOR COUNTING DIFFERENT MICRO-ORGANISMS ISOLATED FROM THE SURFACE OF LIMBURGER CHEESE

3.1. SAMPLING TECHNIQUE

In a preliminary experiment two methods for sampling were applied for counting and isolating micro-organisms from the surface of Limburger cheese.

3.1.1. *The scraping method*

This method was applied by scraping exactly 16 cm^2 of cheese surface until the whole slime layer, which reaches to a depth of 2 to 3 millimeters of the cheese surface, had been collected. A special apparatus was designed to adjust the cheese surface to be scraped. This apparatus, as shown in Plate 1, consisted of a thick metal frame of 10 × 10 cm, through which 4 thin stainless steel wires were stretched cross-wise, giving an inner square of 16 cm^2. By pressing this frame on the cheese, a block of cheese having a square surface of 16 cm^2 was cut out.

3.1.2. *The core method*

The core method was employed by removing a core of cheese with a thickness of about 2 cm. Only the highest 0.5 cm of the cheese core was used for the analysis. A circular surface of 12.57 cm^2 was obtained by this method.

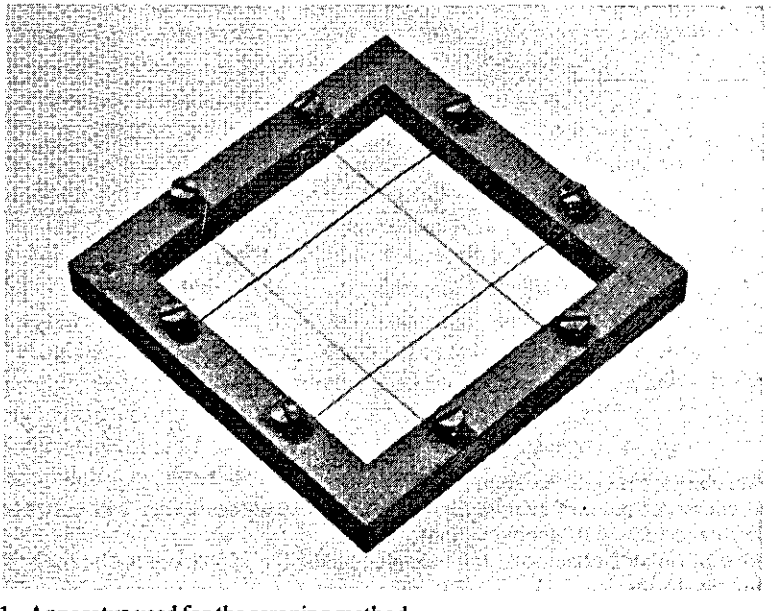


PLATE 1. Apparatus used for the scraping method.

3.1.3. *Results*

3.1.3.1. Total count

With both methods mentioned above, the whole isolated sample was weighed, homogenized in a sterilized mortar and one gram weighed and used for the analysis. Tryptone glucose extract agar (T.G.E.A.) was used as the medium for the total viable count. The results were expressed as total plate count per gram of sample derived from equal areas of cheese surface in order to get as close a comparison, between the two methods, as possible. As it is shown in Tables 1 and 2, it was found that in both experiments A and B the total plate count per gram of sample was higher with the scraping method than with the core method. The values obtained amounted to 2.83×10^{10} and 5.45×10^{10} with the scraping method, and to 1.97×10^{9} and 3.5×10^{9} with the core method in experiments A and B, respectively.

3.1.3.2. Grouping of the isolated micro-organisms

All colonies, growing in a representative sector of the plates, were isolated and purified on yeast extract glucose agar. These isolated pure strains were used for the identification experiment.

3.1.3.2.1. Identification procedure

The isolated strains were tested for the following morphological characteristics:

a. Colour of the colonies on yeast extract glucose agar.

TABLE 1. Comparison between two methods of sampling the surface of Limburger cheese[1] (Exp. A).

Type of organism	Figures expressed as % of the number[2] of isolated organisms							
	Scraping method				Core method			
	C[4]	O	R	G	C	O	R	G
Arthrobacter	58	–	3	9	62.5	–	2.5	7.5
Br. linens	–	16	–	–	–	15	–	–
'Other coryneforms'	9	–	–	–	7.5	–	–	–
Micrococcus	3	–	–	–	–	–	–	–
Sarcina	–	–	–	–	–	–	–	–
'Other rods'[3]	–	–	–	–	–	–	–	–
Yeast	2	–	–	–	5	–	–	–
Total	72	16	3	9	75	15	2.5	7.5

[1] Market cheese, ready for consumption.

[2] Total count per gram of sample derived from equal surface areas: 2.83×10^{10} (scraping method) and 1.97×10^{9} (core method).
Number of isolated organisms: 100 (scraping method) and 80 (core method).

[3] Non-coryneforms.

[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

TABLE 2. Comparison between two methods of sampling the surface of Limburger cheese[1] (Exp. B).

Type of organism	Figures expressed as % of the number[2] of isolated organisms							
	Scraping method				Core method			
	C[4]	O	R	G	C	O	R	G
Arthrobacter	62.5	–	2.5	5	57.1	–	2.4	2.4
Br. linens	–	12.5	–	–	–	11.9	–	–
'Other coryneforms'	8.8	–	–	–	10.7	–	–	–
Micrococcus	2.5	–	–	–	4.8	–	–	–
Sarcina	–	–	–	1.3	–	–	–	2.4
'Other rods'[3]	–	–	–	–	2.1	–	–	–
Yeast	5	–	–	–	7.1	–	–	–
Total	78.8	12.5	2.5	6.3	80.9	11.9	2.4	4.8

[1] Market cheese, ready for consumption.
[2] Total count per gram of sample derived from equal surface areas: 5.45×10^{10} (scraping method) and 3.50×10^{9} (core method).
Number of isolated organisms: 80 (scraping method) and 84 (core method).
[3] Non-coryneforms.
[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

b. Cell form
c. The formation of cocci when grown on casein agar medium.
d. Rod formation when grown on yeast extract glucose agar.

Arthrobacters occurred as rods when grown on yeast extract glucose agar. The rods were of variable length, regularly occurring in V position (snapping) or palisade position. When these strains were grown on casein agar medium, the rods were transformed into coccoid forms. This group included strains of a white, cream, red or greenish-yellow colour.

Br. linens occurred as longer and thinner rods as compared with the arthrobacters. Formation of cocci on casein agar medium was found to be a retarded phenomenon when compared with the quick formation of cocci of the arthrobacters. The colour of the *Br. linens* group was orange; part of the strains required light for pigmentation. Strains belonging to this group were found to resemble the coryneforms in their angular and palisade arrangements of cells.

The group of 'other coryneforms' included those strains having the same morphological implications as the cheese coryneforms but being very late in their transformation from rods into coccoid forms. This group included only strains forming cream-coloured colonies.

The micrococci and sarcina groups as well as the yeasts were recognized microscopically by their cell form. 'Other rods' included those which did not show any of the morphological characteristics of the coryneforms.

3.1.3.2.2. *Results*

The results of the grouping of the isolated strains are shown in Tables 1 and

2. With both methods used and in both experiments A and B the *Arthrobacter* group represented approximately 70% of the total count. In the case of the *Br. linens* group these values varied between 12 and 16%, in the yeast group between 2 and 6% and in the group of 'other coryneforms' it was approximately 9%.

The groups of *Micrococcus*, *Sarcina* and 'other rods' were not playing any important role in the ripening of Limburger cheese and mostly they might have been either contaminants or minor groups.

3.2. The most proper media for counting and isolating different micro-organisms from the surface of Limburger cheese

3.2.1. *Media*

Preliminary experiments showed that two types of media gave the best results for plate count and for the isolation procedures. These two media were the tryptone glucose extract agar (T.G.E.A.), made by supplementing the tryptone glucose extract broth (Oxoid) with 1.2% agar, and the tryptone soya agar (T.S.A.), prepared by adding 1.2% agar to the tryptone soya broth (Oxoid). The pH of both media was adjusted to 7.0. The addition of 4% NaCl to the media was found to have an effect on counting and grouping of the different micro-organisms isolated from the cheese surface.

For the above-mentioned reason it was decided to test both media, T.S.A. and T.G.E.A., for their effect on total plate count and for the grouping of the different micro-organisms within the tested sample. It was also thought to be of some interest to study the effect of both media in the presence and absence of 4% NaCl.

3.2.2. *Results*

Table 3 gives a comparison between the two media, T.G.E.A. and T.S.A., for the total viable count and the grouping of the isolates from the surface of Limburger cheese. Tables 4 and 5 give the response to the addition of 4% NaCl.

3.2.2.1. Total count

Table 3 shows that the tryptone soya agar had a highly beneficial effect on the total plate count when compared with the tryptone glucose extract agar. The total count was more than three times higher when T.S.A. was used (11.0×10^{12} as compared with 3.0×10^{12} per gram on T.G.E.A.).

When 4% NaCl had been added, the total count on both media was nearly doubled (Tables 4 and 5).

The very high values for total count found in this cheese sample may have been due to the occurrence of the majority of micro-organisms in the coccus form.

3.2.2.2. Grouping of the isolated micro-organisms

When the two media used in this experiment were compared for their effect

TABLE 3. Comparison between two types of media for the total count and grouping of micro-organisms isolated from the surface of Limburger cheese[1].

Type of organism	Figures expressed as % of the number[2] of isolated organisms							
	Tryptone soya agar				Tryptone glucose extract agar			
	C[4]	O	R	G	C	O	R	G
Arthrobacter	51.5	–	1.5	4.5	39.3	–	3.6	10.7
Br. linens	–	12.1	–	–	–	5.4	–	–
'Other coryneforms'	18.2	–	–	–	7.1	–	–	–
Micrococcus	3	–	–	–	7	–	–	–
Sarcina	–	–	–	1.5	–	–	–	1.8
'Other rods'[3]	–	–	–	–	3.6	–	–	–
Yeast	6.1	–	1.5	–	14.3	–	7.1	–
Total	78.8	12.1	3	6	71.4	5.4	10.7	12.5

[1] Market cheese, ready for consumption
[2] Total count per gram of scraped surface material: 11×10^{12} (T.S.A.) and 3×10^{12} (T.G.E.A.).
Number of isolated organisms: 66 (T.S.A.) and 56 (T.G.E.A.).
[3] Non-coryneforms.
[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

TABLE 4. Effect of the addition of 4% NaCl to the medium on the total count and grouping of micro-organisms isolated from the surface of Limburger cheese[1].

Type of organism	Figures expressed as % of the number[2] of isolated organisms							
	Tryptone glucose extract agar				Tryptone glucose extract agar + 4% NaCl			
	C[4]	O	R	G	C	O	R	G
Arthrobacter	39.3	–	3.6	10.7	41.9	–	6.5	11.3
Br. linens	–	5.4	–	–	–	9.7	–	–
'Other coryneforms'	7.1	–	–	–	8.1	–	–	–
Micrococcus	7.1	–	–	–	6.5	–	–	–
Sarcina	–	–	–	1.8	–	–	–	3.2
'Other rods'[3]	3.6	–	–	–	1.6	–	–	–
Yeast	14.3	–	7.1	–	9.7	–	1.6	–
Total	71.4	5.4	10.7	12.5	67.8	9.7	8.1	14.5

[1] Market cheese, ready for consumption.
[2] Total count per gram of scraped surface material: 3×10^{12} (T.G.E.A.) and 6.5×10^{12} (T.G.E.A + 4% NaCl).
[3] Non-coryneforms.
[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

TABLE 5. Effect of the addition of 4% NaCl to the medium on the total count and grouping of micro-organisms isolated from the surface of Limburger cheese[1].

Type of organism	Figures expressed as % of the number[2] of isolated organisms							
	Tryptone soya agar				Tryptone soya agar + 4% NaCl			
	C[4]	O	R	G	C	O	R	G
Arthrobacter	48	–	3	9	48.3	–	5	8.3
Br. linens	–	16	–	–	–	16.7	–	–
'Other coryneforms'	10	–	–	–	8.3	–	–	–
Micrococcus	2	–	–	–	–	–	–	–
Sarcina	–	–	–	–	–	–	–	1.7
'Other rods'[3]	–	–	–	–	1.7	–	–	–
Yeast	12	–	–	–	10	–	–	–
Total	72	16	3	9	68.3	16.7	5	10

[1] Market cheese, ready for consumption.
[2] Total count per gram of scraped surface material: 1.83×10^{10} (T.S.A.) and 3.45×10^{10} (T.S.A. + 4% NaCl).
Number of isolated organisms: 100 (T.S.A.) and 120 (T.S.A + 4% NaCl).
[3] Non-coryneforms.
[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

on different groups of isolated strains, T.G.E.A. was found to give higher counts for yeasts than did T.S.A. For the main group, the arthrobacters, T.S.A. gave a percentage of 57.5 of the total count while on T.G.E.A. this figure was 53.6%. A striking difference was shown in the case of the *Br. linens* group and with the group of 'other coryneforms'. On T.S.A., the *Br. linens* group came to 12.1%, on T.G.E.A. to 5.4%. The group of 'other coryneforms' amounted to 18.2% on T.S.A. and 7.1% on T.G.E.A.

When the media had been supplemented with 4% NaCl slightly higher values were obtained with the *Arthrobacter* group, which was met by a decrease of the yeast count. The *Br. linens* percentage of the total was nearly doubled when salt was added to the media (Tables 4 to 10). The group of 'other coryneforms' either did not respond to the addition of salt or became lower.

3.3. DISCUSSION

Two methods were applied for collecting samples from the surface of Limburger cheese, viz. a scraping method and a core method. The total plate count per gram of sample derived from equal areas of cheese surface was found to be higher when estimated with the former than with the latter method. The distribution of different types of micro-organisms was hardly affected by the way of sampling. The differences found in the total count by using the two methods may have been due to the higher amount of cheese material in the samples taken by the core method. For this reason and as it was found to be much easier to

apply the scraping method in covering a larger cheese surface, it was decided to use this method for further studies concerning the sampling of surface growth.

For counting and isolation of the cheese surface micro-organisms, preliminary experiments showed that T.S.A. and T.G.E.A. were the most suitable media. A comparison of the results obtained with the two media showed that, for total viable count, T.S.A. gave more than three times higher values than did T.G.E.A. Of the different groups, *Arthrobacter*, *Br. linens*, and 'other coryneforms', gave higher percentages of the total count on T.S.A. than on T.G.E.A.

The effect of supplementing the media with 4% NaCl was very clear in the total plate counts and also within the grouping of the isolated strains. As it is shown in Tables 4 to 10, the total plate count was about doubled when 4% NaCl was added to the media. The main two groups which were affected by the addition of salt were *Br. linens* and the yeasts. The percentage of colonies belonging to organisms of the *Br. linens* group was nearly doubled in all samples when the media had been supplemented with salt. The reverse was true of the counts of yeast colonies which were lower in the presence of salt. The group of *Arthrobacter* strains responded only slightly to the added salt. The counts of 'other coryneforms' remained either unchanged or were lower upon adding salt.

4. SURVEY OF THE TOTAL COUNTS AND GROUPS OF MICRO-ORGANISMS GROWING ON THE SURFACE OF LIMBURGER CHEESE AND THOSE PRESENT IN THE BRINE AND ON DIFFERENT SHELVES USED FOR CUTTING AND HOLDING THE CHEESE FOR RIPENING

4.1. Introduction

Although preliminary experiments had shown that the *Arthrobacter* group was the predominant group of bacteria on the surface of Limburger cheese, a confirmation of this result was needed before adjusting the identification and isolation procedures concerning this group of organisms. Therefore a complete survey concerning the groups of micro-organisms which could be present and isolated from the cheese surface and the surrounding atmosphere was needed. In this survey isolates were collected from the surface of Limburger cheeses of different ages, from the brine used for the cheese salting, and from different shelves used for laying and for cutting the cheese on.

In order to confirm the previously observed effect of salt on the numbers of micro-organisms growing on the plates, the total plate count was performed on T.S.A. either in the absence or the presence of 4% NaCl. The isolated strains were grown in the light to avoid the absence of the orange pigment in some of the *Br. linens* strains when grown in the dark.

4.2. Results

4.2.1. *Limburger cheese samples of different ages*

Scraped surface material was collected from Limburger cheeses ageing 1,2, 3 or 6 weeks, obtained from different sources. Tables 6 and 7 show the results of the total viable counts and the grouping of the micro-organisms isolated from the cheese surface.

For the total viable counts the values ranged from 2.2×10^{10}, on 1 to 2 weeks old cheese, to 7.3×10^{10} on 6 weeks old Limburger cheese, when 4% NaCl was added to the medium. In the media without added salt, the numbers were 1.45×10^{10} and 6.6×10^{9} in young and old cheese, respectively.

The yeast group made nearly 14% of the total count of young cheese, but was completely absent in older cheeses. On media supplemented with salt the values found were 3.7 and 2.6 on young cheese while no yeasts were found on older cheeses.

The *Arthrobacter* group represented 50.0 and 73.4% of the total count on young and old cheese, respectively. When salt had been added to the media these percentages were 62.2 and 72.0 in young and old cheese, respectively.

The *Br. linens* group was not present on young cheese until the age of approximately 2 weeks. Its percentage amounted to 2.3 and 6.3% in young and old cheese, respectively, while on media with 4% NaCl these values were 3.9 and 14.

TABLE 6. Total count and grouping of micro-organisms isolated from the surface of Limburger cheese.

Sample	Type of organism	Figures expressed as % of the number[1] of isolated organisms									
		Tryptone soya agar					Tryptone soya agar + 4% NaCl				
		C[4]	O	R	G	Total	C	O	R	G	Total
	Arthrobacter	36.1	–	2.8	11.1	70	46.3	–	3.7	12.2	62.2
	Br. linens	–	–	–	–	–	–	–	–	–	–
	'Other coryneforms'	30.6	–	–	–	30.6	25.6	–	–	–	25.6
I[2]	*Micrococcus*	–	–	–	–	–	3.7	–	–	–	3.7
	Sarcina	–	–	–	2.8	2.8	–	–	–	2.4	2.4
	'Other rods'	2.8	–	–	–	2.8	2.4	–	–	–	2.4
	Yeast	11.1	–	2.8	–	13.9	3.7	–	–	–	3.7
	Total	80.6	–	5.6	13.9	100.1	81.7	–	3.7	14.6	100
	Arthrobacter	31.5	–	2.3	11.2	45	50.0	–	7.7	2.6	60.3
	Br. linens	–	2.3	–	–	2.3	–	3.9	–	–	3.9
	'Other coryneforms'	27	–	–	–	27	11.5	–	–	–	11.5
	Micrococcus	10.1	–	–	–	10.1	18	–	–	–	18
II[3]	*Sarcina*	–	–	–	–	–	–	–	–	–	–
	'Other rods'	1.1	–	–	–	1.1	3.9	–	–	–	3.9
	Yeast	13.5	–	–	–	13.5	2.6	–	–	–	2.6
	Mould	–	–	–	1.1	1.1	–	–	–	–	–
	Total	83.2	2.3	2.3	12.3	100.1	86	3.9	7.7	2.6	100.2

[1] Total count per gram of scraped surface material: 1.45×10^{10} (I, T.S.A.), 2.20×10^{10} (I, T.S.A + 4% NaCl), 6.80×10^{9} (II, T.S.A) and 7.2×10^{10} (II, T.S.A + 4% NaCl).
Number of isolated organisms: 72 (I, T.S.A), 82 (I, T.S.A + 4% NaCl), 89 (II, T.S.A) and 78 (II, T.S.A + 4% Na Cl).

[2] I: cheese sample 1–2 weeks old (J. Pinckers, Epen).

[3] II: cheese sample 2 weeks old (P. Pinckers, Neu-Moresnet).

[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

The 'other coryneforms' came to 30.6% of the total count of young cheese and to 20.3% on 6 weeks old cheese when tested on media without added salt. In the presence of 4% NaCl these values were 25.6 to 9.3, respectively.

In this experiment the pigmentation of the colonies was also noted. The yeast group mainly consisted of cream-coloured strains; however, strains with red colonies also occurred. The arthrobacters were white, cream, red or greenish-yellow coloured. In the *Br. linens* group only the orange pigment could be detected. The 'other coryneforms' were all cream-coloured strains.

4.2.2. *The brine samples*

Table 8 shows the results of the analysis of brine samples from different sources. Counted on media without salt, values between 1.52×10^{6} and 1.56×10^{7} for total numbers of micro-organisms per ml of brine were obtained, while on media supplemented with salt these values ranged between 2.42×10^{6} and 4.6×10^{7} per ml.

TABLE 7. Total count and grouping of micro-organisms isolated from the surface of Limburger cheese.

Sample	Type of organism	Figures expressed as % of the number[1] of isolated organisms									
		Tryptone soya agar					Tryptone soya agar + 4% NaCl				
		C[4]	O	R	G	Total	C	O	R	G	Total
I[2]	*Arthrobacter*	54.8	–	–	7.1	61.9	55.3	–	5.3	2.6	63.2
	Br. linens	–	2.4	–	–	2.4	–	5.3	–	–	5.3
	'Other coryneforms'	22.6	–	–	–	22.6	13.2	–	–	–	13.2
	Micrococcus	9.5	–	–	–	9.5	10.5	–	–	–	10.5
	Sarcina	–	–	–	–	–	–	–	–	–	–
	'Other rods'	1.2	–	–	–	1.2	5.3	–	–	–	5.3
	Yeast	–	–	–	–	–	–	–	–	–	–
	Mould	–	–	–	2.4	2.4	–	–	–	2.6	2.6
	Total	88.1	2.4	–	9.5	100	84.3	5.3	5.3	5.2	100.1
II[3]	*Arthrobacter*	63.3	–	6.3	3.8	73.4	62.8	–	7	2.2	72
	Br. linens	–	6.3	–	–	6.3	–	14	–	–	14
	'Other coryneforms'	20.3	–	–	–	20.3	9.3	–	–	–	9.3
	Micrococcus	–	–	–	–	–	4.7	–	–	–	4.7
	Sarcina	–	–	–	–	–	–	–	–	–	–
	'Other rods'	–	–	–	–	–	–	–	–	–	–
	Yeast	–	–	–	–	–	–	–	–	–	–
	Total	83.6	6.3	6.3	3.8	100	76.8	14	7	2.2	100

[1] Total count per gram of scraped surface material: 2.9×10^{10} (I, T.S.A), 3.25×10^{10} (I, T.S.A + 4% NaCl), 6.6×10^{9} (II, T.S.A) and 7.3×10^{10} (II, T.S.A + 4% NaCl).
Number of isolated organisms: 84 (I, T.S.A), 79 (II, T.S.A), 76 (I, T.S.A + 4% NaCl) and 86 (II, T.S.A + 4% NaCl).
[2] I: cheese sample 3 weeks old (J. Pinckers, Epen).
[3] II: cheese sample 6 weeks old (P. Pinckers, Neu-Moresnet, from the cellar).
[4] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

The main groups of micro-organisms found in the brine included: *Arthrobacter*, *Br. linens*, 'other coryneforms', *Micrococcus*, *Sarcina*, 'other rods', yeasts and moulds. The presence of 4% salt in the total counting media gave slightly higher counts of *Arthrobacter*, and considerable larger counts of *Br. linens* and *Micrococcus* (the latter organism was not detected on the plates unless 4% NaCl had been added). The group of 'other coryneforms', that of *Sarcina* and particularly the yeasts occurred in lower numbers on the counting plates when 4% NaCl had been added.

4.2.3. *Scraped material from the shelves*

Tables 9 and 10 show the results of the total counts and the grouping of the micro-organisms isolated from the scraped material of the shelves used for cutting or laying Limburger cheese.

The total counts per gram of scraped material lay between 1.40×10^{5} and 1.19×10^{9} when counted on media without added salt and between 6.50×10^{5} and 2.42×10^{9} on media supplemented with 4% NaCl. The groups of micro-

TABLE 8. Total count and grouping of micro-organisms isolated from the brine.

Sample	Type of organism	Figures expressed % of the number[1] of isolated organisms									
		Tryptone soya agar					Tryptone soya agar + 4% NaCl				
		C[5]	O	R	G	Total	C	O	R	G	Total
	Arthrobacter	36.6	–	5.6	8.5	50.7	31	–	9.5	7.1	47.6
	Br. linens	–	2.8	–	–	2.8	–	14.3	–	–	14.3
	'Other coryneforms'	11.3	–	–	–	11.3	11.9	–	–	–	11.9
	Micrococcus	–	–	–	–	–	14.3	–	–	–	14.3
I[2]	*Sarcina*	–	–	–	8.5	8.5	–	–	–	–	–
	'Other rods'	8.5	–	–	–	8.5	3.6	–	–	–	3.6
	Yeast	14.1	–	2.8	–	16.9	6	–	–	–	6
	Mould	–	–	–	1.4	1.4	–	–	–	2.4	2.4
	Total	70.5	2.8	8.4	18.4	100.1	66.8	14.3	9.5	9.5	100.1
	Arthrobacter	54.8	–	3.2	3.2	61.2	52.8	–	–	–	52.8
	Br. linens	–	–	–	–	–	–	1.4	–	–	1.4
	'Other coryneforms'	6.5	–	–	–	6.5	5.6	–	–	–	5.6
	Micrococcus	–	–	–	–	–	13.9	–	–	–	13.9
II[3]	*Sarcina*	–	–	–	19.4	19.4	–	–	–	9.7	9.7
	'Other rods'	3.2	–	–	–	3.2	–	–	–	–	–
	Yeast	4.8	–	4.8	–	9.6	8.3	–	5.6	–	13.9
	Mould	–	–	–	–	–	–	–	–	2.8	2.8
	Total	69.3	–	8	22.6	99.9	80.6	1.4	5.6	12.5	100.1
	Arthrobacter	33.3	–	3	3	39.3	44.1	–	1.5	4.4	50
	Br. linens	–	3	–	–	3	–	5.9	–	–	5.9
	'Other coryneforms'	12.1	–	–	–	12.1	5.9	–	–	–	5.9
	Micrococcus	–	–	–	–	–	17.7	–	–	–	17.7
III[4]	*Sarcina*	–	–	–	16.8	16.8	–	–	–	5.9	5.9
	'Other rods'	6.1	–	–	–	6.1	2.9	–	–	–	2.9
	Yeast	21.2	–	1.5	–	22.7	8.8	–	2.9	–	11.7
	Total	72.7	3	4.5	19.8	100	79.4	5.9	4.4	10.3	100

[1] Total count per ml of brine: 1.52×10^6 (I, T.S.A), 1.56×10^7 (II, T.S.A), 9.6×10^6 (III, T.S.A), 2.42×10^6 (I, T.S.A + 4% NaCl), 4.07×10^7 (II, T.S.A + 4% NaCl) and 4.6×10^7 (III, T.S.A + 4% NaCl).
Number of isolated organisms: 71, 62, 66 (I, II and III, respectively on T.S.A) and 84, 72 and 68(I, II and III, respectively on T.S.A + 4% NaCl).
[2] I: Brine sample from P. Pinckers, Neu-Moresnet.
[3] II: Brine sample from J. Pinckers, Epen.
[4] III: Brine sample from P. Geelen, Berg en Terblijt.
[5] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

-organisms found on the shelves included: *Arthrobacter*, *Br. linens*, 'other coryneforms', *Micrococcus*, *Sarcina*, 'other rods', yeasts and moulds. The arthrobacters formed the main group (Tables 9 and 10). However, in most cases yeasts occurred in numbers nearly as high as those of the arthrobacters. *Br. linens* occurred in small numbers, sometimes being absent; 'other coryneforms' were found in moderate amounts.

TABLE 9. Total count and grouping of micro-organisms isolated from scraped material from different shelves.

Sample	Type of organism	Figures expressed as % of the number[1] of isolated organisms									
		Tryptone soya agar					Tryptone soya agar ± 4% NaCl				
		C[5]	O	R	G	Total	C	O	R	G	Total
I[2]	*Arthrobacter*	26.9	–	–	10.1	37	31.9	–	2.8	5.6	40.3
	Br. linens	–	–	–	–	–	–	–	–	–	–
	'Other coryneforms'	12.7	–	–	–	12.7	16.7	–	–	–	16.7
	Micrococcus	–	–	–	–	–	–	–	–	–	–
	Sarcina	–	–	–	3.8	3.8	–	–	–	1.4	1.4
	'Other rods'	2.5	–	–	–	2.5	–	–	–	–	–
	Yeast	20.3	–	10.1	–	30.4	16.7	–	8.3	–	25
	Mould	2.5	–	1.3	10.1	13.9	2.8	–	–	13.9	16.7
	Total	64.9	–	11.4	24	100.3	68.1	–	11.1	20.9	100.1
II[3]	*Arthrobacter*	25.8	–	–	–	25.8	40.8	–	–	–	40.8
	Br. linens	–	–	–	–	–	–	–	–	–	–
	'Other coryneforms'	13.6	–	–	–	13.6	7.9	–	–	–	7.9
	Micrococcus	–	–	–	–	–	7.9	–	–	–	7.9
	Sarcina	–	–	–	6.1	6.1	–	–	–	4	4
	'Other rods'	4.6	–	–	–	4.6	5.3	–	–	–	5.3
	Yeast	28.8	–	12.1	–	40.9	15.8	–	13.2	–	29
	Mould	–	–	–	9.1	9.1	–	–	–	5.3	5.3
	Total	72.8	–	12.1	15.2	100.1	77.7	–	13.2	9.3	100.2
III[4]	*Arthrobacter*	28.4	–	2.7	5.4	36.5	40.7	–	7.4	6.2	54.3
	Br. linens	–	2.7	–	–	2.7	–	7.4	–	–	7.4
	'Other coryneforms'	13.5	–	–	–	13.5	8.6	–	–	–	8.6
	Micrococcus	–	–	–	–	–	6.2	–	–	–	6.2
	Sarcina	–	–	–	2.7	2.7	–	–	–	–	–
	'Other rods'	6.8	–	–	–	6.8	–	–	–	–	–
	Yeast	16.2	–	14.9	–	31.1	12.4	–	7.4	–	19.8
	Mould	–	–	–	6.7	6.7	–	–	–	3.7	3.7
	Total	64.9	2.7	17.6	14.8	100	67.9	7.4	14.8	9.9	100

[1] Total count per gram of scraped surface material: 1.40×10^5 (I), 2.46×10^8 (II) and 1.19×10^9 (III) on T.S.A and 6.50×10^5 (I), 3.75×10^8 (II) and 2.42×10^9 (III) on T.S.A + 4% NaCl. Number of isolated organisms: 79, 66, 74 (I, II and III, respectively on T.S.A) and 72, 76, 81 (I, II and III, respectively on T.S.A + 4% NaCl).

[2] I: Shelves used for cutting the cheeses.

[3] II: Shelves holding one week old cheese.

[4] III: Shelves in the ripening room holding no cheese at the sampling time.

[5] Colour: C, cream + white; O, orange; R, red; G, greenish-yellow.

TABLE 10. Total count and grouping of micro-organisms isolated from scraped material from shelves and part of the ripening room wall in which the shelves were fixed.

Sample	Type of organism	Figures expressed as % of the number[1] of isolated organisms									
		Tryptone soya agar					Tryptone soya agar ± 4% NaCl				
		C[5]	O	R	G	Total	C	O	R	G	Total
	Arthrobacter	17.4	–	7	11.6	36	17.4	–	17.4	8.7	43.5
	Br. linens	–	2.3	–	–	2.3	–	4.4	–	–	4.4
	'Other coryneforms'	25.6	–	–	–	25.6	17.4	–	–	–	17.4
	Micrococcus	–	–	–	–	–	–	–	–	–	–
I[2]	*Sarcina*	–	–	–	1.2	1.2	–	–	–	–	–
	'Other rods'	4.7	–	–	–	4.7	2.9	–	–	–	2.9
	Yeast	14	–	8.1	–	22.1	17.4	–	8.7	–	26.1
	Mould	–	–	–	8.1	8.1	–	–	–	5.8	5.8
	Total	61.7	2.3	15.1	20.9	100	55.1	4.4	26.1	14.5	100.1
	Arthrobacter	24.3	–	4.1	10.8	39.2	40.3	–	9.7	4.8	54.8
	Br. linens	–	5.4	–	–	5.4	–	16.1	–	–	16.1
	'Other coryneforms'	16.2	–	–	–	16.2	9.7	–	–	–	9.7
	Micrococcus	–	–	–	–	–	–	–	–	–	–
II[3]	*Sarcina*	–	–	–	4.1	4.1	–	–	–	–	–
	'Other rods'	2.7	–	–	–	2.7	–	–	–	–	–
	Yeast	18.9	–	9.5	–	28.4	16.1	–	3.2	–	19.3
	Mould	–	–	–	4.1	4.1	–	–	–	–	–
	Total	62.1	5.4	13.6	19	100.1	66.1	16.1	12.9	4.8	99.9
	Arthrobacter	43.7	–	4.2	11.3	59.2	47.5	–	7.5	7.5	62.5
	Br. linens	–	–	–	–	–	–	2.5	–	–	2.5
	'Other coryneforms'	16.9	–	–	–	16.9	17.5	–	–	–	17.5
	Micrococcus	–	–	–	–	–	7.5	–	–	–	7.5
III[4]	*Sarcina*	–	–	–	4.2	4.2	–	–	–	–	–
	'Other rods'	5.6	–	–	–	5.6	7.5	–	–	–	7.5
	Yeast	8.5	–	–	–	8.5	–	–	–	–	–
	Mould	–	–	–	5.6	5.6	–	–	–	2.5	2.5
	Total	74.7	–	4.2	21.1	100	80	2.5	7.5	10	100

[1] Total count per gram of scraped surface material: 4.80×10^6 (I), 7.95×10^8 (II) and 9.70×10 (III) on T.S.A and 6.15×10^6 (I), 1.42×10^9 (II) and 4.78×10^8 (III) on T.S.A + 4% NaCl. Number of isolated organisms: 86, 74, 71 (I, II and III, respectively on T.S.A) and 69, 62, 80 (I, II and III, respectively on T.S.A + 4% NaCl).

[2] I: Shelves used for cutting the cheeses.

[3] II: Shelves in the ripening room holding no cheese at the sampling time.

[4] III: Part of the ripening room walls in which the shelves were fixed.

[5] Colour: C, cream + white; O, ogange; R, red; G, greenish-yellow.

The presence of 4% NaCl in the medium largely depressed the numbers of yeast colonies; the 'other coryneforms' were mostly also depressed. Arthrobacters and particularly *Br. linens* occurred in larger numbers on media supplemented with salt.

4.3. Discussion

In the literature the significance of *Br. linens* as one of the surface organisms of Limburger cheese is well documented. This bacterium has usually been found to be present in large numbers in the slimy growth on the surface of this particular type of cheese (Wolff, 1909; Steinfatt, 1930; Kelly, 1937 and Albert et al., 1944). Yale (1943) was the first to mention that the predominant organism isolated from surface-ripened cheeses was a Gram-positive, non-chromogenic, non-spore-forming rod which produced a marked alkaline reaction in litmus milk and did not liquefy gelatin.

Mulder et al. (1966), in their investigation of the relationship between *Br. linens* and bacteria of the genus *Arthrobacter*, studied the occurrence of the coryneform bacteria on cheese surfaces. On Limburger cheese they found that approximately 90% of the total surface flora were coryneforms. From 10 to 25% of the total flora were orange coryneforms, 65–80% grey-white and 2.0 to 3.0% yellow coryneforms.

Preliminary studies of the author showed that the main micro-organisms to be found on Limburger cheese surface included: arthrobacters, *Br. linens*, 'other coryneforms' and yeasts. To confirm these results, a more extensive survey was made which included the surface flora of Limburger cheese as well as the micro-organisms of the brine and of the shelves of the ripening room.

From the results obtained (Tables 6 and 7) it appeared that on young cheeses the main three groups of micro-organisms were yeasts, arthrobacters and 'other coryneforms'. On older cheeses the yeasts had disappeared and the 'other coryneforms' had declined to a lower percentage. The arthrobacters continued to be the predominant micro-organisms on the cheese surface. The *Br. linens* group was not present in very young cheese, but its percentage increased upon ageing. However, this group did not exceed a percentage of 14%, a value found on 6 weeks old cheese when tested on a medium containing 4% NaCl. These results are in agreement with those found by Mulder et al. (1966). It should be stressed that the percentage of colonies belonging to organisms of the *Br. linens* group was always higher when counted on media with 4% NaCl than on media without this amount of added salt, a phenomenon which was also found in the group of *Micrococcus*. Yeast colonies were present in lower percentages when the agar media had been supplemented with salt. This was also true of the *Sarcina* group.

The brine flora consisted of the same series of micro-organisms as found on the cheese surface except for the presence of moulds which were nearly absent on the cheese surface. The main group of organisms was that of the arthrobacters followed by that of yeasts, *Sarcina* and 'other coryneforms'. When 4% NaCl

had been added to the counting media, *Br. linens* and *Micrococcus* appeared in moderate numbers, whereas they were absent *(Micrococcus)* or nearly absent *(Br. linens)* on agar media without added salt.

The organisms isolated from the scraped material of the shelves were mainly arthrobacters and yeasts with small numbers of *Br. linens* and 'other coryneforms'.

5. MORPHOLOGICAL CHARACTERS AND NUTRITIONAL REQUIREMENTS OF THE MICRO-ORGANISMS ISOLATED FROM THE SURFACE OF LIMBURGER CHEESE

5.1. INTRODUCTION

The morphological characters and the nutritional requirements of the micro-organisms isolated from the surface of Limburger cheese have been studied by several workers (WOLFF, 1909, 1910; STEINFATT, 1930; KELLY, 1937; KELLY and MARQUARDT, 1939; YALE, 1943; MULDER, 1964 and MULDER et al., 1966). There is no complete agreement between the different authors as to the taxonomical position of the organisms concerned. Therefore, it was decided by the present author to run further ecological and taxonomical examinations (dealing with morphological and nutritional aspects) using 251 representative strains isolated from the surface of Limburger cheeses of different ages and thought to be responsible for the ripening process. These strains represented the main three groups, viz. *Arthrobacter*, *Brevibacterium linens* and 'other coryneforms', which were found to be present during the ripening of Limburger cheese. It should be kept in mind that the name *Arthrobacter* is used in the present work as well as in other publications (MULDER and ANTHEUNISSE, 1963; MULDER, 1964 and MULDER et al., 1966) for a type of cheese coryneform bacteria possessing the morphological characters (rods of irregular form and size in the young stage, cocci upon ageing) of this genus which is mainly including soil coryneforms. As several differences, mainly physiological, exist between these cheese coryneforms and the arthrobacters from soil, MULDER et al. (1966) suggested that the cheese coryneforms should not be placed in the genus *Arthrobacter*. No further examinations were carried out with the other groups of micro-organisms like *Micrococci*, *Sarcinae*, 'other rods', yeasts and moulds.

The 251 strains were classified as follows:

I. *Arthrobacter* group
   a. Cream and white-coloured (62)
   b. Grey-white (42)
   c. Red (36)
   d. Greenish-yellow (33)

II. *Brevibacterium linens* group
   a. Strains with an orange colour in both, dark and light (28)
   b. Strains with an orange colour in the light only (24)

III. Group of 'other coryneforms' cream-coloured (26)

5.2. MORPHOLOGICAL CHARACTERS OF THE ISOLATED GROUPS OF STRAINS

Earlier workers described *Br. linens* as the main ripening agent in Limburger cheese. They described this bacterium as a short, unbranched rod without any of the implicalions of the coryneform bacteria (BERGEY's Manual, 1957).

STADHOUDERS and MULDER (1958) observed that *Br. linens* resembled the corynebacteria in their angular and palisade arrangements of the cells. MULDER et al. (1966) stated the close relationship between orange cheese coryneforms of the *Br. linens* type, and the grey-white cheese arthrobacters.

Bacteria of the genus *Arthrobacter* are characterized mainly by their cell morphology. Like the other members of the *Corynebacteriaceae*, they show a marked diversity of cell form and size. In the young stages they often occur as rods of irregular shape and length and they are sometimes branched. Upon ageing, the cultures largely consist of oval or coccoid cells.

MULDER et al. (1966) found that the grey-white cheese arthrobacters, generally formed shorter rods and had a more pronounced tendency to form cocci as compared with soil arthrobacters. Most of their cheese strains occurred in the coccus state after 24 hr and often even after 12 hr of incubation at 30 °C. As for the orange-pigmented cultures of the *Br. linens* type, they found them forming slender rods of irregular shape, frequently showing snapping division, palisade arrangement of cells or flocculent growth. The tendency of these bacteria to form cocci was much less pronounced and in this respect they more closely resembled the soil arthrobacters than the grey-white coryneforms from cheese.

5.2.1. *The Arthrobacter group*

5.2.1.1. The cream and white-coloured strains

Preliminary experiments showed that the morphology of cheese arthrobacters depends on the age of the culture, the nutritional conditions and the incubating temperature. Plate 2 (A-D) is showing a cream-coloured *Arthrobacter* strain grown on a rich (yeast-extract glucose agar) and a poor medium (casein agar) and at low and high temperatures (15 and 30 °C), respectively. When the strain was cultivated for 24 hr at 30 °C on the rich medium (A) it formed short and thick rods. When grown on the poor medium for 5 days at 30 °C (B) it had formed coccoid cells.

When the strain growing on the rich medium was incubated at a low temperature (15 °C) for 24 hr (C) the rods became longer. When this culture was incubated at a higher temperature (30 °C) for 24 hr (D), the rods had been transformed to cocci.

5.2.1.2. Grey-white strains

Plate 3 is showing a grey-white *Arthrobacter* strain growing for 24 hr at 30 °C on a yeast extract glucose agar (A) and for 5 days on a casein agar medium at 30 °C (B). It is clear that this group possesses the same morphological characters. as the group of cream and white-coloured arthrobacters. They generally had also the same characteristics as to the rod-coccus transformation.

5.2.1.3. The red strains

The morphological characters of this group of strains are shown in Plate 4. After 24 hr of growth on the rich medium (Y.E.G.A.) at 30 °C (A) the rods were predominant. When grown for 5 days at 30 °C on the poor medium (casein

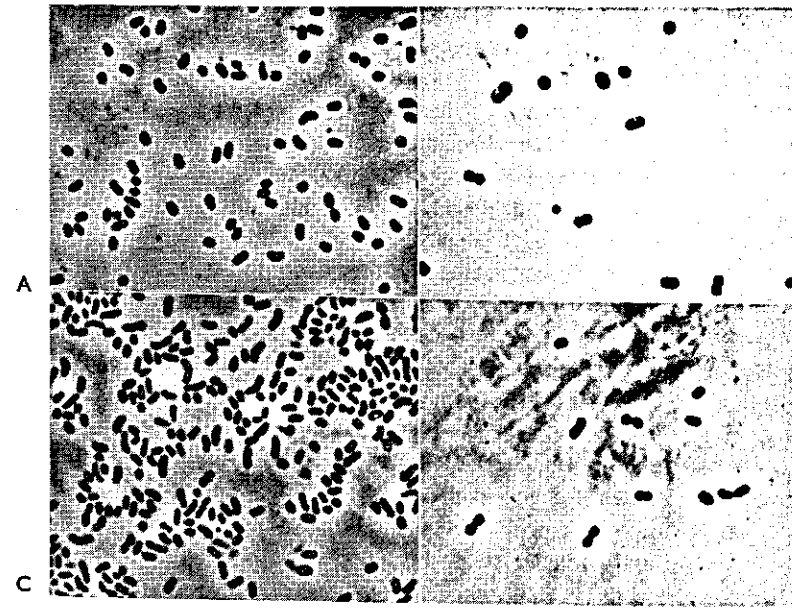


PLATE 2. Cream-coloured *Arthrobacter* strain No. 62 (× 1625)
A. On a rich medium after 24 hr at 30 °C
B. On a poor medium after 5 days at 30 °C
C. On a rich medium after 24 hr at 15 °C
D. C after 24 hr at 30 °C

agar) the rods became shorter and the coccoid forms appeared and predominated (B).

5.2.1.4. Greenish-yellow strains

Plates 5 and 6 are showing the morphological characters of two strains of this group grown under different nutritional conditions and at different temperatures of incubation.

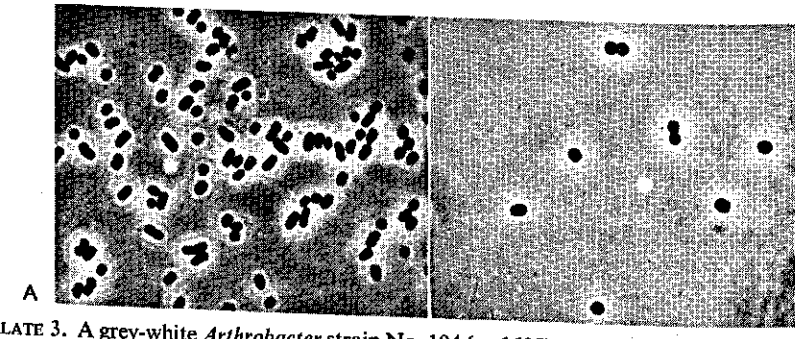


PLATE 3. A grey-white *Arthrobacter* strain No. 104 (× 1625)
A. On a rich medium after 24 hr at 30 °C
B. On a poor medium after 5 days at 30 °C

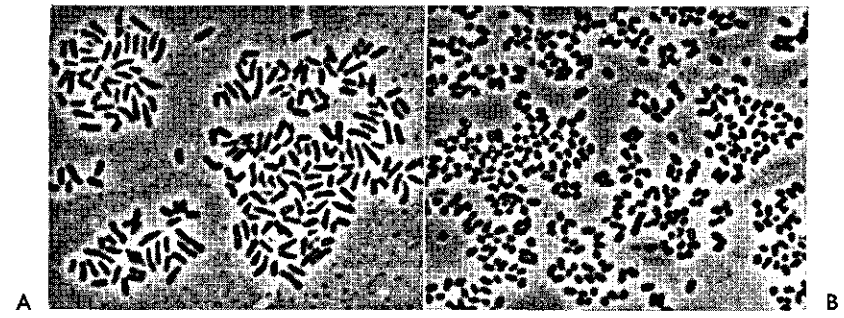


PLATE 4. A red *Arthrobacter* strain No. 140 (× 1625)
A. On a rich medium after 24 hr at 30 °C
B. On a poor medium after 5 days at 30 °C

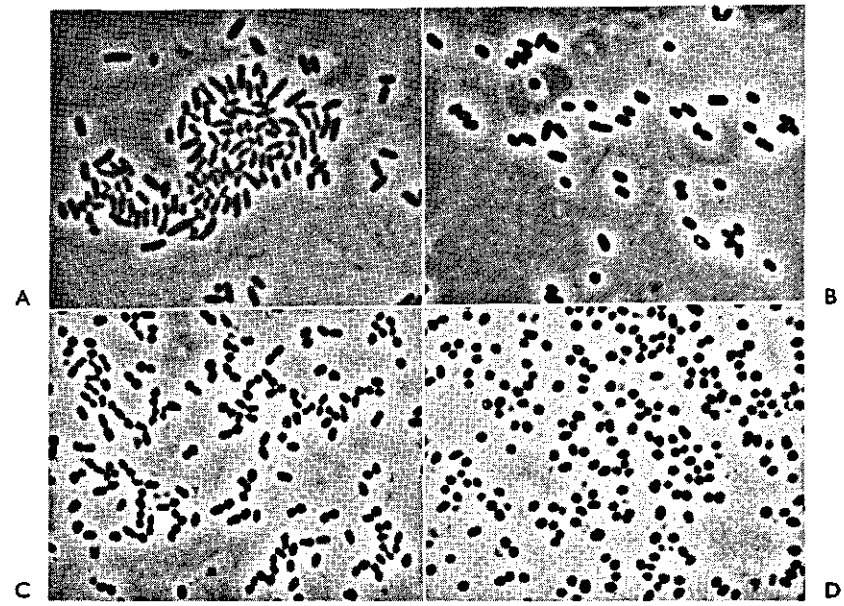


PLATE 5. A greenish-yellow *Arthrobacter* strain No. 171 (× 1625) grown on a rich medium:
A. After 24 hr at 15 °C
B. After 12 hr at 30 °C
C. After 24 hr at 30 °C
D. After 48 hr at 30 °C

Plate 5 shows the life cycle of a greenish-yellow strain when grown on yeast extract glucose agar for 24 hr at 15 °C (A) and then incubated at 30 °C for 12 (B), 24 (C) and 48 hr (D). It is clear that the long rods became shortened and thicker upon ageing until the coccus stage predominated all over the culture.

Plate 6 is showing the effect of temperature and nutritional conditions on the rod-coccus transformation of another greenish-yellow strain. When the organism was grown at 15 °C for 24 hr (A) on the poor medium the rods were slender and long as compared with those grown for 12 hr on the rich medium at 15 °C (E). When grown for 24 hr on the rich medium at 30 °C, the rods were thicker

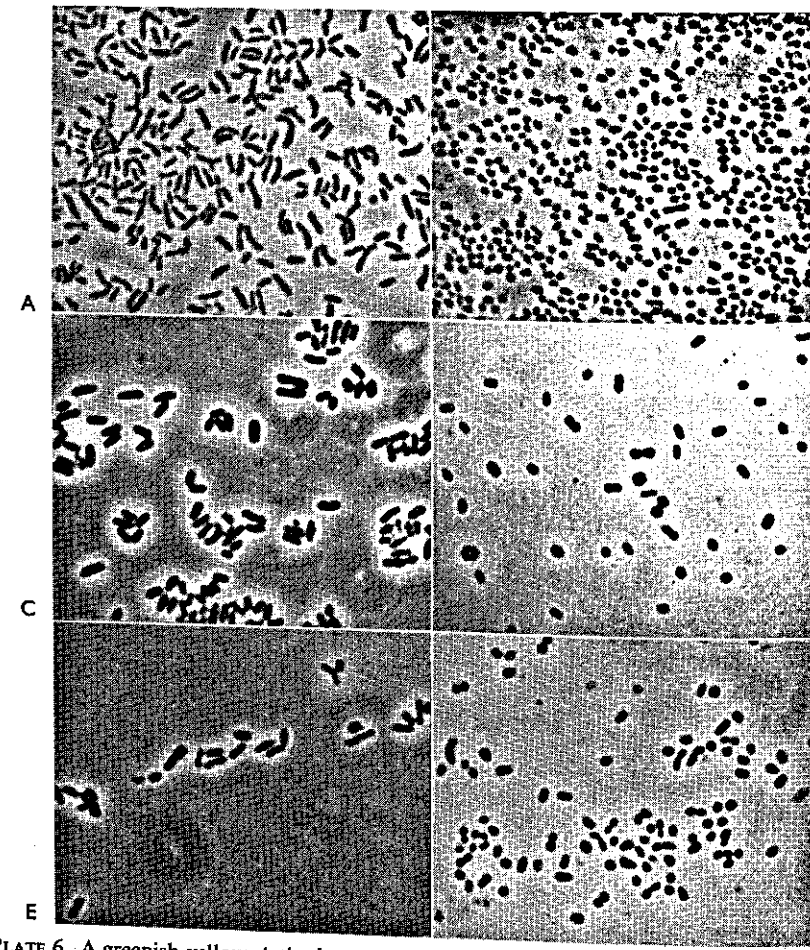


PLATE 6. A greenish-yellow *Arthrobacter* strain No. 173 (× 1625)
A. After 24 hr on a poor medium at 15 °C
B. After 5 days on a poor medium at 30 °C
C. After 24 hr on a rich medium at 30 °C
D. 24 hr after inoculation from A on a rich medium at 30 °C
E. After 12 hr at 15 °C on a rich medium
F. E after 6 hr on a rich medium at 30 °C

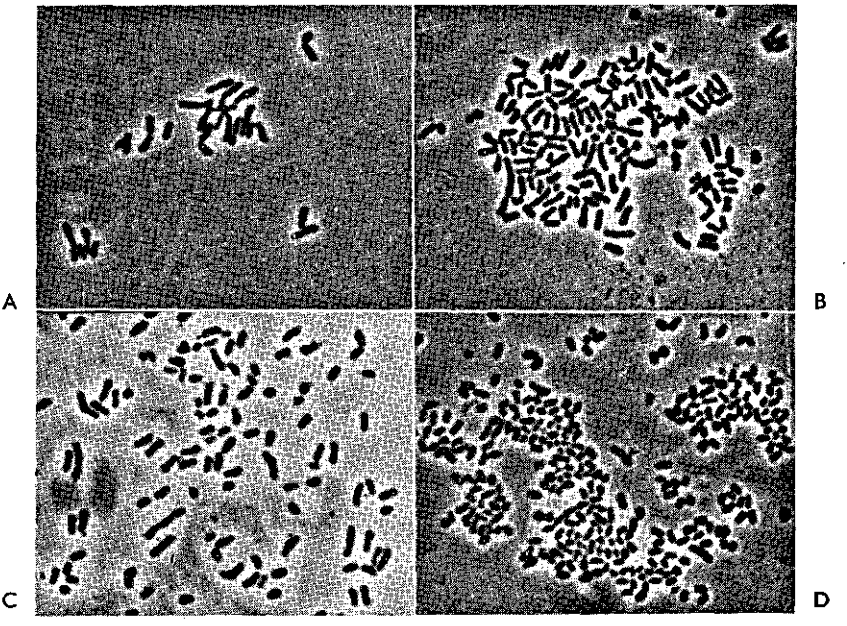


PLATE 7. Two orange cheese strains (*Br. linens* type) No. 200 and 204 (× 1625)
A. Strain 200 after 24 hr on a rich medium at 30 °C
B. After 5 days on a poor medium at 30 °C
C. Strain 204 after 24 hr on a rich medium at 15 °C
D. C, after 24 hr on a rich medium at 30 °C

and shorter as compared with (A) and (E). The coccoid forms were larger when inoculation material from A was grown on the rich medium for 24 hr (D) than when the organism was grown for 5 days in poor medium (B). Many of these strains occurred in the coccus state after 24 hr and often even after 6 hr (F) of incubation.

5.2.2. *Br. linens group*

In this experiment use was made of 2 orange strains isolated from the surface of Limburger cheese (Plate 7) and 4 strains from the American Type Culture Collection (Plate 8). The latter were ATCC 9172, ATCC 9174, ATCC 9175 and ATCC 8377. Plate 7 shows the effect of temperature and nutritional conditions on the morphology of the orange cheese strains. When grown on a rich medium at 30 °C or 15 °C for 24 hr, rods were predominating (A + C). They were relatively long and slender and of irregular shape. The ability of forming cocci (B, D) in this group of strains was less pronounced as compared with the group of arthrobacters.

Plate 8 shows the strains of the American Type Culture Collection grown on a rich medium for 24 hr at 15 °C and then incubated at 30 °C for another 24 hr.

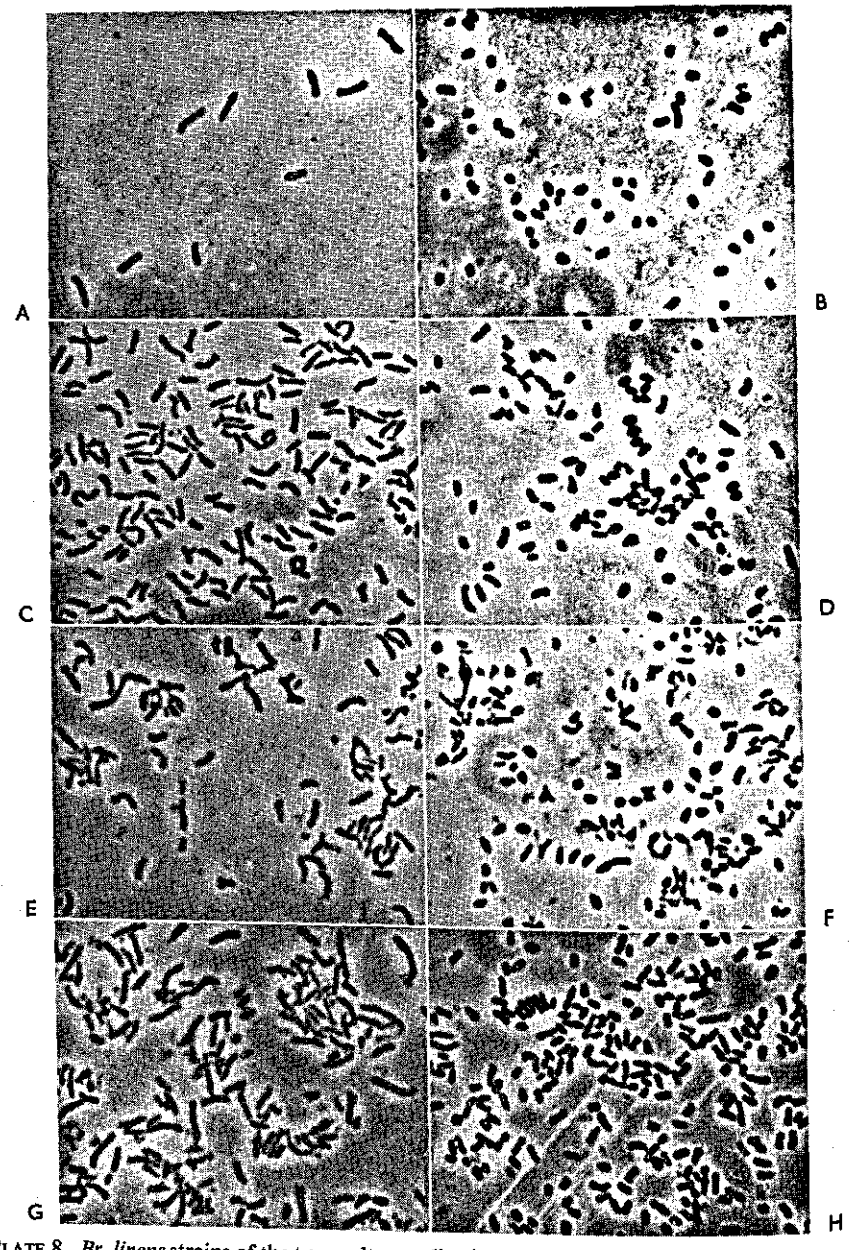


PLATE 8. *Br. linens* strains of the type culture collection (× 1625)
A and B, ATCC 9172
C and D, ATCC 9174
E and F, ATCC 9175
G and H, ATCC 8377
A, C, E and G, after 24 hr on a rich medium at 15 °C
B, D, F and H, the same cultures after another 24 hr at 30 °C

It is clear that these strains form slender rods showing snapping division and palisade arrangement. Branching rods were also often found in this group of organisms (C, E and G). The strains differed in their ability of coccus formation, as seen in B and H. In general the tendency to form coccoids was less pronounced as compared with the *Arthrobacter* strains.

5.2.3. *The group of 'other coryneforms'*

Plate 9 shows the morphological characters of a representation of this group

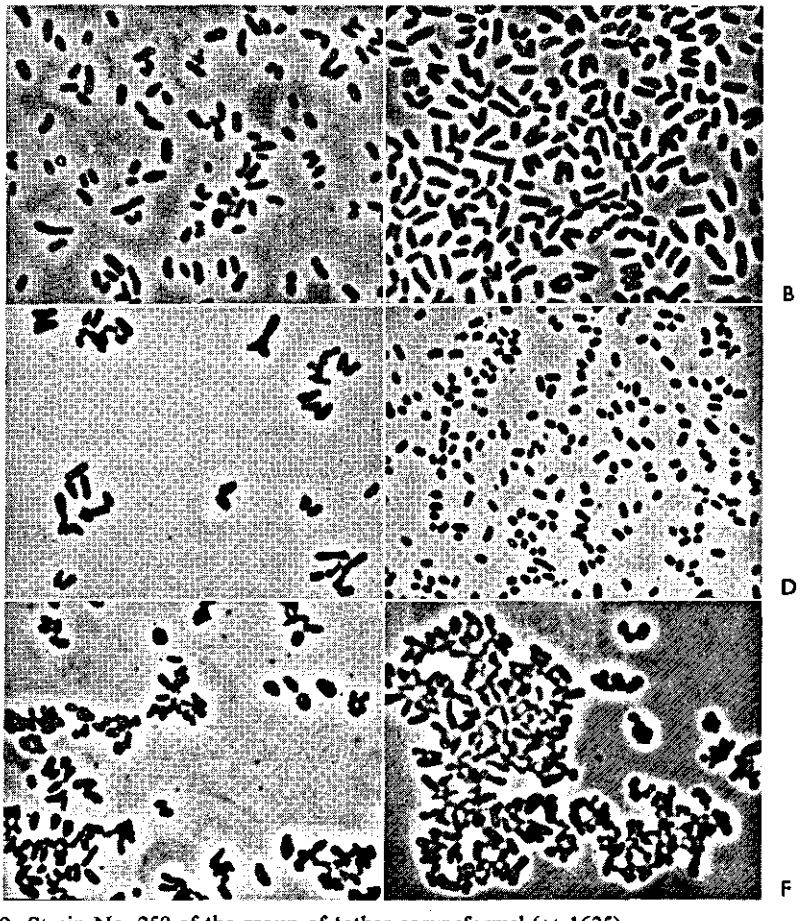


PLATE 9. Strain No. 250 of the group of 'other coryneforms' (× 1625)
A. After 24 hr on a rich medium at 15 °C
B. A after another 24 hr at 30 °C
Corynebacterium bovis (ATCC 13722) (× 1625)
C. After 24 hr on a rich medium at 15 °C
D. C after another 24 hr at 30 °C
Corynebacterium bovis (NCTC 3224) (× 1625)
E. After 24 hr on a rich medium at 15 °C
F. E after another 24 hr at 30 °C

of strains, isolated from the cheese surface (A + B), and those of two type culture collection strains of *Corynebacterium bovis*, which were included for comparison. The isolated cheese strain showed a very strongly retarded tendency of forming cocci. The rods were long and thick with all the implications of corynebacteria. One of the type culture collection strains, viz. *C. bovis*, ATCC 13722, proved to have the morphological characters of cheese arthrobacters as shown in C + D. The other strain (NCTC 3224) was similar to the cheese strain in its tendency to strongly retarded coccus formation (E + F).

5.3 Effect of light on pigmentation of the isolated strains

To find the effect of light on pigmentation of the bacteria, a comparison was made between growth and pigment formation in the dark and in the light. The 251 isolated strains were inoculated in duplicate on yeast extract glucose agar. One set of cultures was exposed to light (Philips TL 40W/34), by incubating the isolates in an illuminated incubator at 25 °C, the other set was incubated in the same incubator, but covered with a wooden box to keep them in the dark.

A comparison of the colour of both sets was made after 10 days of incubation. The results of this experiment are shown in Table 11.

5.3.1. *Arthrobacter group*

The white, cream, grey-white and red *Arthrobacter* strains were not affected by light in the course of pigmentation. All of these strains, 140, were found to have the same colour in both dark and light.

For the greenish-yellow arthrobacters, a clear and pronounced effect of light on their pigmentation was noticed. When grown in the dark a grey-white or light cream-yellow colour was obtained, while in the light the colonies formed were greenish-yellow.

Table 11. Effect of light on the pigmentation of the groups of isolated strains.

Type of organism	Number of strains examined	Colour of colonies grown in the	
		Dark	Light
Arthrobacter			
Cream	62	Cream	Cream
Grey-white	42	Grey-white	Grey-white
Red	36	Red	Red
	11	Light cream-yellow	Greenish-yellow
Greenish-yellow	22	Grey-white	Greenish-yellow
Br. linens			
Orange	28	Orange	Orange
Cream	24	Cream	Orange
Other coryneforms			
Cream	26	Cream	Cream

5.3.2. *Br. linens group*

This group of strains could be divided into two groups according to the effect of light on their pigmentation. The first group included 28 strains which were not affected by light, the orange colour developing under both light and dark conditions. The second group of 24 strains included cultures that required light to induce the orange pigment, in the dark a cream colour was obtained.

5.3.3. *The group of 'other coryneforms'*

The 26 strains belonging to this group were not affected by light; the colonies had a cream colour in the dark as well as in the light.

5.4. Salt tolerance of the isolated coryneforms

The representative strains were incubated at 30°C in Erlenmeyer flasks containing yeast extract glucose medium and different amounts of sodium chloride. The pH of the medium was adjusted to 7.0. The cultures were aerated on a gyratory shaker. The results of this experiment, recorded after 31 days of incubation, are shown in Table 12. Growth was estimated by measuring the turbidity of the culture compared with that of an uninoculated blank.

5.4.1. *Arthrobacter group*

All of the 173 *Arthrobacter* strains tolerated a concentration up to 9% of NaCl in the medium. The 33 greenish-yellow strains tolerated 12% NaCl whereas only about one third of the cream + white and red strains and one fifth of the grey-white strains were able to grow at such a salt concentration. No growth was recorded with 16 and 20% salt.

Table 12. Effect of salt on the growth of the isolated cheese coryneforms.

Type of organism	Number of tested strains	Number of strains growing in the presence of added salt (%)							
		0	2	4	6	9	12	16	20
Arthrobacter									
Cream	62	62	62	62	62	62	22	–	–
Grey-white	42	42	42	42	42	42	8	–	–
Red	36	36	36	36	36	36	12	–	–
Greenish-yellow	33	33	33	33	33	33	33	–	–
Br. linens									
Orange[1]	28	28	28	28	28	28	28	12	–
Cream[2]	24	24	24	24	24	24	24	6	–
'Other coryneforms' (cream)	26	26	26	26	26	26	4	–	–

[1] In the dark and in light.
[2] In the dark, orange in light.

5.4.2. *Br. linens group*

The 52 strains of the *Br. linens* type grew in the yeast extract glucose medium in the presence of 12% NaCl. With 16% salt only 12 of the 28 orange strains and 6 of the 24 white strains were able to grow. None of the strains was able to grow with 20% NaCl in the medium.

5.4.3. *The group of 'other coryneforms'*

This group followed the same trend as the grey-white arthrobacters. The strains tolerated a salt concentration up to 9%, but when the concentration was raised to 12% only 4 strains out of 26 could stand it. At concentrations of 16 and 20% of NaCl, no growth was obtained.

5.5. Gram stain

According to Conn and Dimmick (1947), soil arthrobacters would be Gram-negative in the rod form and Gram-positive in the coccus form. Mulder and Antheunisse (1963), see also Mulder (1964), studied the Gram-reaction of a large number of arthrobacters from different origin in both rod and coccus forms. They found that the majority of the *Arthrobacter* strains isolated from cheese were Gram-positive in both rod and coccoid forms.

In the present experiment, the rods used for the Gram staining were derived from a 24 hr old culture grown at 25 °C on a yeast extract glucose agar medium and the cocci from a 10 days old culture grown at 30 °C on a casein agar medium.

All the 251 isolated strains were found to be Gram-positive in both the rod and the coccus stages.

5.6. Nutritional requirements

5.6.1. *Utilization of carbon compounds*

Table 13 shows the utilization of different carbon compounds by the different isolated strains.

5.6.1.1. Glucose

All of the 251 strains were able to utilize glucose as the only carbon source in the medium.

5.6.1.2. Sucrose

Only 6 strains belonging to the grey-white and greenish-yellow arthrobacters, were able to utilize sucrose. The rest of the arthrobacters, the *Br. linens* and the 'other coryneforms' were unable to utilize sucrose as the only carbon source.

5.6.1.3. Lactose

Only 1/5 of the cream + white-coloured arthrobacters were able to utilize lactose. Of the grey-white, red and greenish-yellow strains, a larger percentage

TABLE 13. Utilization of carbon compounds by the isolated cheese coryneforms.

Type of organism	Number of tested strains	Number of strains utilizing				Catalase test		Number of strains liquefying gelatin
		Glucose	Sucrose	Lactose	Lactate	+	–	
Arthrobacter								
Cream	62	62	–	12	62	62	–	4
Grey-white	42	42	4	20	42	42	–	42
Red	36	36	–	16	36	36	–	16
Greenish-yellow	33	33	2	14	33	33	–	33
Br. linens								
Orange[1]	28	28	–	2	28	28	–	28
Cream[2]	24	24	–	1	24	24	–	24
'Other coryneforms' (cream)	26	26	–	–	26	26	–	–

[1] In the dark and in light.
[2] In the dark, orange in light.

(approximately 40%) were able to utilize this sugar (Table 13). Only 3 of the 52 strains of *Br. linens* were able to utilize lactose, whereas none of the 'other coryneforms' was able to use it.

5.6.1.4. Lactate

All the isolated strains were able to utilize lactate.

5.6.2. *Catalase test*

All of the isolated 251 strains were found to be catalase-positive.

5.6.3. *Gelatin liquefaction*

For detecting the proteolytic activity of the various strains, the gelatin stab method was used.

5.6.3.1. *Arthrobacter*

From Table 13 it will be seen that there are two groups of arthrobacters in reference to their proteolytic activity. All the grey-white and greenish-yellow coryneforms were able to liquefy gelatin. Of the second group, including the cream-coloured and red arthrobacters, only 4 strains out of 62 of the former and 16 strains out of 36 of the latter were able to liquefy gelatin.

5.6.3.2. *Br. linens* strains

All of the 52 isolated strains were able to liquefy gelatin.

5.6.3.3. 'Other coryneforms'

None of the isolated strains belonging to this group was able to liquefy gelatin. In this respect they resemble most of the cream-coloured arthrobacters.

5.6.4. *Utilization of nitrogen compounds and requirement of vitamins*

The utilization of different nitrogen compounds and the vitamin requirements are important characters in the classification of arthrobacters. According to CONN and DIMMICK (1947), the soil arthrobacters would be able to utilize inorganic nitrogen compounds in the absence of vitamins. This statement was not always confirmed by other workers (TAYLOR, 1938; JENSEN, 1934; MORRIS, 1960; CHAN and STEVENSON, 1962; VELDKAMP, 1962, 1963; MULDER, 1964 and MULDER et al., 1966).

MULDER and ANTHEUNISSE (1963) and MULDER (1964) tested the nitrogen and vitamin requirements of a large number of coryneform bacteria from different sources. They found that nearly all the *Arthrobacter* strains isolated from soil were able to utilize inorganic nitrogen, either in the presence or absence of vitamins. *Arthrobacter* strains isolated from activated sludge reacted like the soil arthrobacters. Of 46 cheese strains, they found 13 to have the same nitrogen and vitamin requirements as the soil strains, 11 utilized ammonium nitrate when a vitamin mixture had been added and 22 required Casamino acids either with or without vitamins.

After keeping their cheese arthrobacters for a number of years at room temperature on yeast extract glucose agar slopes, MULDER et al. (1966) found that several strains had lost the ability to utilize ammonium nitrate and had to be supplied with a mixture of an ammonium salt and glutamic acid.

TABLE 14. Utilization of nitrogen and vitamins by the isolated cheese coryneforms.

Type of organism	Number of tested strains	Number of strains growing on			
		$(NH_4)_2SO_4$		Casamino acids[1]	
		No vitamins	Vitamin[4] mixture	No vitamins	Vitamin[4] mixture
Arthrobacter					
Cream	62	20	22	12	8
Grey-white	42	9	13	14	6
Red	36	2	3	11	20
Greenish-yellow	33	–	4	7	22
Br. linens					
Orange[2]	28	2	2	10	14
Cream[3]	24	1	2	10	11
'Other coryneforms' (cream)	26	–	–	7	19

[1] Strains growing only in the presence of Casamino acids.
[2] In the dark and in light.
[3] In the dark, orange in light.
[4] Strains growing only in the presence of vitamins.

In the present investigation the 251 isolated strains were tested for their ability to grow on inorganic and organic nitrogen either without or in the presence of vitamins. The results of this experiment are shown in Table 14.

5.6.4.1. The arthrobacters

The cream and white-coloured arthrobacters could be divided into three groups as to their nitrogen and vitamin requirements. About one third of the tested strains were able to utilize ammonium sulphate only, while another third needed a vitamin mixture for the utilization of inorganic nitrogen. The remaining 20 strains of this type required Casamino acids, either without or in the presence of vitamins. About 50% of the grey-white strains were able to utilize inorganic nitrogen, either with or without vitamins, while the other half required Casamino acids, either with or without vitamins. Only a few strains of the red and greenish-yellow arthrobacters were able to utilize inorganic nitrogen, viz. 5 out of 36 strains of the red group and 4 of the 33 greenish-yellow organisms. The majority of these coloured strains required Casamino acids either with or without vitamins.

5.6.4.2. The group of *Br. linens*

This group was found to behave similarly to the red as well as to the greenish-yellow arthrobacters. Only 7 out of 52 strains were able to utilize inorganic nitrogen either with or without vitamins. The majority of the strains required organic nitrogen either with or without vitamins.

5.6.4.3. The group of 'other coryneforms'

None of the strains of this group was able to utilize inorganic nitrogen. More than 2/3 of the strains in addition to organic nitrogen required vitamins for growth.

5.7. Discussion

Mulder et al. (1966) found that the surface flora of Limburger cheese consisted for 65 to 80% of grey-white coryneforms and for 9 to 24% of orange coryneforms of the *Br. linens* type. According to these authors both types of cheese coryneforms are able to tolerate large amounts of salt and, under certain conditions, even require relatively high concentrations of it. All but one of their orange strains tested required organic nitrogen. The nitrogen nutrition of their grey-white strains was found to be intermediate between that of the orange coryneforms and that of the soil arthrobacters. Ammonium nitrogen was assimilated by approximately 85% of the tested strains. This occurred both with and without amino acids and/or vitamins. Of the two types of their cheese coryneforms, a small group of the grey-white type only was highly proteolytic; the majority were inactive in this respect. The bacteria of the second group, including cultures of *Br. linens* from type culture collections, were moderately proteolytic.

In the present investigation, 251 strains isolated from the surface of Limburger cheeses of different ages, were tested for their morphological characters and their nutritional requirements. All the tested strains were found to be Gram-positive in both coccus and rod stages. This is in complete agreement with the findings of MULDER (1964). All of the isolated strains were found to tolerate high salt concentrations. The greenish-yellow arthrobacters and the *Br. linens* strains were able to tolerate 12 and some even 16% of NaCl. The cream and white-coloured, grey-white and red arthrobacters and the 'other coryneforms' were somewhat less salt-tolerant. This salt tolerance is not surprising because of the fact that Limburger cheese is heavily salted which brings about a selection of salt-tolerant micro-organisms growing on the surface of the cheese.

As to the pigmentation of the isolated strains, an interesting phenomenon concerning the effect of light on pigmentation was observed. About 66% of the greenish-yellow arthrobacters needed light to induce pigmentation. Grey-white colonies were formed when these strains grew in the dark. Approximately 45% of the *Br. linens* strains required light for the development of the orange pigment. No light effect was observed in the case of the cream and white-coloured, grey-white and red arthrobacters and the strains of the 'other coryneforms'. This light effect on pigmentation of part of the *Br. linens* strains was also found by MULDER et al. (1966).

As for the utilization of carbon compounds, all the strains utilized both glucose and lactate as the only carbon source. This was not true of sucrose, which was utilized by only few strains (6 out of 251). Lactose was utilized by approximately one third of the strains tested. These results agreed with those found by MULDER (1964).

All the isolated strains were found to be catalase-positive.

A large difference in proteolytic activity of the various groups of coryneform bacteria was found. The greenish-yellow strains were very highly proteolytic while the grey-white arthrobacters and the *Br. linens* strains were moderately proteolytic. The cream + white-coloured arthrobacters were almost entirely inactive, while about 45% of the red strains were either moderately or slightly proteolytic. The 'other coryneforms' were non-proteolytic.

The nitrogen nutrition of the isolated coryneform bacteria was found to be an important factor in differentiating these organisms. The red and greenish-yellow arthrobacters, the *Br. linens* strains and the 'other coryneforms' were largely unable to utilize inorganic nitrogen. They required Casamino acids either with or without vitamins. About 50% of the grey-white arthrobacters and about 70% of the cream + white-coloured ones were able to utilize ammonium sulphate in the presence or absence of vitamins. Evidence in support of these results could be derived from the investigations on cheese coryneforms made by MULDER and ANTHEUNISSE (1963), MULDER (1964) and MULDER et al. (1966).

The most important differential character in classifying the groups of isolated micro-organisms was their cell form. The group of *Arthrobacter* is easily and quickly transformed from the rod in to the coccus form upon ageing (mostly after

24 or 48 hr), or under poor nutritional conditions. The rods of the *Arthrobacter* group were mostly short and thick. Coccoids were found to be the predominant cells in ageing cultures. The rods of the *Br. linens* strains were slender, relatively long and sometimes branched. The transformation of rods into cocci was found to be a much retarded process. In the group of 'other coryneforms' this process was even more retarded or entirely absent. All the 251 isolated strains as well as those from type culture collections showed, in the rod stage, the morphological implications of the coryneform bacteria: straight, slightly bent, swollen or club-shaped cells, sometimes developing filaments and true branching. Snapping division and angular cell arrangement (palisade arrangement) are regular features.

6. CHEMICAL AND MICROBIOLOGICAL ANALYSIS OF LIMBURGER CHEESE IN THE COURSE OF RIPENING

6.1. Introduction

Ripening of cheese, in general, is a complicated process, which includes the partial degradation of the main components of the cheese, viz. protein and fat. Earlier workers have shown that the breakdown of casein during the ripening proceeds under the influence of rennet enzymes, bacterial enzymes and the enzymes present in the original milk. As a result of these processes a complex mixture of polypeptides (proteoses, peptones), amino acids, amines and ammonia is formed. In Limburger cheese, where the casein is extensively degradated, the biochemical processes going on during ripening are only poorly known. The same is true of the role which the various types of micro-organisms of the surface flora are playing in the ripening process.

This part of the work was designed to obtain more basic informations as to the chemistry and microbiology of the ripening process in Limburger cheese. For that purpose, cheeses, made under commercial conditions, were analysed just before salting, immediately after salting (over night) and 5, 9, 14, 20, 27 and 35 days after salting. In addition to the chemical compostion of the cheese, the composition of the surface flora was estimated.

6.2. Experimental results

6.2.1. *Moisture*

The moisture content of the cheese decreased when the ripening progressed (experiment A, Table 15, Fig. 1). This loss of water was much more pronounced during the first 9 days of ripening (about 9%) than during the rest of the ripening time (approximately 8% in 26 days). A similar trend was found to occur in experiment B (Table 15, Figure 1) where the losses of water were about 8% during the first 9 days after manufacture and about 10% during the rest of the ripening period.

The final moisture content of the ripe cheeses was 43.82% in experiment A and 44.12% in B.

6.2.2. *Salt and salt/water ratio*

The salt percentage and the salt/water ratio increased on cheese ageing. This was due to the loss of moisture of the cheese. The salt percentage reached values of 3.11 in experiment A and 2.80 in B, at the end of 35 days of ripening (Table 15 and Fig. 2). The salt/water ratio was 7.11 and 6.35% in experiments A and B, respectively.

6.2.3. *Total nitrogen*

The percentage of the total nitrogen in the cheese increased as ripening progressed. Like the increased salt content, this depended on the loss of moisture.

TABLE 15. Chemical analysis of Limburger cheese in the course of ripening.

Experiment	Age of cheese[1]	As % of cheese					Soluble nitrogen	Amino acid nitrogen	Salt %	Salt/water ratio	pH
		Moisture[2]	Total nitrogen	Apparent protein	Soluble nitrogen	Amino acid nitrogen[4]	in % of total nitrogen				
A	Fresh cheese	65.33	2.21	14.10	N.D[3]	N.D	–	–	N.D	–	5.40
	After salting	61.02	2.48	15.85	N.D	N.D	–	–	2.08	3.41	5.10
	5 days	57.46	2.71	17.30	0.36	0.13	13.43	4.65	2.21	3.85	5.30
	9 days	52.04	3.06	19.50	0.66	0.24	21.66	7.82	2.49	4.78	5.60
	14 days	48.95	3.25	20.76	1.12	0.47	34.27	14.47	2.78	5.67	6.00
	20 days	46.83	3.39	21.63	1.76	0.92	51.92	27.02	2.92	6.24	6.00
	27 days	44.79	3.53	22.53	2.37	1.41	67.15	39.99	3.09	6.90	6.30
	35 days	43.82	3.71	23.69	3.15	1.71	84.89	46.11	3.11	7.11	6.60
B	Fresh cheese	66.12	2.14	13.65	N.D	N.D	–	–	N.D	–	5.60
	After salting	62.73	2.41	15.35	N.D	N.D	–	–	1.83	2.92	5.30
	5 days	59.51	2.61	16.67	0.29	0.16	11.17	6.12	1.99	3.35	5.50
	9 days	54.39	2.94	18.78	0.65	0.29	22.12	9.85	2.24	4.12	5.70
	14 days	50.48	3.20	20.38	1.01	0.58	31.64	18.22	2.48	4.92	5.90
	20 days	47.95	3.36	21.42	1.70	1.45	50.71	34.16	2.56	5.34	6.05
	27 days	45.34	3.53	22.50	2.21	1.52	62.56	42.99	2.69	5.93	6.15
	35 days	44.12	3.67	23.41	2.82	1.83	76.83	49.77	2.80	6.35	6.35

[1] The first sample was taken just before salting, the second after salting the cheese in the brine (over night).
[2] Calculated on cheese.
[3] Not determined.
[4] Including ammoniacal nitrogen.

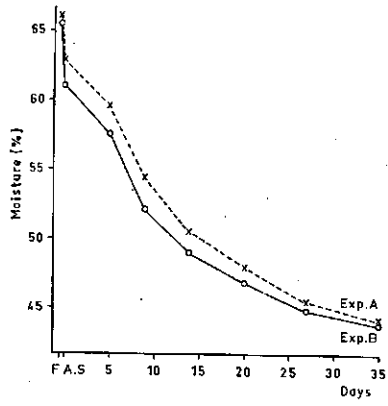


FIG. 1. Moisture content of cheese during ripening.

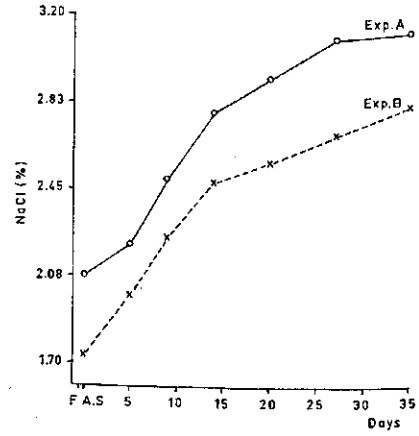


FIG. 2. Salt content of cheese during ripening.

When calculated on total solids, both salt and total nitrogen content of the cheese remained practically constant during the ripening period. The percentage of total nitrogen in ripe cheese, 35 days after salting, was 3.71 in experiment A and 3.67 in B, corresponding with an apparent protein content of 23.69 and 23.41%, respectively (Table 15 and Fig. 3).

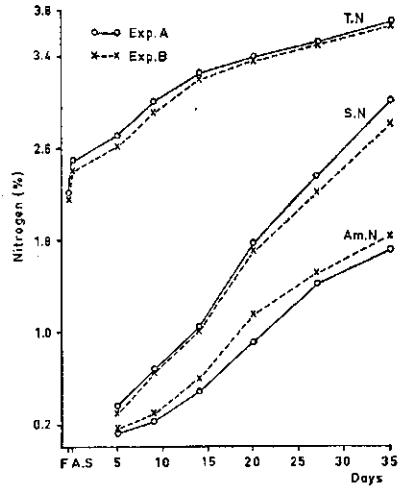
6.2.4. *Soluble nitrogen*

As shown in Table 15, the rate of proteolysis in both experiments was lower in the first stage of ripening than when ripening had progressed. In the period between 5–9 days of cheese age about 8% of the total nitrogen was transformed into the soluble phase in experiment A and about 11% in B. Between 9 and 14 days of cheese age the amounts transformed into soluble nitrogen were nearly 13% in A and 9.5% in B, while between 14 and 20 days of cheese age these values were 17.5% and 19%, respectively. From the same table it will be seen that 84.9% of the total nitrogen in experiment A and 76.8% in B were in the soluble phase at the end of 35 days of ripening (see also Fig. 3).

6.2.5. *Amino acid nitrogen*

The amino acid nitrogen content of the cheese rapidly increased in the course

FIG. 3. Nitrogen distribution in cheese during ripening.



of ripening, mainly due to the breakdown of protein and peptides. The strongest increase of amino acid nitrogen occurred in the period between 14 and 27 days of cheese age (25% from the total nitrogen in A and 24% in B). At the age of 35 days 46.11% of the total nitrogen in experiment A and 49.77% in B were found as amino acid nitrogen (Table 15 and Fig. 3).

6.2.6. *The pH*

The pH of the cheese gradually increased with ripening up to 6.60 in experiment A and 6.35 in B (Table 15 and Fig. 4). The drop in the pH value in both experiments occurred during the salting process and undoubtedly was due to the exchange of H ions of the protein for Na ions.

The cheese sample used for the pH estimation was taken from a whole mixed cheese. It should be kept in mind that ripening proceeds from the outside to-

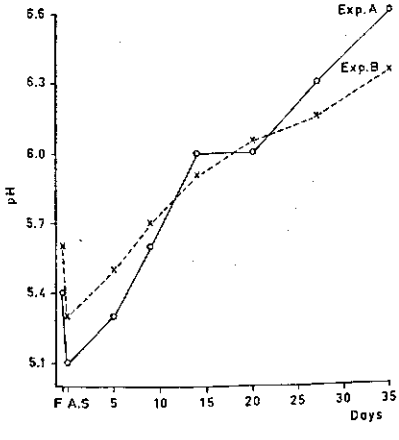


FIG. 4. The pH of cheese during ripening.

wards the cheese centre, so that the pH values recorded here are representing averages of higher surface pH values and lower ones in the centre of the cheese (Fig. 4).

6.2.7. *Total viable count*

The highest number of micro-organisms in the surface layer of ripening Limburger cheese was found from the 20th until the 27th day after salting (Table 16). The fluctations of the total counts during the different stages of ripening were presumably due to the frequent changes in the composition of the surface flora. The salting process brought about a drop of the total count in both experiments. After salting, the total viable counts increased gradually and steadily. In the period between 9–14 days of cheese age there was a slight drop in the total counts in both experiments which could have been due to the disappearance of the yeasts, followed by the increase of other organisms. The highest count per gram of scraped material of cheese surface was attained after 27 days in experiment A (5.50×10^{10}) and after 20 days in experiment B (4.70×10^{9}).

6.2.8. *Grouping of the micro-organisms of the cheese surface in the course of ripening*

(a). Lactic acid bacteria:

This group of micro-organisms was found to be nearly the sole group in fresh cheese after manufacture (97.4% of the total count in experiment A and 100% in experiment B; cf. Table 16). Upon salting its numbers quickly decreased to 8 and 19.4% of the total flora in experiments A and B, respectively.

In 5 days old cheese the surface flora did not contain this group anymore and throughout the rest of the ripening period no lactic acid bacteria were detected in the cheese surface flora.

(b). Yeasts:

The number of yeasts attained its maximum 5 days after salting when in both experiments the whole surface flora was found to consist of these organisms. On 9 days old cheeses the number of yeasts had dropped to 42.5% of the total surface flora in experiment A and to 52.6% in B. On 14 days old cheeses the yeasts had disappeared and kept absent until the end of the ripening period.

(c). Arthrobacters:

This group was present in nearly all ripening stages, in both experiments, except after 5 days of ripening. In the first experiment it was even detected on fresh cheese where it amounted to 2.6% of the total flora. The highest number of arthrobacters in both experiments was found 14 days after salting, viz. 97.6% of the total flora in experiment A and 91.4% in experiment B. When ripening proceeded, this group decreased to 78.4 and 62.7% of the total flora, respectively, after a ripening period of 35 days.

(d). *Br. linens:*

Bacteria of this group appeared for the first time immediately after salting the cheese when they made up 24.0 and 15.3% of the total surface flora in ex-

TABLE 16. Total counts and groups of micro-organisms in the surface layer of Limburger cheese during ripening.

Experiment	Age of cheese[1]	Total count per gram of scraped surface material	Number of strains examined	Type of organism (as % of the total count)						
				Lactic acid bacteria	Yeast	*Arthro-bacter*	*Br. linens*	'Other coryne-forms'	*Sarcina*	Mould
A	Fresh cheese	5.56×10^7	78	97.4	–	2.6	–	–	–	–
	After salting	2.38×10^7	75	8.0	8.0	36.0	24.0	18.7	4.0	1.3
	5 days	1.19×10^9	75	–	100.0	–	–	–	–	–
	9 days	5.54×10^{10}	80	–	42.5	57.5	–	–	–	–
	14 days	4.45×10^9	82	–	–	96.7	2.4	–	–	–
	20 days	5.22×10^{10}	90	–	–	93.3	6.7	–	–	–
	27 days	5.50×10^{10}	90	–	–	88.9	11.1	–	–	–
	35 days	3.10×10^{10}	88	–	–	78.4	21.6	–	–	–
B	Fresh cheese	4.20×10^7	66	100.0	–	–	–	–	–	–
	After salting	2.41×10^7	72	19.4	20.8	30.6	15.3	11.1	2.8	–
	5 days	1.48×10^8	84	–	100.0	–	–	–	–	–
	9 days	4.41×10^9	76	–	52.6	47.4	–	–	–	–
	14 days	1.81×10^9	70	–	–	91.4	8.6	–	–	–
	20 days	4.70×10^9	68	–	–	80.9	19.1	–	–	–
	27 days	4.60×10^9	80	–	–	73.8	26.2	–	–	–
	35 days	3.17×10^9	75	–	–	62.7	37.3	–	–	–

[1] The first sample was taken just before salting, the second after salting the cheese in the brine (overnight).

periments A and B, respectively. No organisms of this type were detected on 5 and 9 days old cheese, which could mean that their occurrence on the cheese surface immediately after salting was due to the presence of the brine flora on the cheese surface at the time of sampling. On 14 days old cheese, strains of *Br. linens* reappeared in the surface layer and their numbers gradually increased upon ageing. On 35 days old cheeses they constituted 21.6 and 37.3% of the total surface flora in experiments A and B, respectively.

(e). 'Other coryneforms':

This group in both experiments was detected only once, viz. immediately after salting in the brine which may have been responsible for the presence of this group on the cheese surface. Throughout the ripening period these organisms were not found anymore on the cheese surface.

(f). Sarcinae:

These bacteria represented a minor group occurring only in samples taken immediately after salting which presumably have contained part of the brine flora.

(g). Moulds:

These organisms were found in the surface layer of only one cheese sample immediately after salting. They were not present in any of the samples throughout the ripening period.

6.3. Discussion

Throughout the ripening process of cheese, breakdown of protein and transformation of the products of hydrolysis are considered as the main indications for ripening. In Limburger cheese these processes mainly depend on the action of micro-organisms occurring in the surface layer of the cheese. This activity for a large degree is affected by the type of micro-organism occurring in dominating numbers in the surface flora. Since the composition of the surface flora depends on the moisture content, the salt/water ratio and the pH of the cheese, these factors in an indirect way are controlling the ripening of the cheese. In addition to this indirect effect, moisture content, salt/water ratio and pH, together with the products of protein degradation, are determining the quality of the final ripened product.

In agreement with literature recordings, the pH of the cheeses studied was found to increase gradually with ripening. The slight drop of pH upon salting was not due to a microbial process, but depended on the exchange of H ions for Na ions. The pronounced rise of pH during the first 9 days of the ripening period was connected with the occurrence of yeasts as the dominating micro-organism in the surface layer of the cheese. It is thought that these organisms consume the lactate formed from lactose by the lactic acid bacteria, thus decreasing the acidity of the cheese. Subsequent rises of pH, which are correlated with the development of a highly proteolytic surface flora, are thought to have been brought about by the breakdown of amino acids and the formation of ammonia.

The moisture content of the cheese decreased when the ripening progressed. This decrease was most remarkable during the first stages of ripening, but it was less pronounced with ageing. This result was expected, it is in agreement with results obtained in earlier investigations.

As a result of the loss in moisture, the salt content and salt/water ratio of the cheese increased. When calculated as a percentage of the total solids, the values of salt, salt/water ratio and total nitrogen content of the cheese were practically constant.

The values of protein breakdown increased along with ripening. The rate of proteolysis was low in the early days of ripening, but after about one week of cheese age the values for soluble nitrogen and amino acid nitrogen increased at higher rates until the age of approximately 20 days when this rate slowed down. In the first stages of ripening, soluble nitrogen was formed at a higher rate than amino acid nitrogen. This difference might be explained by the fact that soluble nitrogen is produced by the action of milk proteases, rennet enzymes and bacterial enzymes, while the formation of amino acids in cheese mainly depends on bacterial enzymes (STADHOUDERS, 1959). At the age of 35 days, the cheeses were heavily proteolysed and partly liquefied. They were ready for consumption after a ripening period of 20 to 27 days, depending on the degree of maturation. When incubated for 35 days in the ripening room, the cheese was overripened, and soluble and amino acid nitrogen calculated, as a percentage of the total nitrogen, attained values of 84.89 and 46.11, respectively (Table 15).

As for the cheese surface flora, it was found that the frequent changes in its composition in the course of ripening, resulting from salting, change of pH etc., strongly affected the total counts obtained at the different stages of ripening. The salting process brought about a drop in the total count. This was explained by the adverse effect of the brine on lactic acid bacteria which was the predominant group in fresh cheese. In samples taken immediately after salting, the composition of the cheese surface flora resembled that of the brine. However, after five days these organisms had disappeared from the cheese surface except yeasts which were present as the sole micro-organism. After salting, the total viable count of the surface flora increased gradually and steadily. This was followed by another drop in the period between 9 and 14 days of cheese age. This drop was due to the replacement of the predominant yeasts by arthrobacters. The total count reached its highest value at the age of about 3 weeks.

As for the main groups of organisms found in the cheese surface flora, it may be stated that yeasts, arthrobacters, and *Br. linens* were the main ripening agents in Limburger cheese. Yeasts were predominating until the age of about 9 days when they were replaced by the arthrobacters. The latter kept their predominance until the end of 35 days of cheese age. The most surprising result was that concerning the group of *Br. linens*. In the literature this group is always described as the main ripening agent in Limburger cheese. This statement was not confirmed in the present study. The cheese was ready for consumption at the age of 20 to 27 days whereas, the *Brevibacterium linens* group attained its highest number in 27 days old cheese (26.3 % of total count, exp. B). In exp. A this value

was only 11.1% of the total count (Table 16). It seems more likely that the predominating group of arthrobacters is playing the most important role in the ripening of this particular type of cheese. MULDER et al. (1966) found the same predominance of the arthrobacters in two samples of ripened Limburger cheese.

7. PIGMENTATION AND PROTEOLYTIC ACTIVITY OF MICRO-ORGANISMS REPRESENTING THE SURFACE FLORA OF LIMBURGER CHEESE DURING DIFFERENT RIPENING STAGES

7.1. INTRODUCTION

Limburger cheese is a surface-ripened cheese which is mainly characterized by its reddish-orange colour and distinct smell, due to the extensive degradation of the curd. Preliminary experiments showed that many strains of the surface flora, which is mainly responsible for the ripening of this cheese, were pigmented, the colour ranging from white or cream to red, orange or greenish-yellow. These pigmented strains were found to have a pronounced effect on the colour of the surface of the cheese which is an important factor as to the commercial value of the cheese. This character together with the degree of softness, the smell and the taste of the cheese are determining the cheese quality and the rate of ripeness.

For these reasons it was decided to check the strains isolated from the surface of Limburger cheese, for pigmentation and proteolytic activity.

7.2. PIGMENTATION OF BACTERIA ISOLATED FROM RIPENING LIMBURGER CHEESE

In respect of pigmentation, the isolated strains were grown under both light and dark conditions. The experiment was carried out in the same way as described in 5.2. Light was found in preliminary experiments to be a very important factor for several coryneform bacteria that needed it for inducing the pigment (see Chapter 5(5.2.) and MULDER et al., 1966). Tables 17 and 18 show the pigmentation of the bacteria isolated from the surface of Limburger cheese.

7.2.1. *Fresh cheese*

The cheese in this stage showed a clear white curd.

The majority of the micro-organisms isolated from this cheese, which were almost entirely lactic acid bacteria, formed white colonies. None of these white strains was affected by light.

7.2.2. *Cheese immediately after salting*

The cheese at this stage was still white but shadowed with a light cream colour.

The majority of organisms in both experiments A and B formed white colonies in the dark as well as in the light. This group of white-coloured organisms included the lactic acid bacteria, about half of the yeasts and a part of the arthrobacters (cf. Table 16). Organisms with cream-coloured, grey, yellow, greenish-yellow and orange colonies also occurred. The group of cream-coloured organisms consisted for a large part of yeasts. The remaining organisms of this group and about all organisms of the other coloured groups were coryneform bacteria (cf. Table 16). It is interesting to note that a number of

TABLE 17. Pigmentation of micro-organisms isolated from the surface of Limburger cheese during ripening (Exp. A).

Age of cheese[1]	Number of strains examined	Incubated in	Colour of colonies (as % of the total count)							
			White	Cream	Grey	Red	Yellow	Greenish--yellow	Orange	Reddish--orange
Fresh cheese	78	Dark	97.4	–	–	–	–	2.6	–	–
After salting	75		33.3	14.7	12.0	–	4.0	4.0	13.3	18.7[4]
5 days	75		53.3	46.7	–	–	–	–	–	–
9 days	80		32.5[2]	33.8[3]	20.0	2.5	–	11.3	–	–
14 days	82		12.2	22.0	22.0	20.7	–	20.7	2.4	–
20 days	90		37.8	24.4	14.4	7.8	–	11.1	4.5	–
27 days	90		50.0	33.3	5.6	4.4	–	–	6.7	–
35 days	88		42.1	29.5	8.0	4.6	–	2.3	13.6	–
Fresh cheese	78	Light	97.4	–	–	–	–	2.6	–	–
After salting	75		33.3	4.0	4.0	–	4.0	12.0	24.0	18.7
5 days	75		53.3	46.7	–	–	–	–	–	–
9 days	80		32.5	33.8	11.3	2.5	–	20.0	–	–
14 days	82		12.2	22.0	22.0	20.7	–	20.7	2.4	–
20 days	90		37.8	22.2	10.0	7.8	–	15.6	6.7	–
27 days	90		50.0	28.9	5.6	4.4	–	–	11.1	–
35 days	88		42.1	21.6	5.7	4.6	–	4.6	21.6	–

[1] The first sample was taken just before salting, the second after salting the cheese in the brine (overnight).
[2] 22.5% of the total flora were white yeasts and 10.0% white bacteria.
[3] 20.0% of the total flora were cream yeasts and 13.8% cream bacteria.
[4] Very light reddish-orange colour.

TABLE 18. Pigmentation of micro-organisms isolated from the surface of Limburger cheese during ripening (Exp. B).

Age of cheese[1]	Number of strains examined	Incubated in	Colour of colonies (as % of the total count)							
			White	Cream	Grey	Red	Yellow	Greenish-yellow	Orange	Reddish-orange
Fresh cheese	66		100.0	–	–	–	–	–	–	–
After salting	72		38.9	24.0	8.3	–	2.8	5.6	8.3	11.1[4]
5 days	84		42.9	57.1	–	–	–	–	–	–
9 days	76		26.3[2]	26.3[3]	23.7	7.9	–	15.8	–	–
14 days	70	Dark	14.3	21.4	25.7	17.1	–	17.1	4.3	–
20 days	68		29.4	30.9	11.8	8.8	–	8.8	10.3	–
27 days	80		37.5	36.3	7.5	3.8	–	–	15.0	–
35 days	75		40.0	29.3	9.3	–	–	–	21.3	–
Fresh cheese	66		100.0	–	–	–	–	–	–	–
After salting	72		38.9	18.1	2.8	–	2.8	11.1	15.3	11.1
5 days	84		42.9	57.1	–	–	–	–	–	–
9 days	76		26.3	26.3	11.8	7.9	–	27.6	–	–
14 days	70	Light	14.3	17.1	17.1	17.1	–	25.7	8.6	–
20 days	68		29.4	22.1	5.9	8.8	–	14.7	19.1	–
27 days	80		37.5	25.0	5.0	3.8	–	2.5	26.3	–
35 days	75		40.0	13.3	5.3	–	–	4.0	37.3	–

[1] The first sample was taken just before salting, the second after salting the cheese in the brine (overnight).
[2] Yeasts.
[3] Yeasts.
[4] Very light reddish-orange colour.

strains with grey colonies in the dark, formed greenish-yellow colonies in the light while several cream-coloured strains formed an orange pigment when growing in light.

7.2.3. *5 days old cheese*

In this stage of ripening the cheese was cream-coloured with a heavy sticky surface growth.

All the organisms isolated from this very young cheese were yeasts. Two types of strains were distinguished, white and cream ones. The pigmentation of none of these two types was affected by light.

7.2.4. *9 days old cheese*

In this stage the cheese attained a red shade on its cream-coloured surface.

More than 50% of the isolated organisms of this 9 days old cheese formed white or cream-coloured colonies, the majority of which were yeasts. The remaining organisms were red arthrobacters which occurred only in low numbers as compared with the groups of grey and greenish-yellow arthrobacters. When exposed to light, a considerable number of the grey arthrobacters developed the greenish-yellow pigment, suggesting a close relationship of the organisms of both coloured groups. The group of red arthrobacters was detected for the first time at this stage of ripening. The red pigment was developed in the light as well as in the dark.

7.2.5. *14 days old cheese*

The cheese surface at this stage of ripening had a light red colour.

The group of white coryneform bacteria which was small at the 9th day of ripening, gradually increased in numbers upon disappearance of the yeasts, reaching a value of 12–14% of the total counts in this type of cheese. The group of cream-coloured coryneforms had increased to approximately 20% of the total counts of which only few strains developed an orange pigment when grown in the light. The grey and greenish-yellow coryneforms had attained their maximum numbers throughout the ripening period of the cheese (together about 40% of the total counts, cf. Tables 17 and 18). The red-coloured coryneforms also had reached their highest numbers at this stage of cheese ripening (approximately 18% of the total counts). The group of orange coryneforms, *Br. linens* type, started to make a part of the cheese surface flora at this stage of ripening.

7.2.6. *20 days old cheese*

The cheese at this stage of ripening had a more pronounced and deeper red-coloured surface as compared with that of the previous ripening stage.

The white, cream and orange coryneforms occurred in larger numbers than on the cheese of the previous ripening stage, whereas the grey, greenish-yellow and red strains had fallen to much lower values (from 40 to about 20% in the case of grey + greenish-yellow strains and from 19 to about 8% in the case of the red arthrobacters).

7.2.7. *27 days old cheese*

In this stage of ripening the red colour of the cheese surface had again deepened while it had attained an orange shade.

The trend of increase in numbers of the white, cream-coloured and orange coryneforms as observed in the previous sample was continued in this 27 days old cheese (between 40 and 50% of the total counts consisted of white coryneforms and approximately 34% of cream-coloured strains when the latter had grown in the dark and about 27% when grown in the light). The group of orange coryneforms (*Br. linens* type) comprised approximately 18% of the total count when the strains had grown in the light and 11% in the dark showing that approximately 40% of this group of strains required light for changing the colour of the colonies from cream to orange. The drop in the numbers of grey, greenish-yellow and red coryneforms, which had been noted in the previous cheese sample, had continued in the 27 days old cheese (cf. Tables 17 and 18).

7.2.8. *35 days old cheese*

The cheese surface was still red-coloured with a deeper orange shade than that of the previous sample.

The percentage of white and cream-coloured coryneforms had slightly decreased. Nevertheless, the group of white arthrobacters still comprised the highest numbers of bacteria on the cheese surface (about 40% of the total counts). The number of cream-coloured coryneforms was nearly 30% of the total count when grown in dark. In the light this number was considerably lower, approximately 18%, owing to the fact that many strains of this group had formed the orange pigment and therefore had to be transferred to the *Br. linens* group. The grey, greenish-yellow and red-coloured coryneforms were still present in low numbers. The only group of bacteria which showed a pronounced increase was that of the orange-coloured coryneforms, *Br. linens*, which had attained values of about 18% of the total in the dark and 30% in the light.

7.3. Proteolytic activity of the micro-organisms isolated from ripening Limburger cheese

Table 19 shows the proteolytic activity of the micro-organisms representing the surface flora at different stages of ripening. Organisms giving clear zones in milk agar plates after 5 days of incubation at 25 °C were considered to be highly proteolytic, those giving such zones after 10 days moderately proteolytic, after 21 days weakly proteolytic, while those which gave no change after one month of incubation were considered to be non-proteolytic.

7.3.1. *Fresh cheese*

At this stage the cheese surface flora consisted almost entirely of non-proteolytic organisms.

TABLE 19. Distribution of micro-organisms with different proteolytic activity in the surface layer of ripening Limburger cheese.

Experiment	Age of cheese[1]	Number of strains examined	% of the strains being			
			Highly proteolytic	Moderately proteolytic	Weakly proteolytic	Non-proteolytic
A	Fresh cheese	78	2.6	–	–	97.4
	After salting	75	20.0	20.0	4.0	56.0
	5 days	75	–	–	–	100.0
	9 days	80	32.5	–	–	67.5
	14 days	82	41.5	2.4	–	56.1
	20 days	90	25.6	6.7	–	67.8
	27 days	90	11.1	5.6	–	83.3
	35 days	88	10.2	21.6	–	68.2
B	Fresh cheese	66	–	–	–	100.0
	After salting	72	13.9	15.3	2.8	68.1
	5 days	84	–	–	–	100.0
	9 days	76	23.7	–	7.9	68.4
	14 days	70	25.7	8.6	–	65.7
	20 days	68	11.8	19.1	–	69.1
	27 days	80	7.5	26.3	–	66.3
	35 days	75	9.3	37.3	–	53.3

[1] The first sample was taken just before salting, the second after salting in the brine (overnight).

7.3.2. *Cheese immediately after salting*

More than 50% of the total surface flora were found to belong to the non-proteolytic group (the lactic acid bacteria, yeasts, 'other coryneforms' and a part of the *Arthrobacter* strains). The rest of the strains isolated from this cheese were either highly, moderately or weakly proteolytic (cf. Table 19).

7.3.3. *5 days old cheese*

The entire surface flora consisted of yeasts which had no proteolytic activity.

7.3.4. *9 days old cheese*

Two main groups of organisms were detected at this stage of ripening. The non-proteolytic group, including mainly the yeasts and some *Arthrobacter* strains, made up nearly 70% of the total counts. The second group, the highly proteolytic one, consisting of arthrobacters, made up nearly 30% of the total count.

7.3.5. *14 days old cheese*

In this stage of ripening the percentage of highly proteolytic strains of the total count was the highest of the entire ripening period (nearly 35%). The moderately proteolytic organisms (the *Br. linens* strains) started to appear at this stage of ripening. They made up about 5% of the total count. The rest of

the strains isolated from this cheese surface, about 60% of the total count, were non-proteolytic. Both the highly proteolytic and the non-proteolytic strains belonged to the *Arthrobacter* group. The white and those cream-coloured arthrobacters which are not affected by light were non-proteolytic, whereas the grey and greenish-yellow ones were highly proteolytic.

7.3.6. *20 days old cheese*

The non-proteolytic strains (calculated as % of the total count) in this stage of ripening had increased to nearly 70%. The moderately proteolytic strains, still consisting only of *Br. linens*, made up about 13% of the total count. The highly proteolytic group, comprising the grey and the greenish-yellow arthrobacters, decreased to approximately 18% of the total count.

7.3.7. *27 days old cheese*

The non-proteolytic group of organisms (comprising the white and those cream-coloured strains which were not affected by light) had increased to about 75% of the total count. The moderately proteolytic group had increased to approximately 16% of the total count. The group of highly proteolytic organisms, still decreasing, had attained a value of about 9%.

7.3.8. *35 days old cheese*

The group of moderately proteolytic organisms *(Br. linens)* had increased to nearly 30% of the total count. The group of highly proteolytic organisms had hardly changed as compared with the previous cheese sample. The group of non-proteolytic strains was slightly lower.

7.4. Discussion

Earlier workers and preliminary experiments of the present author had shown the presence of micro-organisms with different pigmentation growing on the surface of Limburger cheese. The presence of the orange-coloured strains of the *Br. linens* type is well documented in the literature. Wolff (1909) stated the presence of reddish-yellow, brown-yellow and orange-yellow micro-organisms on surface-ripened cheeses of the Limburger type. Yale (1943) observed the predominance of non-chromogenic organisms in the flora of various types of surface-ripened cheeses, followed by organisms of the *Br. linens* type, the colour of which ranged from light cream to deep orange. Mulder et al. (1966) studied the pigmentation of coryneform bacteria isolated from the surface of different cheeses, including Limburger, and distinguished two groups, i.e. grey-white and orange-pigmented strains. They also showed the dependence of about 50% of their orange strains on light for developing the orange pigment. Preliminary experiments of the present author confirmed this observation concerning the light effect on pigmentation of part of the orange-coloured strains and extended it to greenish-yellow strains part of which also require light for pigmentation (see Chapter 5, 5.2.).

Since pigmentation is an important factor in the production of Limburger cheese and also a specific character in the classification of the flora growing on the cheese surface, this phenomenon was studied in some detail.

The colour of the cheese surface ranged from white in fresh cheeses to reddish-orange in 35 days old cheeses. As for the surface flora, the following colours could be detected in the course of ripening: white, cream, grey, red, yellow, greenish-yellow, orange and reddish-orange.

The occurrence of these colours in the different groups of micro-organisms isolated from the cheese surface is as follows:

a. The group of yeasts included strains forming cream and white colonies.
b. The group of the arthrobacters included strains with white, cream, grey, red or greenish-yellow colour.
c. The group of *Br. linens* included strains of cream and orange colours when the bacteria had grown in the dark, and light to deep orange-coloured strains when grown in light.

Light effect on pigmentation was detected in the group of grey arthrobacters and the group of *Br. linens*. A number of grey arthrobacters were developing a greenish-yellow pigment when grown in light. As recorded earlier, part of the *Br. linens* type of strains were light-dependant for inducing the orange pigment. No light effect was detected in the group of the red arthrobacters. These results showed the same trend as found in Chapter 5 (5.2.) and it confirmed and extended the findings of MULDER et al. (1966).

The presence and the distribution of strains belonging to different pigmentation groups in the surface flora of ripening Limburger cheese is an interesting and important phenomenon in the production of this particular type of cheese. The white and cream-coloured strains predominated on the cheese surface throughout the ripening period of 35 days. The grey and greenish-yellow arthrobacters came second after the previous group until the age of 20 days, after which they decreased in number. The red arthrobacters attained their maximum numbers on the cheese surface at the age of 14 days, making the third group after the white-cream and grey-greenish-yellow groups. This red group decreased in number throughout the rest of the ripening period. The orange-coloured strains of the *Br. linens* type showed up on the surface of about 2 weeks old cheese and increased in numbers throughout the rest of the cheese ripening so that they became the second group on the surface of cheese ripened for 27 and 35 days.

It may be stated that the colour of the cheese surface is a combination of the colours of the above-mentioned strains joined with the colour of the cheese curd. The only indication as to the effect of light on pigment formation of ripening Limburger cheese was found when a few cheeses were brought to a completely dark room in the curing cellar at the age of 9 days and kept under completely dark conditions (see Plate 10) until the end of the ripening period. These cheeses had a more pronounced red colour as compared with cheeses kept under normal ripening conditions (many times per day the light was left on in the cellar). This is thought to be due to the absence of light-induced pigmen-

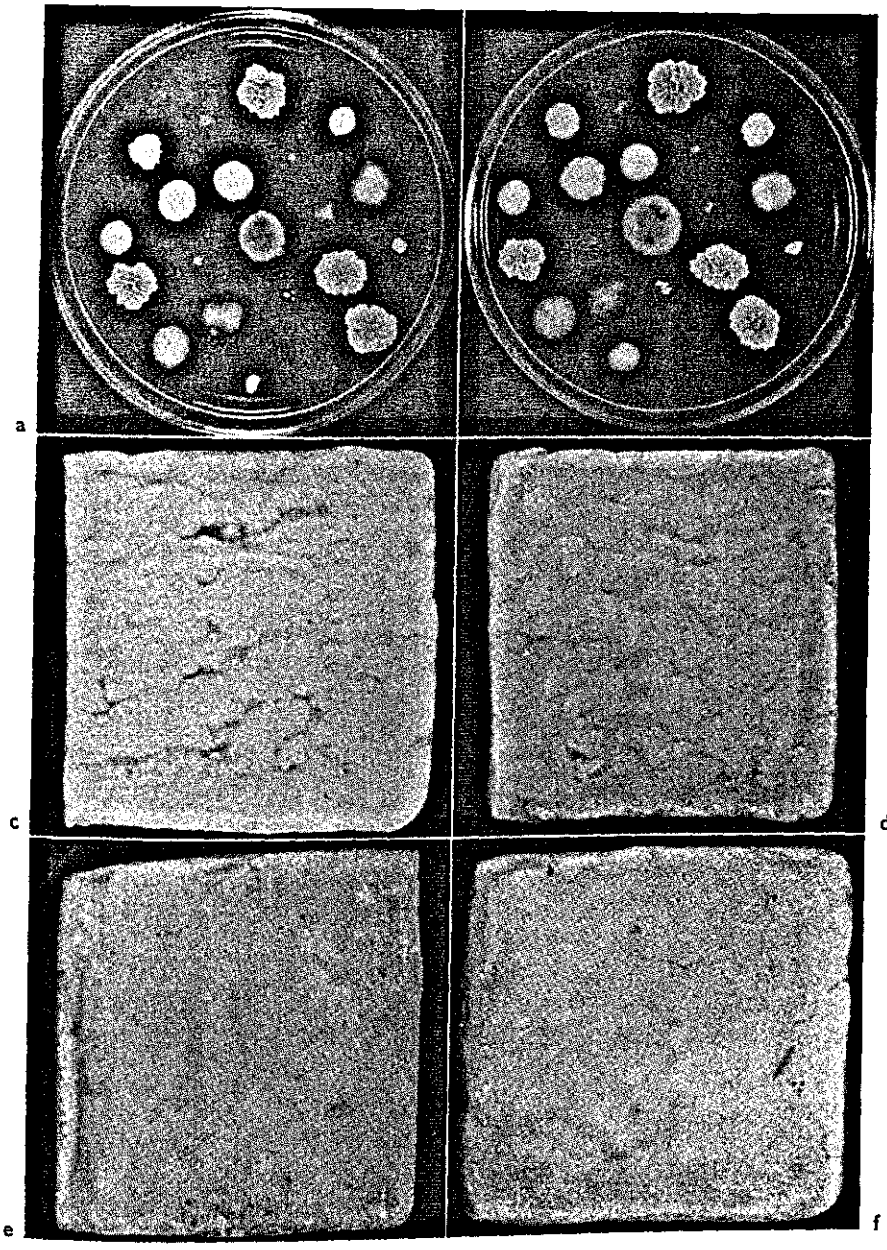


PLATE 10. Effect of light on the colour of coryneform bacteria, growing on yeast extract glucose agar (a and b), and on the colour of ripening Limburger cheese made under commercial conditions (c and d, 14 days old, e and f, 27 days old); a, c and e kept in the light, b, d and f kept in the dark.

tation of greenish-yellow and orange coryneforms whose pigment would have reduced the pigmentation of the red arthrobacters on the cheese surface.

As to the proteolytic activity of different strains of cheese surface bacteria, it was found that in the period of 9 to 20 days of cheese age the percentage of highly proteolytic strains of the total count was highest of the entire ripening period. The grey and greenish-yellow arthrobacters were the strains with the highest proteolytic activity found in the surface flora, followed by strains of the *Br. linens* type and the red arthrobacters. The majority of the white and cream arthrobacters had no proteolytic activity. The same was true of the yeasts. MULDER et al. (1966) found that the largest group of their grey-white cheese coryneforms was non-proteolytic, but their proteolytic ones were more strongly proteolytic than the orange coryneforms of the *Br. linens* type, an observation, which is in agreement with the findings of the present investigation.

8. GROWTH AND ACTION OF SEPARATE MICRO-ORGANISMS ON STERILIZED CHEESE SLICES AND IN CASAMINO ACIDS-CONTAINING MEDIA

8.1. INTRODUCTIONAL

In order to gain more information concerning the part played by micro-organisms representing the surface flora in the ripening process of Limburger cheese, a study was made of the growth of a number of such organisms, on sterilized slices of cheese. This group of representative micro-organisms was also grown in a Casamino acids-containing medium of the following composition: Casamino acids (Oxoid), 3 g; glucose, 10 g; and the mineral nutrient medium, 1000 ml; pH: 7.0. The proteolytic activity of this group of isolated strains was estimated by determining the amount of soluble nitrogen and amino acid nitrogen after growing the organisms on the sterilized cheese slices. Colour, growth density, pH, and organoleptic features were recorded in this experiment. A comparison was made between the amino acid pattern of the cheese slices after 3 weeks of incubation with the different strains and that of the Casamino acids-containing media after 10 days of inoculation with these organisms.

The inoculation material used in this experiment was grown in 100 ml of the yeast extract glucose medium for 48 hr at 25 °C using a gyratory shaker. The cells were collected by centrifugation, washed three times and then diluted in 5 ml of distilled water. From this dilution, 0.5 ml was used as inoculation material for the sterilized cheese slices and the Casamino acids media. The inoculum was distributed over the surface of the sterilized cheese slices by means of a sterilized bent glass rod. The plates in groups of six were then wrapped in cellophane paper, and placed in an incubator with a high level of humidity at 25 °C for 3 weeks. The inoculated Casamino acids media, contained in Erlenmeyer flasks, were shaken for 10 days at 25 °C on a gyratory shaker.

8.2. THE STRAINS USED FOR INOCULATION

Twenty strains isolated from the surface of Limburger cheese in the course of the ripening were used for the inoculation of the cheese slices and the Casamino acids-containing media. The main characters of this group of strains are contained in Table 20. The group of *Arthrobacter* strains included 13 strains of which the numbers 7, 8, 9 and 10 were red, 11, 12, 13 and 14 grey, 15, 16, 17 and 18 cream-coloured, and 19 and 20 greenish-yellow. The group of *Br. linens* organisms included 4 strains, 3, 4, 5 and 6, of which strain 4 was light-dependent for pigmentation. Two yeast strains were used, viz. numbers 21 and 22.

TABLE 20. Characters of the strains used for inoculation.

Type of organism	Colour when grown on				Growth on								Gelatin liquefaction
	Yeast extract glucose agar		Tryptone soya agar		Glucose	Sucrose	Lactose	Lactate	$(NH_4)_2SO_4$ vitamin mixture		Casamino acids vitamin mixture		
	Light	Dark	Light	Dark					0	+	0	+	
Arthrobacter													
Strain 7	R.O[1]	Red	Orange	Cream	+	−	+	+	−	−	+	+	+
8	Red	Red	Cream	Cream	+	−	+	+	−	−	+	+	+
9	Red	Red	Cream	Cream	+	−	−	+	−	−	+	+	+
10	Red	Red	Cream	Cream	+	−	−	+	−	−	−	+	+
11	Grey	Grey	Grey	Grey	+	−	−	+	−	+	+	+	+
12	Grey	Grey	Grey	Grey	+	−	−	+	−	−	+	+	+
13	Grey	Grey	Grey	Grey	+	−	+	+	−	−	+	+	+
14	Grey	Grey	Grey	Grey	+	−	−	+	−	+	−	+	+
15	Cream	Cream	Cream	Cream	+	−	+	+	−	−	+	+	+
16	Cream	Cream	Cream	Cream	+	−	−	+	−	−	+	+	−
17	Cream	Cream	Cream	Cream	+	−	+	+	−	−	+	+	−
18	Cream	Cream	Cream	Cream	+	−	−	+	−	−	+	+	−
19	G.Y[2]	G.Y	G.Y	G.Y	+	−	+	+	−	−	+	+	+
20	G.Y	Grey	G.Y	Grey	+	−	−	+	−	−	−	+	+
Br. linens													
Strain 3	Orange	Orange	Orange	Orange	+	−	−	+	−	−	+	+	+
4	Orange	Cream	Orange	Cream	+	−	−	+	−	−	+	+	+
5	Orange	Orange	Orange	Orange	+	−	−	+	−	−	−	+	+
6	Orange	Orange	Orange	Orange	+	−	−	+	−	−	−	+	+
Yeast													
Strain 21	Cream	Cream	Cream	Cream	+	N.D	+	+	N.D	N.D	+	+	−
22	Cream	Cream	Cream	Cream	+	N.D	+	+	N.D	N.D	+	+	−

[1] R.O = Reddish-orange.
[2] G.Y = Greenish-yellow.

8.3. Results

8.3.1. *Properties of the cheese slices*

8.3.1.1. The pH

Table 21 gives the pH values of the cheese slices and the Casamino acids-containing media inoculated with different strains after different periods of incubation. The cream-coloured *Arthrobacter* strains caused a drop in the pH of the cheese slices after one week of incubation, which was followed by a quick rise throughout the rest of the incubation time. In the Casamino acids-containing medium two trends occurred viz. one, including 2 strains, which raised the pH of the media to a high value, and a second causing a pronounced drop in pH (see also Fig. 5).

The group of grey arthrobacters showed either a slight increase or decrease in the pH values when grown on cheese slices for one week, after which the pH increased steadily with most of the strains. When grown in the Casamino acids-containing medium half of the strains caused a considerable increase in the pH of the media while the other half gave a slight decrease after 10 days of incubation (see also Fig. 6).

All the red arthrobacters increased the pH when grown on cheese slices for 3 weeks, while in the Casamino acids medium the pH either increased or slightly decreased (Fig. 7). Both greenish-yellow arthrobacters caused a decrease in the

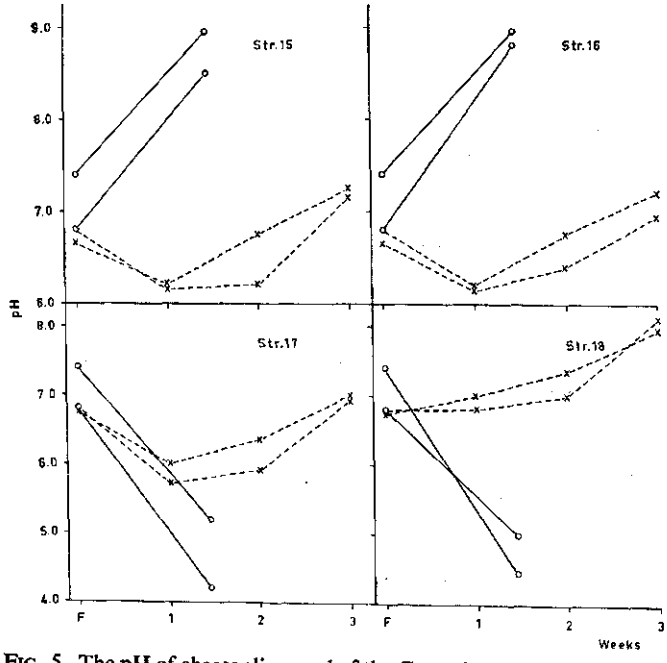


FIG. 5. The pH of cheese slices and of the Casamino acids-containing media due to the growth of different strains.

TABLE 21. The pH of cheese slices and Casamino acids-containing media inoculated with different strains after different periods of growth.

Type of organism	pH[1] after growing on cheese slices for			pH[1] after growing on Casamino acids media for 10 days	
	1 week[2]	2 weeks	3 weeks	I[3]	II[4]
Arthrobacter					
Cream					
Strain 15	6.15	6.50	7.15	8.60	8.95
16	6.15	6.60	7.05	8.80	8.95
17	5.85	6.10	6.95	4.20	5.20
18	6.90	7.15	8.00	5.10	4.45
Grey					
Strain 11	6.80	6.95	7.15	4.90	5.10
12	7.10	7.55	8.45	9.20	9.00
13	6.25	6.50	6.95	9.00	8.70
14	6.30	6.70	7.40	6.70	7.00
Red					
Strain 7	6.90	7.10	8.30	7.70	8.60
8	7.10	7.20	8.05	6.60	6.90
9	6.90	7.20	8.00	6.70	7.40
10	6.50	6.75	7.05	6.70	6.80
Greenish-yellow					
Strain 19	6.20	6.65	7.00	4.90	4.95
20	6.15	6.70	7.80	5.80	5.25
Br. linens					
Strain 3	7.20	7.70	8.30	7.60	8.60
4[5]	7.30	7.35	7.40	7.20	8.10
5	6.95	7.15	8.00	6.90	7.60
6	6.95	7.10	7.80	8.00	8.30
Yeast					
Strain 21	5.85	6.45	7.60	7.50	7.90
22	6.30	6.80	7.10	6.15	6.90

[1] Averages of data from duplicate experiments.
[2] pH of 6.75 just before inoculation.
[3] pH of 6.80 just before inoculation.
[4] pH of 7.40 just before inoculation.
[5] Orange in light, cream in the dark.

pH after one week of growth on cheese slices which was followed by an increase after 3 weeks of incubation. In Casamino acids media, both strains caused a considerable decrease in the pH after 10 days of incubation (Fig. 8).

All the *Br. linens* strains raised the pH when grown either on cheese slices or in the Casamino acids media (Fig. 9).

The yeast strains decreased the pH after 1 week of growth on the cheese slices which was followed by an increase after 3 weeks. When grown in the Casamino acids-containing medium, one strain raised the pH while the other slightly decreased it.

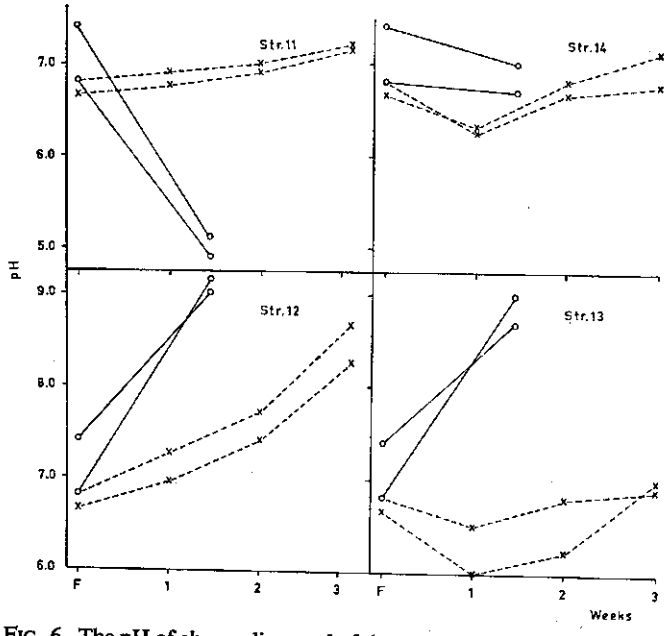


FIG. 6. The pH of cheese slices and of the Casamino acids-containing media due to the growth of different strains.

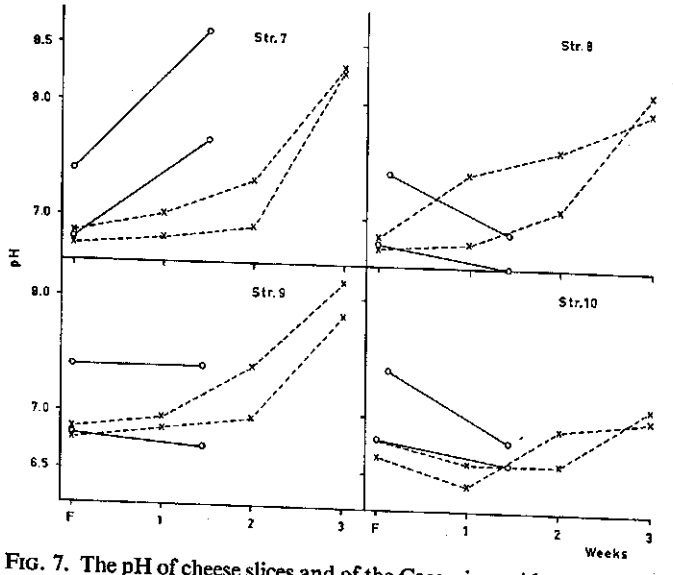


FIG. 7. The pH of cheese slices and of the Casamino acids-containing media due to the growth of different strains.

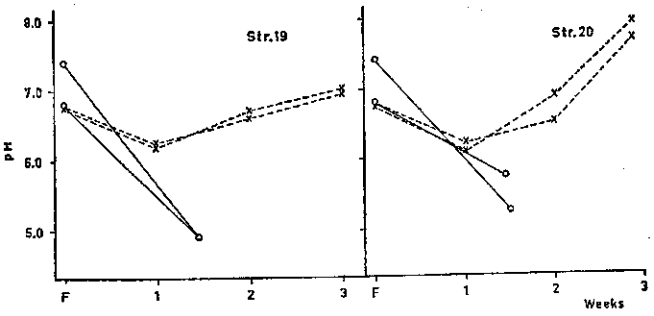


FIG. 8. The pH of cheese slices and of the Casamino acids-containing media due to the growth of different strains.

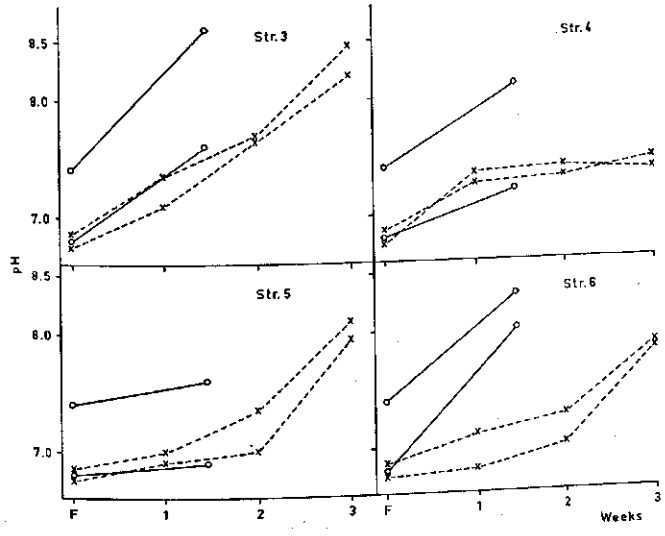


FIG. 9. The pH of cheese slices and of the Casamino acids-containing media due to the growth of different strains.

8.3.1.2. Soluble nitrogen

Soluble nitrogen expressed as percentage of the total nitrogen in the cheese slices after different periods of incubation with the different strains is given in Table 22. It will be seen that cream-coloured arthrobacters in general caused only a slight increase of the soluble nitrogen of the cheese slices as compared with the rest of the arthrobacters. Only strain 15 caused a moderate increase in soluble nitrogen. Most of the red, grey and greenish-yellow arthrobacters and the *Br. linens* organisms strongly hydrolysed the protein and in most cases more than 3/4 of the total nitrogen was in the soluble state after 3 weeks incubation. Strains 12 and 14, grey, 7 and 8, red, and 20, greenish-yellow arthrobacters and

TABLE 22. Soluble nitrogen of the cheese slices as percentage of the total nitrogen after different periods of incubation with the different strains.

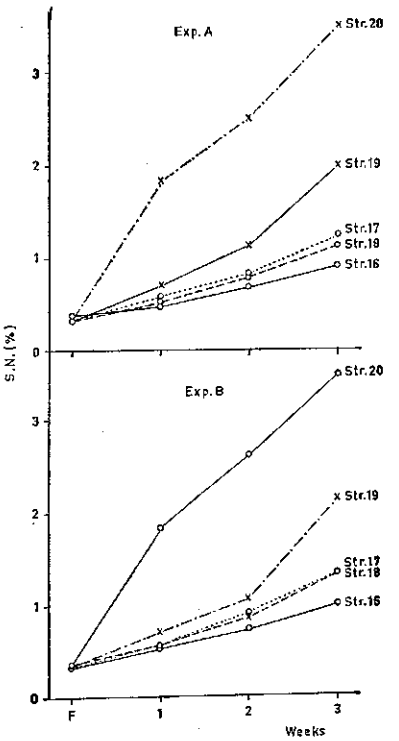
Type of organism	Soluble nitrogen as percentage of total nitrogen after							
	Fresh cheese		1 week		2 weeks		3 weeks	
	I	II	I	II	I	II	I	II
Arthrobacter								
Cream								
Strain 15	8.81	8.16	19.02	19.92	40.18	39.05	51.84	50.64
16	8.81	8.16	11.48	12.62	16.61	17.44	22.23	24.23
17	7.84	8.22	13.81	12.82	19.47	21.06	29.20	30.84
18	7.84	8.22	12.88	13.12	19.21	19.70	26.60	30.91
Grey								
Strain 11	8.81	8.16	13.33	15.04	27.27	32.88	44.56	48.60
12	8.81	8.16	43.83	43.48	58.92	57.15	85.21	84.17
13	8.81	8.16	14.94	14.65	36.05	34.30	59.45	58.70
14	8.81	8.16	35.39	36.23	51.35	56.39	78.89	77.27
Red								
Strain 7	8.80	8.89	37.94	35.88	49.53	44.72	83.67	76.43
8	8.80	8.89	43.87	40.38	59.37	51.83	88.01	79.21
9	8.80	8.89	19.77	19.06	47.16	45.44	63.36	64.19
10	8.81	8.16	16.08	14.70	38.51	37.33	52.10	49.78
Greenish-yellow								
Strain 19	7.84	8.22	16.86	16.25	27.07	24.37	47.68	49.56
20	7.84	8.22	42.94	42.76	59.50	60.78	83.68	81.28
Br. linens								
Strain 3	8.80	8.89	40.45	35.91	53.49	47.81	79.79	75.40
4	8.80	8.89	20.29	18.31	48.90	45.01	61.69	57.30
5	8.80	8.89	39.78	35.93	51.87	50.17	82.83	81.30
6	8.80	8.89	23.03	21.25	49.80	42.02	68.10	63.89
Yeast								
Strain 21	7.84	8.22	10.21	9.62	14.26	13.73	19.21	18.49
22	7.84	8.22	8.67	7.56	13.78	11.69	16.91	16.85

strains 3 and 5 of the *Br. linens* type, were able to hydrolyse the cheese extensively, about 80% or more of the total nitrogen being in the soluble phase. When the yeasts were grown on cheese slices only about 17% of the total nitrogen was found in the soluble form (see also Figures 10, 11, 12 and 13).

8.3.1.3. Amino acid nitrogen

The data of the amino acid nitrogen analyses (including ammoniacal nitrogen) as percentage of the total nitrogen are shown in Table 23. The majority of the cream-coloured arthrobacters caused a slight increase in the amino acid nitrogen content when grown on cheese slices for 3 weeks. Most of the grey arthrobacters caused a moderate increase in the free amino acid nitrogen with the exception of strain 12 which gave a very high increase. The same trend was observed in the red and greenish-yellow arthrobacters and in *Br. linens* where most of the strains had brought about a moderate amino acid content except strains 8, red, and 20, greenish-yellow arthrobacters which were similar to strain 12 of the grey

FIG. 10. Soluble nitrogen as % of the cheese slices, inoculated with different strains, after different periods of incubation.



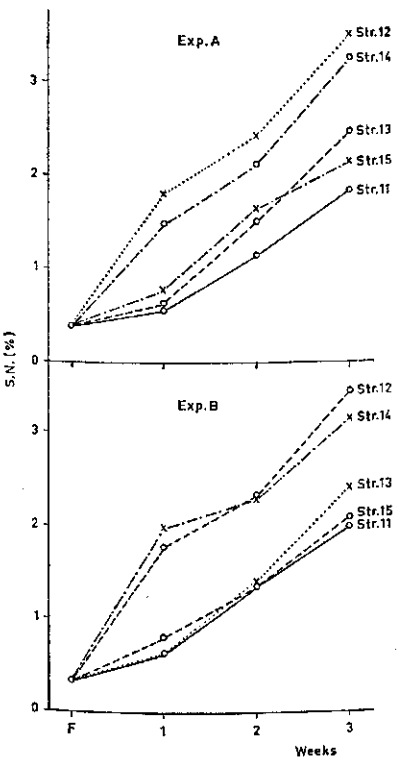


FIG. 11. Soluble nitrogen as % of the cheese slices, inoculated with different strains, after different periods of incubation.

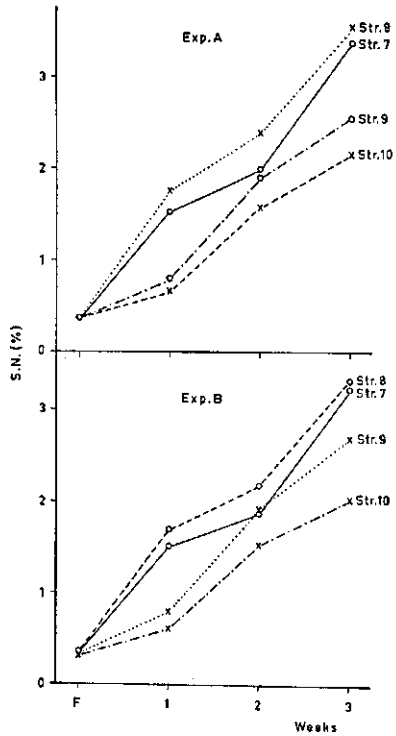


FIG. 12. Soluble nitrogen as % of the cheese slices, inoculated with different strains, after different periods of incubation.

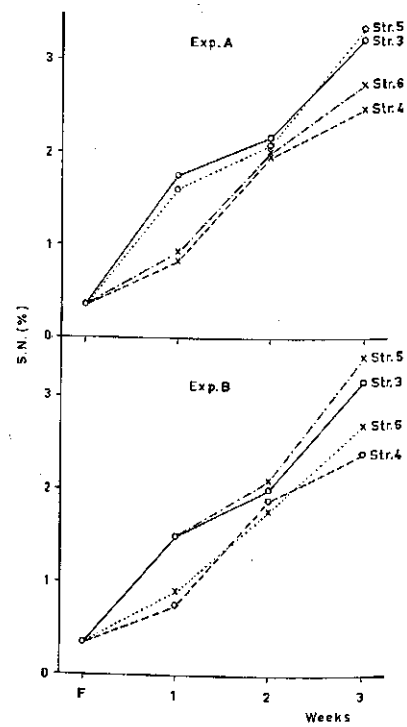


FIG. 13. Soluble nitrogen as % of the cheese slices, inoculated with different strains, after different periods of incubation.

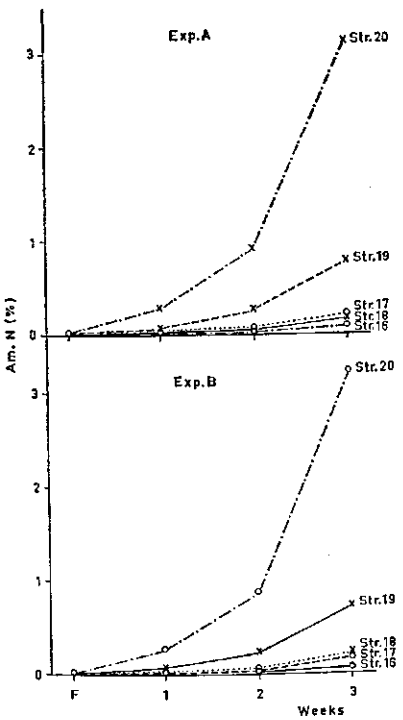


FIG. 14. Amino acid nitrogen, including ammoniacal nitrogen, as % of the cheese slices, inoculated with different strains, after different periods of incubation.

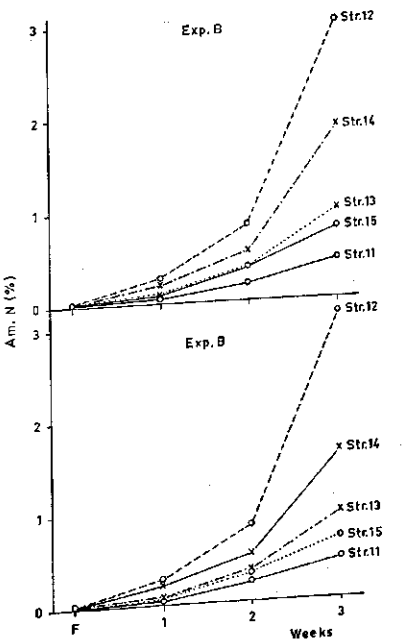


FIG. 15. Amino acid nitrogen, including ammoniacal nitrogen, as % of the cheese slices, inoculated with different strains after different periods of incubation.

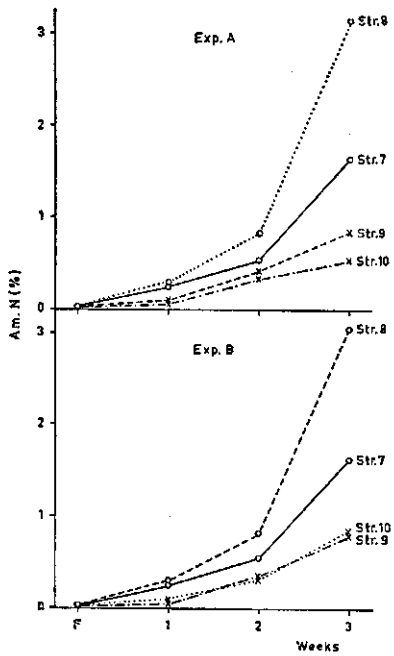


FIG. 16. Amino acid nitrogen, including ammoniacal nitrogen, as % of the cheese slices, inoculated with different strains, after different periods of incubation.

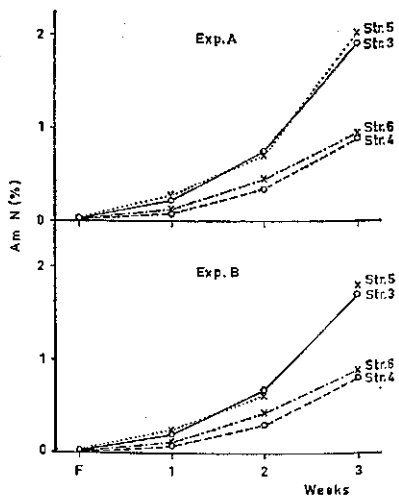


FIG. 17. Amino acid nitrogen, including ammoniacal nitrogen, as % of the cheese slices, inoculated with different strains, after different periods of incubation.

arthrobacters. The yeasts only very slightly increased the amino acid nitrogen content when grown on the cheese slices for 3 weeks (see also Figures 14, 15, 16 and 17).

8.3.1.4. Growth

As to the rate of growth on the cheese slices the *Arthrobacter* strains could be divided into 3 groups, viz. (a) including most of the cream-coloured strains and one greenish-yellow strain growing moderately, (b) half of the grey and the red strains growing copiously and (c) the rest of the grey, red and greenish-yellow

TABLE 23. Amino acid nitrogen[1] of the cheese slices as percentage of the total nitrogen after different periods of incubation with the different strains.

Type of organism	Amino acid nitrogen as percentage of the total after							
	Fresh cheese		1 week		2 weeks		3 weeks	
	I	II	I	II	I	II	I	II
Arthrobacter								
Cream								
Strain 15	0.46	0.42	1.73	1.57	8.39	7.30	18.88	16.10
16	0.46	0.42	0.58	0.56	1.05	0.96	2.35	1.98
17	0.45	0.42	0.88	0.75	2.04	1.89	5.02	4.65
18	0.45	0.42	0.69	0.56	1.52	1.56	4.74	5.16
Grey								
Strain 11	0.46	0.42	0.58	0.51	4.45	4.70	10.05	10.51
12	0.46	0.42	6.59	6.52	19.73	20.16	73.61	76.21
13	0.46	0.42	1.75	1.54	8.49	8.18	33.47	22.08
14	0.46	0.42	4.84	4.70	12.70	12.27	39.99	39.25
Red								
Strain 7	0.45	0.40	6.03	5.61	13.56	12.79	40.68	38.31
8	0.45	0.40	7.03	6.92	20.54	19.39	77.62	71.91
9	0.45	0.40	1.65	1.28	9.77	8.10	20.79	19.18
10	0.46	0.42	1.48	1.57	7.71	7.89	18.78	19.99
Greenish-yellow								
Strain 19	0.45	0.42	1.68	1.49	6.66	5.77	18.10	17.06
20	0.45	0.42	6.66	6.33	21.81	20.42	73.76	75.14
Br. linens								
Strain 3	0.45	0.40	5.28	4.71	17.80	16.23	47.16	40.28
4	0.45	0.40	1.79	1.59	7.93	7.18	21.76	18.99
5	0.45	0.40	6.08	5.49	17.17	14.78	49.83	43.87
6	0.45	0.40	2.27	2.02	10.52	9.86	23.03	21.01
Yeast								
Strain 21	0.45	0.42	0.52	0.51	1.44	1.33	2.32	2.59
22	0.45	0.42	0.47	0.49	0.99	1.19	1.61	1.70

[1] Including ammoniacal nitrogen.

strains growing very heavily (Table 24). The *Br. linens* organisms were all growing very heavily on the cheese slices after 3 weeks of incubation, while the yeasts grew copiously.

8.3.1.5. Smell of proteolysis

After 3 weeks of incubation, all the red, half of the grey and half of the greenish-yellow strains produced a pronounced smell of proteolysis in the plates. The other half of the grey arthrobacters were moderately smelling, which was true also of strain 15 of the cream-coloured arthrobacters. Most of the cream-coloured strains and strain 19 of the greenish-yellow arthrobacters produced only a slight smell of proteolysis (Table 24). Half of the *Br. linens* strains gave a heavy smell of proteolysis while in the other half it was moderate. No smell of proteolysis was detected when the yeast strains were grown on the cheese slices

TABLE 24. Growth and organoleptic features of cheese slices after different periods of incubation with the different strains.

Type of organism	Growth[1] after			Smell[2] of proteolysis after			Cheese liquefaction[3] after			Smell[4] of ammonia after		
	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
Arthrobacter												
Cream												
Strain 15	+	+	++	1	1	2	−	−	+	−	−	+
16	+	+	+	−	−	1	−	−	−	−	−	−
17	+	+	+	−	−	1	−	−	−	−	−	−
18	+	+	+	−	−	1	−	−	−	−	−	−
Grey												
Strain 11	+	+	++	−	1	2	−	−	−	−	−	+
12	++	+++	+++	1	2	3	−	+	++	−	+	+
13	++	++	++	1	1	2	−	−	+	−	−	+
14	++	+++	+++	1	2	3	−	+	++	−	+	+
Red												
Strain 7	+	++	+++	1	2	3	—	+	++	−	+	+
8	++	++	+++	1	2	3	−	+	++	−	+	+
9	+	++	+++	−	2	3	−	—	+	−	−	+
10	+	+	++	−	2	3	−	−	+	−	−	+
Greenish-yellow												
Strain 19	+	+	+	−	1	1	−	−	−	−	−	−
20	++	+++	+++	1	3	3	−	+	++	−	+	+
Br. linens												
Strain 3	++	++	+++	1	2	3	−	+	+	−	+	+
4	++	++	+++	−	1	2	−	−	+	−	−	+
5	+	++	+++	1	2	3	−	+	+	−	+	+
6	+	++	+++	−	1	2	−	−	+	−	−	+
Yeast												
Strain 21	++	++	++	−	−	−	−	−	−	−	−	−
22	++	++	++	−	−	−	−	−	−	−	−	−

[1] +, moderate; ++, copious; +++, very heavy.
[2] −, absent; 1, light; 2, moderate; 3, heavy.
[3] −, absent; +, partly; ++, heavy.
[4] −, absent; +, present.

for 3 weeks. The data of Table 24 clearly show that after one week of growth the smell of proteolysis was either absent or light, while upon ageing it deepened.

8.3.1.6. Liquefaction of the cheese

After 3 weeks of growth, the yeasts and most of the cream-coloured arthrobacters had been unable to cause any cheese liquefaction whereas most of the grey and red arthrobacters, even after 2 weeks, had liquefied the cheese slices either heavily or moderately. Of the greenish-yellow arthrobacters, strain 19 was unable to liquefy the cheese whereas strain 20 had heavily liquefied it. All the *Br. linens* organisms showed a moderate liquefaction (Table 24).

8.3.1.7. Smell of ammonia

No strain was able to produce a smell of ammonia after 1 week of growth on the cheese slices. After 2 weeks some strains belonging to the grey, red, and greenish-yellow arthrobacters and the *Br. linens* group had been able to produce a clear smell of ammonia in the cheese plates. All the grey, red, half of the greenish-yellow and strain 15 of the cream-coloured arthrobacters produced this smell of ammonia only after 3 weeks of growing on the cheese slices. The rest of the cream-coloured, 3 strains, and the greenish-yellow arthrobacters, 1 strain, and the yeasts were unable to produce a clear smell of ammonia in the cheese plates.

8.3.1.8. Colour

Table 25 shows the data about the colour of the cheese slices after different periods of incubation. The group of cream-coloured arthrobacters gave cream-coloured cheese slices throughout the incubating period of 3 weeks. The grey arthrobacters started with a cream-coloured surface with a light grey shade and reached grey after 3 weeks of growth. The group of red arthrobacters, except strain 7 which brought about a light orange colour, gave light red-coloured cheese slices after 3 weeks. The greenish-yellow strains included two types, strain 19, causing a greenish-yellow cheese surface and strain 20 which started with a greenish-yellow colour with a red shade after 1 week of growth and reached a red colour after 3 weeks of incubation.

The *Br. linens* organisms after 2 weeks of incubation gave an orange colour to the cheese surface, except with strain 4 where the colour came to light orange.

The yeasts, like the cream-coloured arthrobacters, gave cream-coloured cheese slices.

8.4. Qualitative chromatographic analysis of the samples

At the end of the growing periods mentioned before (Tables 21, 22 and 23) the cheese slices and the Casamino acids media were analysed chromatographically for amino acids (qualitatively). Ethanol, 95%, was added to the filtered cheese extracts and the filtrates of the Casamino acids-containing media in the proportion of 5 to 1, followed by centrifugation. The clear solution was evapo-

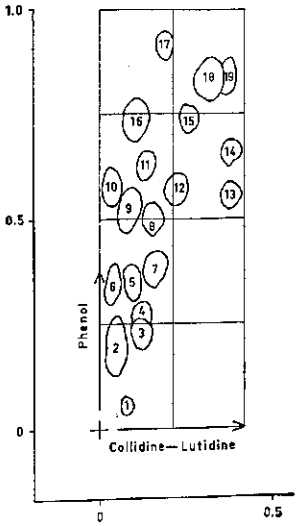
TABLE 25. Colour of cheese slices after different periods of incubation with the different strains.

Type of organism	Colour after		
	1 week	2 weeks	3 weeks
Arthrobacter			
Cream			
Strain 15			
16			
17	Cream	Cream	Cream
18			
Grey			
Strain 11	Cream	Cream with light grey shade	Cream with light grey shade
12	Cream with grey shade	Cream with grey shade	Grey
13			
14			
Red			
Strain 7	Cream with light orange shade	Cream with an orange shade	Light orange
8			
9	Cream with red shade	Light red	Light red
10			
Greenish-yellow			
Strain 19	Cream with yellow shade	Greenish-yellow	Greenish-yellow
20	Greenish-yellow with red shade	Red	Red
Br. linens			
Strain 3	Light orange	Orange	Orange
4	Cream	Light orange	Light orange
5	Light orange	Orange	Orange
6	Cream	Orange	Orange
Yeast			
Strain 21	Cream	Cream	Cream
22			

rated using a rotary evaporator and then diluted to the required dilution for the chromatographic analysis (see Chapter 2: 2.2.4. and 2.3.). The degree of dilution of the extracts of the cheese slices varied from: undiluted (strains 16, 17, 18, 21 and 22) to a dilution of 1: approximately 50 (strains 12 and 20). In the case of the Casamino acids-containing media all the samples (inoculated ones) br ought on the chromatograms comprised the same amount of medium) (diluted 1:10). For comparison, two cheese samples, made under commercial conditions, at the ages of 14 and 27 days were also analysed. Figure 18 shows the position of the different amino acids of a standard mixture on the chromatogram. Plate 11 gives the chromatogram of acid-hydrolysed casein (Casamino acids 'Oxoid'), Plate 12 the chromatograms of the extract of the two samples of Limburger cheese, and Plates 13 to 22 the results obtained with the cheese slices and the Casamino acids-containing media.

FIG. 18. The position of the different amino acids on the chromatogram.

1. Aspartic acid
2. Glutamic acid
3. Serine
4. Glycine
5. Asparagine
6. Lysine
7. Threonine
8. Alanine
9. Glutamine
10. Arginine
11. Histidine
12. Methionine
13. Tyrosine
14. Tryptophan
15. Valine
16. γ-Aminobutyric acid
17. Proline
18. Leucines
19. Phenylalanine



A comparison of Plates 11 and 12 shows that the occurrence of free amino acids in the ripening Limburger cheese sharply differs from that in the acid-hydrolysed casein. The amino acids of the higher area of the cheese chromatograms, mainly leucines, valine, γ-aminobutyric acid and alanine occurred in concentrations comparable to those of the chromatogram of hydrolysed casein. The rest of the amino acids occurred in much lower concentrations, presumably due to microbial transformation during the ripening period.

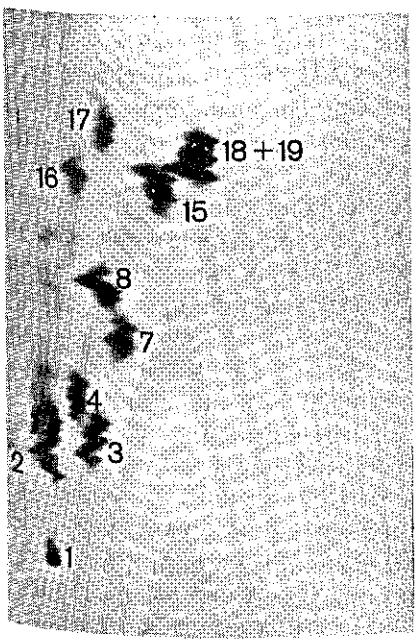


PLATE 11. Chromatogram of the uninoculated Casamino acids-containing medium (diluted 1:20).

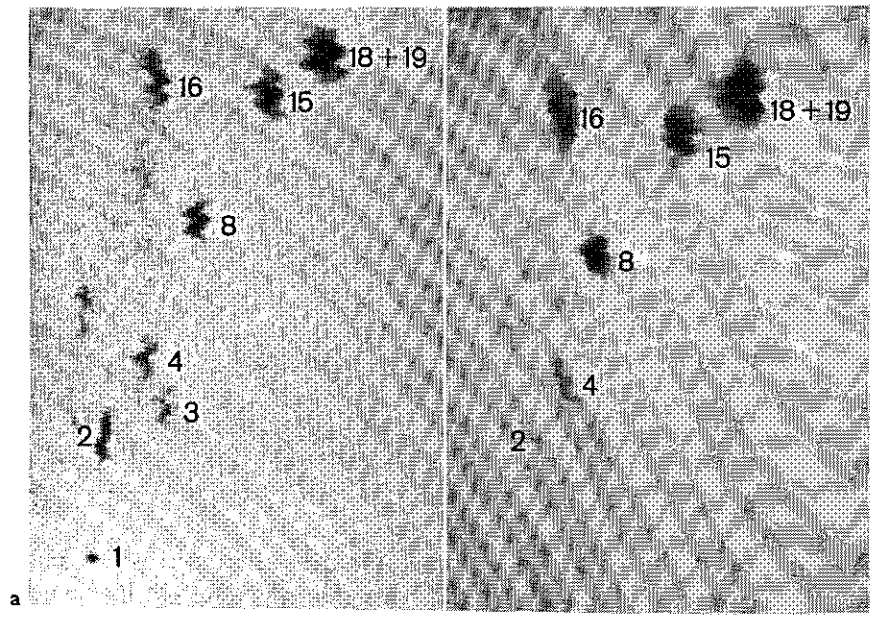


PLATE 12. Chromatograms of the extract of 14 days (a) and 27 days (b) old Limburger cheese. Dilution of cheese extract: a, 1:10 and b, 1:25.

8.4.1. *The Arthrobacter group*

8.4.1.1. Cream-coloured strains

This group of organisms includes strains 15, 16, 17 and 18. As shown in Figures 14 and 15, the formation of free amino acids in the cheese slices by these strains was very poor. Chromatograms obtained from these cheese slices showed that the amino acids of the lower part of the chromatogram (particularly aspartic acid, glutamic acid, serine and glycine) occurred in relatively high concentration (Plate 13, a).

The chromatograms of the Casamino acids-containing medium after 10 days growth with strains of this group (Plate 13, b), were comparable with that of the blank (Plate 11) as to the relative presence of the different amino acids, showing that these organisms had no preference for a particular amino acid.

8.4.1.2. Grey strains

This group of cheese coryneforms comprises highly proteolytic strains (12 and 14) and moderately proteolytic strains (11 and 13). Formation of amino acids in the cheese slices by the former two strains was very high as compared with that by the latter two strains (Fig. 15).

The chromatograms obtained from the cheese slices inoculated with either strain 12 or 14 (Plate 14, a) were more or less resembling that of the hydrolysed casein (Plate 11). However, the leucines and valine occurred in relatively higher concentrations.

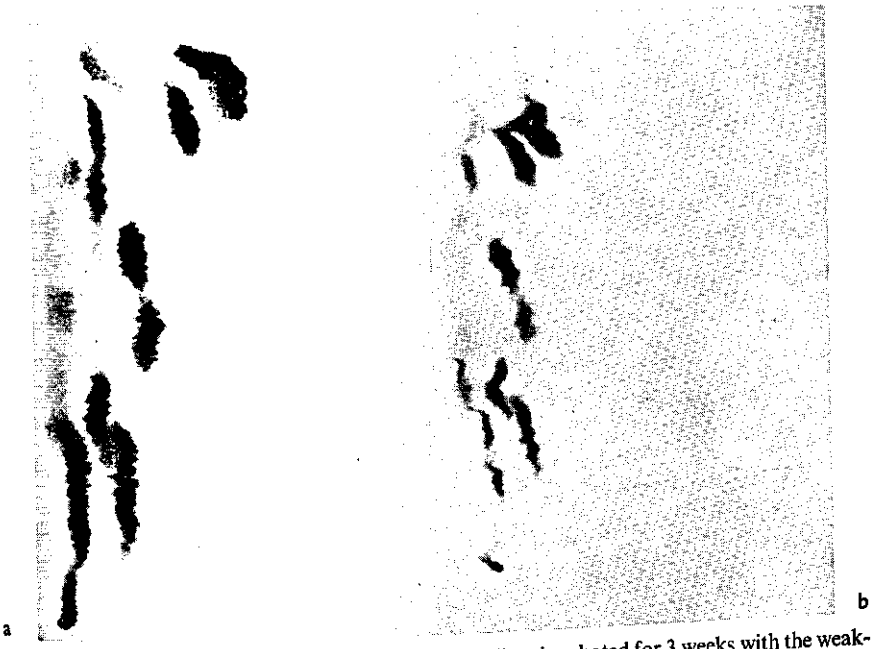


PLATE 13. Chromatograms of the extract of cheese slices incubated for 3 weeks with the weakly proteolytic cream-coloured *Arthrobacter* strain (15) (a, diluted 1:5) and of the Casamino acids-containing media incubated for 10 days with the same organism (b).

The chromatograms of the used Casamino acids-containing medium (Plate 14, b) were comparable with that of the uninoculated blank.

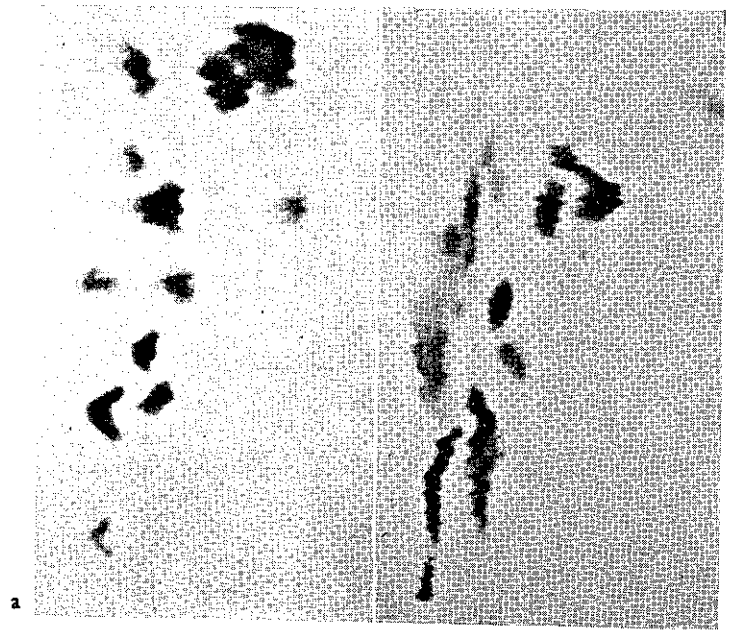
The chromatograms obtained from the cheese slices inoculated with strains 11 and 13 (Plate 14, c) were completely different from those obtained with strains 12 and 14. The amino acids of the lower part of the chromatogram viz. aspartic acid, glutamic acid, serine and glycine had almost entirely disappeared whereas γ-aminobutyric acid was present in relatively much higher concentrations.

The chromatograms of the Casamino acids-containing medium in which strains 11 and 13 had grown (Plate 14, d) indicated a pronounced decrease of all the amino acids except alanine.

8.4.1.3. Red strains

This group of coryneforms also includes highly proteolytic strains (7 and 8) and moderately proteolytic strains (9 and 10). Formation of amino acids was very high in the cheese plates inoculated with strain 8, high with strain 7 and moderate with strains 9 and 10 (Fig. 16).

The chromatograms obtained from the cheese slices inoculated with the different strains were all more or less similar. In comparison with the chromatogram of the hydrolysed casein, serine and particularly threonine occurred in relatively low concentrations (Plate 15, a).



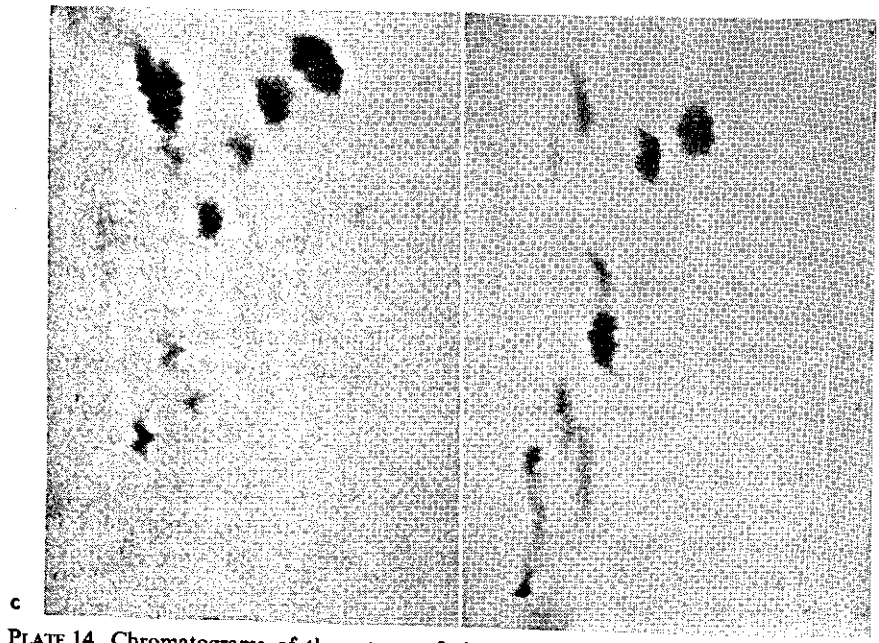


PLATE 14. Chromatograms of the extract of cheese slices incubated for 3 weeks with the strongly proteolytic grey strain (14) (a, diluted 1:30), the moderately proteolytic grey strain (13) (c, diluted 1:15) and of the Casamino acids-containing medium incubated for 10 days with strain 14 (b) and strain 13 (d).

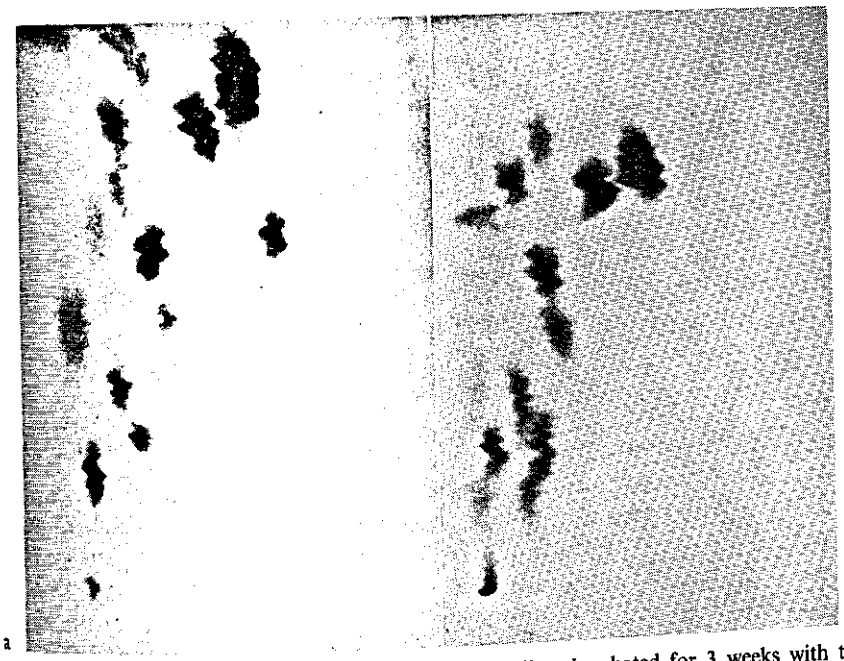


PLATE 15. Chromatograms of the extract of cheese slices incubated for 3 weeks with the strongly proteolytic red strain (8) (a, diluted 1:50) and of the Casamino acids-containing medium incubated for 10 days with the same organism (b).

In the case of the used Casamino acids-containing medium, the chromatograms resembled that of the uninoculated blank, with the exception of a somewhat lower concentration of threonine (Plate 15, b).

8.4.1.4. Greenish-yellow strains

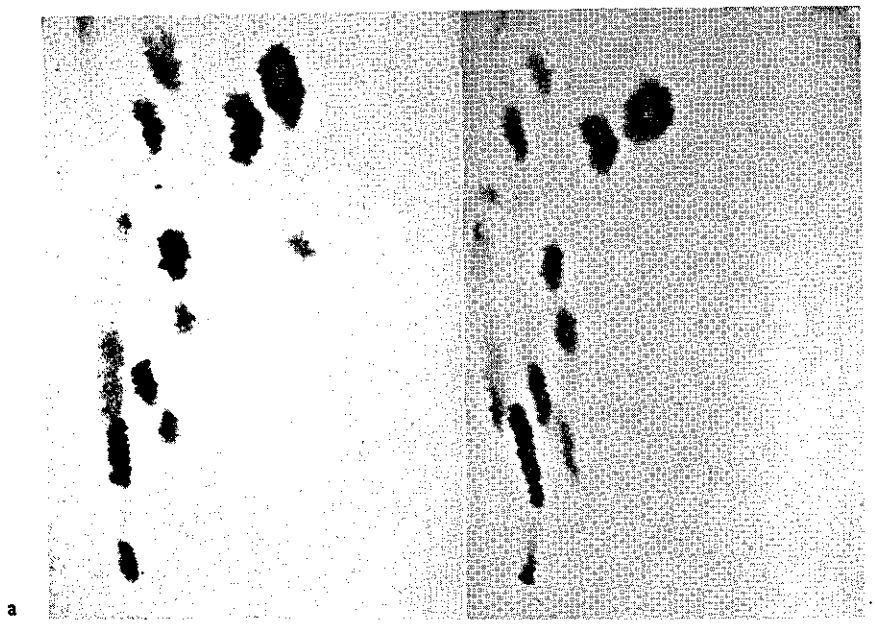
This group of coryneform bacteria includes one highly proteolytic strain (20) and one moderately proteolytic strain (19). The former produced very high amounts of amino acids in the cheese slices, the latter moderate amounts (Fig. 14).

The chromatograms obtained from the cheese slices inoculated with the highly proteolytic strain 20 (Plate 16, a) resembled that of the casein hydrolysate (Plate 11) with the exception of low concentrations of serine and threonine.

In the case of the Casamino acids-containing medium, lower concentrations of serine and higher concentrations of glutamic acid occurred (Plate 16, b).

The chromatograms obtained from the cheese slices containing the moderately proteolytic strain 19 (Plate 16, c) resembled the chromatograms obtained from 14 days old Limburger cheese (Plate 12) with the exception of lower concentrations of valine and γ-aminobutyric acid and the presence of threonine, which was nearly absent in the sample of Limburger cheese.

The chromatograms of the used Casamino acids-containing medium resembled those obtained from the cheese plates inoculated with strain 19,



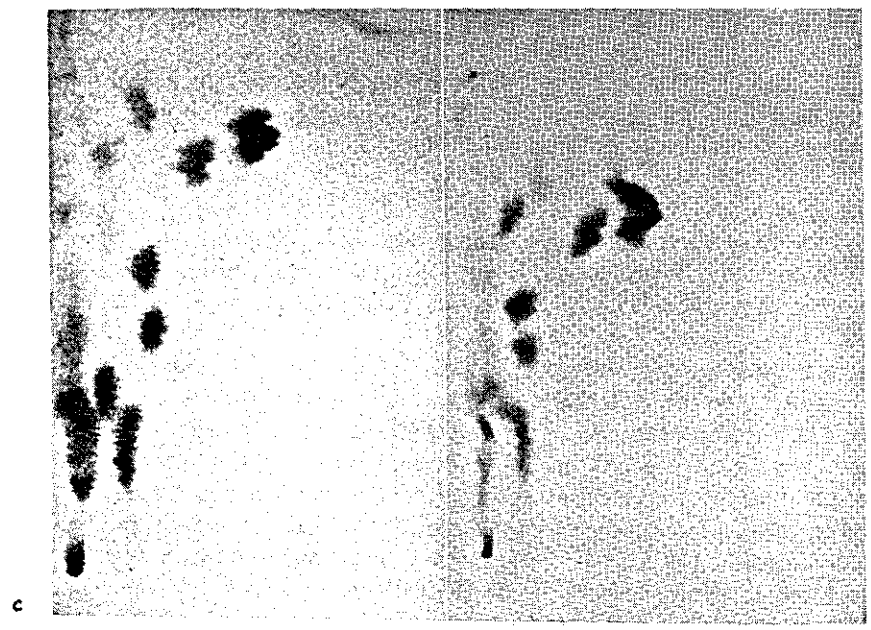


PLATE 16. Chromatograms of the extract of cheese slices incubated for 3 weeks with the strongly proteolytic greenish-yellow strain (20) (a, diluted 1:50), the moderately proteolytic greenish-yellow strain (19) (c, diluted 1:5) and of the Casamino acids-containing medium incubated for 10 days with strain 20 (b) and strain 19 (d).

indicating a pronounced tendency of this strain to transform most of the amino acids present with the exception of leucine (Plate 16, d).

8.4.2. *The group of Br. linens strains*

This group includes strongly proteolytic strains (3 and 5) and moderately proteolytic ones (strains 4 and 6). The amino acid production in cheese slices was high in the presence of strains 3 and 5 and moderate with strains 4 and 6 (Fig. 17).

The chromatograms obtained from the cheese slices inoculated with strains 3 and 6 resembled that of the hydrolysed casein with the exception of a reduced concentration of threonine (Plate 17, a).

The picture of the used Casamino acids medium resembled that of the cheese slices (Plate 17, b).

The chromatograms of the cheese slices inoculated with the strongly proteolytic strain 5 were more alike those derived from Limburger cheese with the exception of lower concentrations of γ-aminobutyric acid and higher concentrations of glutamic acid (Plate 17, c).

In the case of he Casamino acids-containing medium inoculated with strain 5, the chromatograms obtained were comparable with that of the uninoculated blank (Plate 17, d).

The free amino acid composition of cheese slices inoculated with strain 4 (Plate 18, a) resembled that obtained from 14 days old Limburger cheese with the exception of higher concentrations of aspartic acid, glutamic acid and serine.

In the case of the used Casamino acids-containing medium, the chromatograms obtained showed low concentrations of the amino acids on the lower part of the chromatogram except aspartic acid (Plate 18, b) a picture which is almost similar to that of 14 days old Limburger cheese.

8.4.3. Yeasts

This group of micro-organisms were practically free from proteolytic activity. The production of free amino acids during their 3 weeks growth on cheese slices was very low (Table 23).

The chromatograms obtained with both strains of yeast were similar. They resembled the chromatograms of 14 days old Limburger cheese with the exception of higher concentrations of glutamic acid and threonine and the absence of serine (Plate 19, a).

The chromatograms of the used Casamino acids-containing medium resembled that of the uninoculated blank (Plate 19, b).

8.5. Growth and cell yield of different bacteria in media with single amino acids

In this experiment 8 cheese coryneforms were grown in media containing one amino acid as the main organic compound. As many of the strains showed a

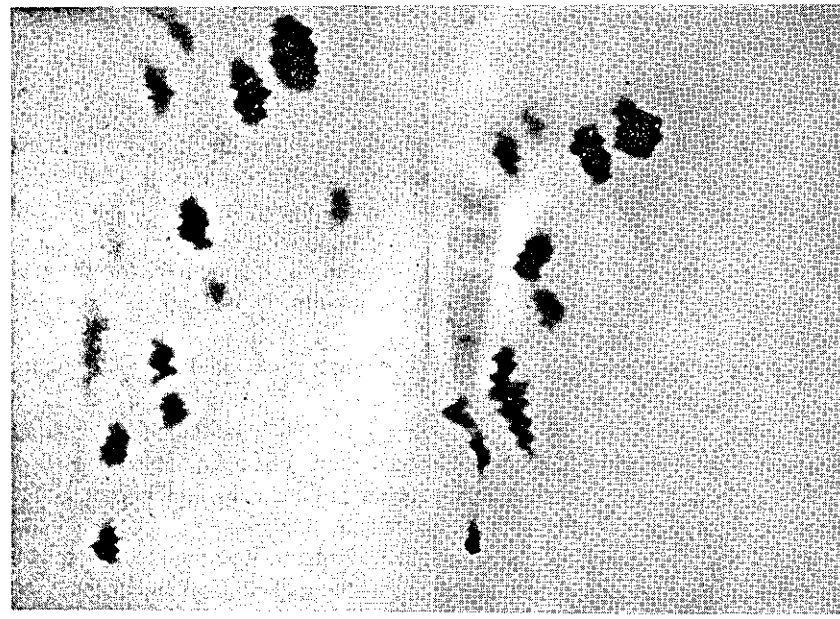
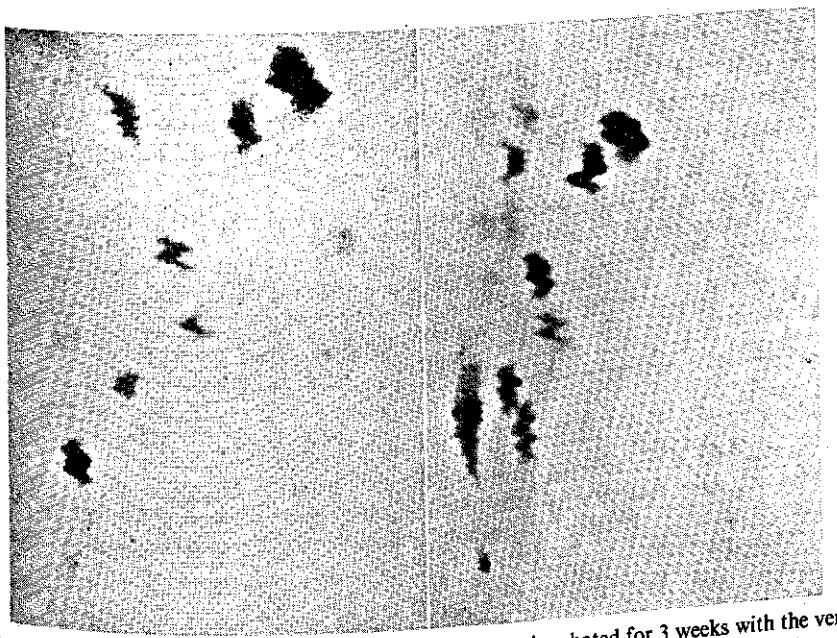


PLATE 17. Chromatograms of the extract of cheese slices incubated for 3 weeks with the strongly proteolytic *Br. linens* strain (3) (a, diluted 1:30), and (5) (c, diluted 1:30) and of the Casamino acids-containing medium incubated for 10 days with strain 3 (b) and strain 5 (d).

a b

PLATE 18. Chromatograms of the extract of cheese slices incubated for 3 weeks with the moderately proteolytic *Br. linens* strain (4) (a, diluted 1:10) and of the Casamino acids-containing medium incubated for 10 days with the same organism.



a b

PLATE 19. Chromatograms of the extract of cheese slices incubated for 3 weeks with the very weakly proteolytic yeast strain (21) (a, not diluted) and of the Casamino acids-containing medium incubated for 10 days with the same organism.

growth response to small amounts of additional $(NH_4)_2$ SO_4, the mineral medium used in this experiment was supplied with 50 mg glucose, 25 mg $(NH_4)_2$ SO_4 and 300 mg amino acid per 100 ml of medium.

The bacteria used in this experiment belonged to grey, strains 13 and 14, red, strains 7 and 8, and greenish-yellow arthrobacters, strains 19 and 20, and to the *Br. linens* type, strains 3 and 4. Those strains were chosen according to the clear effect they had shown when grown in Casamino acids-containing medium.

8.5.1. *Growth*

Table 26 shows the growth of the different strains in the presence of different amino acids. It is clear that glutamic acid was utilized by nearly all the strains, with growth ranging from slight (strains 8 and 20) to heavy (strains 3, 4, 13 and 14). Strains 4 and 13 were able to grow with most of the tested amino acids. Only strain 13 was able to utilize leucine; most of the strains failed to grow in the presence of this amino acid. Glycine was not utilized by most of the strains and many strains were also unable to utilize alanine. Plate 20 shows the effect of the different strains on the different amino acids. An interesting result was obtained with strain 8 when grown on glutamic acid and with strain 4 when grown on aspartic acid. In both cases the presence of another amino acid on the chromatogram was recognized (in the case of strain 4 this was glutamic acid).

8.5.2. *Cell yield*

In a subsequent experiment, cell yield was determined of these coryneforms (strain 4, 7 and 13) grown in a mineral medium supplied with glucose, $(NH_4)_2$. SO_4, serum of young cheese and a single amino acid.

The results of this experiment, recorded in Table 27, show that the amino acids decomposed by the coryneform bacteria, have increased cell yield. This increase, in general, was relatively small while in some cases cell yield was

TABLE 26. Growth of cheese coryneforms in media containing $(NH_4)_2SO_4$ and different amino acids.

Type of organism	Growth[1] in the presence of					
	Glutamic acid	Aspartic acid	Alanine	Serine	Glycine	Leucine
Arthrobacter						
Strain 7	++	+	+	+	±	−
8	±	−	±	−	−	−
13	+++	++	±	+++	−	+++
14	+++	−	±	−	−	−
19	+	−	±	+	−	±
20	±	−	−	±	−	±
Br. linens						
Strain 3	+++	±	−	−	−	±
4	+++	++	++	+++	±	−

[1] −, Nil; ±, slight; +, moderate; ++, good; +++, heavy.

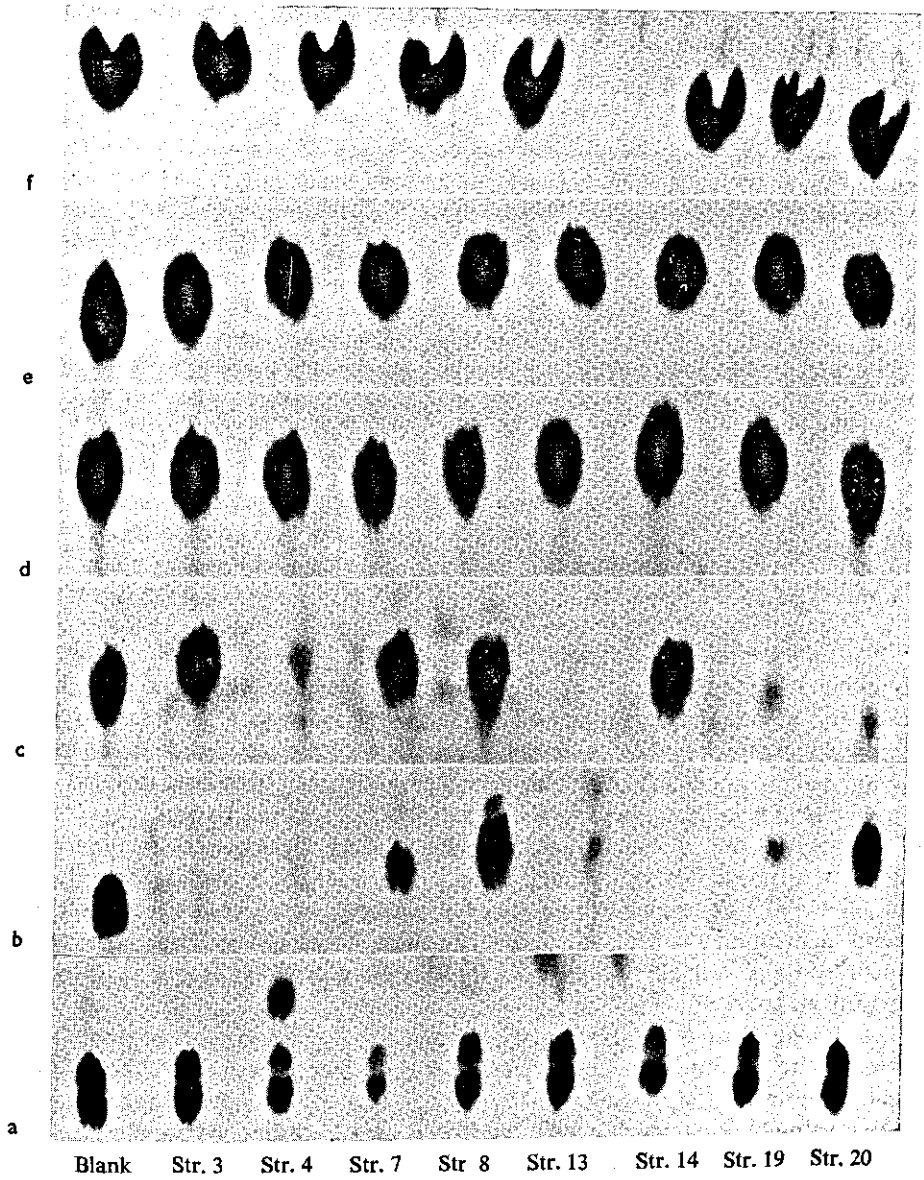


PLATE 20. Chromatograms obtained after the growth of different strains of cheese coryneforms in media containing single amino acid: aspartic acid, a; glutamic acid, b; serine, c; glycine, d; alanine, e; and leucine, f.

TABLE 27. Cell yield of different strains in media containing $(NH_4)_2SO_4$ and one amino acid or a mixture of amino acids.

Amino acid	Cell yield (mg dry wt/100 ml of medium[1]) with strain		
	4	7	13
Glutamic acid	53.5	54.0	27.0
Aspartic acid	39.0	28.0	47.5
Alanine	47.5	38.5	5.0
Glycine	19.0	22.5	7.0
Leucine	21.5	28.5	45.5
Mixture[2]	25.5	44.5	27.0

[1] Containing 25 mg $(NH_4)_2SO_4$, 25 mg glucose, 2 ml of young cheese serum and 300 mg amino acid.

[2] Containing 25 mg $(NH_4)_2SO_4$, 25 mg glucose, 2 ml of young cheese serum and 60 mg of each amino acid.

hardly improved inspite of the ability of the organism concerned to decompose the amino acid.

8.6. DISCUSSION

To study the effect of different micro-organisms, isolated from the surface of ripening Limburger cheese, on the ripening process, experiments have been carried out with sterilized slices of cheese inoculated with a number of representative organisms. The 20 strains tested, belonged to different types of *Arthrobacter* (the cream-coloured, grey, red and greenish-yellow groups), the orange *Br. linens* group, and furthermore included two yeast strains.

The inoculated cheese slices, contained in Petri dishes, were incubated at 25°C in an incubator with a high level of humidity for 3 weeks. Separate experiments with the same series of micro-organisms were run in culture solutions supplied with acid-hydrolysed casein (Casamino acids). The latter series were included to learn the preferential decomposition of different amino acids by the organisms tested.

Although most of the micro-organisms made a good growth on the cheese slices, there was a pronounced difference in the way in which the different pure cultures affected the cheese. After 3 weeks of incubation, the yeasts and most of the cream-coloured arthrobacters had been unable to liquefy the cheese. This was in contrast to most of the strains of the other *Arthrobacter* groups, which showed a moderate and often a pronounced liquefaction of the cheese slices. Owing to this fact, the yeasts and most of the cream-coloured arthrobacters had caused only a slight increase of the soluble and of the amino acid nitrogen as compared with most of the grey, red and greenish-yellow arthrobacters and the strains of the *Br. linens* group which had hydrolysed about 70 and in some cases even more than 80% of the protein at the end of a 3 weeks period.

Qualitative chromatography revealed a clear difference as to the relative amounts of different amino acids in the soluble fractions obtained from cheese

slices incubated with different bacteria. The chromatograms obtained from cheese cultures with the weakly proteolytic cream-coloured *Arthrobacter* strains contained relatively higher values of the amino acids, aspartic acid, glutamic acid, serine and glycine when compared with the chromatogram of a diluted mixture of Casamino acids. This could mean that these amino acids were formed by these organisms in larger amounts, or that the other amino acids were consumed in larger amounts. The results obtained with the Casamino acids-containing media would not confirm the latter hypothesis. The chromatograms of such a medium in which the cream-coloured strains had grown for 10 days were comparable with the uninoculated blank, indicating no preference for the decomposition of particular amino acids by the bacteria of this group.

In the case of the highly proteolytic grey, red and greenish-yellow arthrobacters and most of the *Br. linens* strains, the chromatograms of the amino acids of the liquefied cheese slices more or less resembled that of the Casamino acids mixture with the exception of the leucines and valine occurring in higher concentrations and serine and threonine in lower concentrations.

The chromatograms obtained from the cheese slices inoculated with the moderately proteolytic strains of the grey and greenish-yellow arthrobacters and of the *Br. linens* strains differed from those of the highly proteolytic strains. They more or less resembled the chromatograms obtained with ripening Limburger cheese, showing strongly reduced concentrations of aspartic acid, glutamic acid, serine and glycine, as compared with chromatograms of the diluted Casamino acids mixture. This type of chromatogram indicates the relatively strong decomposition of the latter amino acids, an explanation which was partly confirmed by the results of the experiments with the Casamino acids-containing media in which nearly all of amino acids were shown to be decomposed by this type of micro-organism.

To obtain more information as to the ability of the representative strains to decompose and utilize various amino acids, 8 of the strains used in the experiments with cheese slices were grown in a medium containing a number of single amino acids as the main organic compound. In addition to growth response, the consumption of the amino acids was determined. Comparison of the results of this experiment with those of the experiment with Casamino acids-containing media showed that the highly proteolytic strains, 7 and 8 (red), 14 (grey), 20 (greenish-yellow) and 3 (orange) had a much poorer ability to decompose the amino acids as this was true of the moderately proteolytic strains, 13 (grey), 19 (greenish-yellow) and 4 (orange). Amino acids which were more easily decomposed in this experiment (aspartic acid, serine and glutamic acid) were also decomposed more readily in both the Casamino acids-containing media and the inoculated cheese slices, as compared with the more resistant amino acids glycine, alanine and leucine.

Although the colour of the cheese slices, in general, was in accordance with the colour of the bacterial strains as observed on culture media, exceptions occurred (the greenish-yellow strain 20 after giving a light greenish-yellow colour with red shade to the cheese surface, turned to red in later stages).

9. THE AMINO ACID CONTENT OF LIMBURGER CHEESE

9.1. INTRODUCTION

TUCKY and SAHASRABUDHE (1957) studied the ripening of Limburger cheese by determining the quantities of individual amino acids liberated throughout the ageing period. They found that the ripening proceeds more rapidly in the rind portion than in the interior. The difference in amino acid content between the rind and the centre of the cheese represented a quantitative rather than a qualitatieve relationship. They found no correlation between the presence of any amino acid and the characteristic flavour development in the cheese. TUCKY and SAHASRABUDHE made their Limburger cheese by inoculating the cheese surface with a yeast culture, isolated from good quality commercial Limburger cheese, followed by inoculation with a culture of *Br. linens* (ATCC 9174) and cured at 50 to 52 °F.

However, the present investigation has shown that organisms of the *Br. linens* type can not be considered as the main ripening agent in this particular type of cheese as they make a late appearance in the surface flora. These *Br. linens* types of organisms may play a part in the ripening of Limburger cheese but in any case they are not the sole ripening flora.

The results of TUCKY and SAHASRABUDHE may be compared with those found in the present investigation after growing some organisms of the *Br. linens* (strains 3 and 6) type on the surface of cheese slices. After three weeks of growth all the amino acids present in casein were found in the free state and the chromatograms obtained (cf. Plate 17a) were almost similar to that obtained from a solution of Casamino acids (Plate 11).

Since a complete study concerning the amino acid content of commercial Limburger cheese during ripening has never been made, this part of the present work was undertaken to make a quantitative analysis of different amino acids in Limburger cheese during the different stages of ripening.

The samples of the cheese, which was made under commercial conditions were taken immediately after salting and after 5, 9, 14, 20, 27 and 35 days of ripening. They were analysed quantitatively for free amino acids.

9.2. EXPERIMENTAL

Cheese samples were prepared for the amino acid analysis as mentioned in Chapter 2 (2.2.4.). A volume of 1 to 2 ml was used for the analysis. The amino acids were assayed after deproteinization with 4% sulfosalicylic acid (4 ml/ml sample) and centrifugation. The analyses were made with the aid of a Biocal 200 amino acid analyser following the procedure of MOORE and STEIN (1954). Acid and neutral amino acids were eluted from a column containing 52 × 0.9 cm Biorad A6 spherical ion exchange resin. The basic amino acids were separated on a 22 × 0.9 cm Biorad A5 ion exchange resin column.

Plates 21 and 22 show the position of the separated amino acids as to the

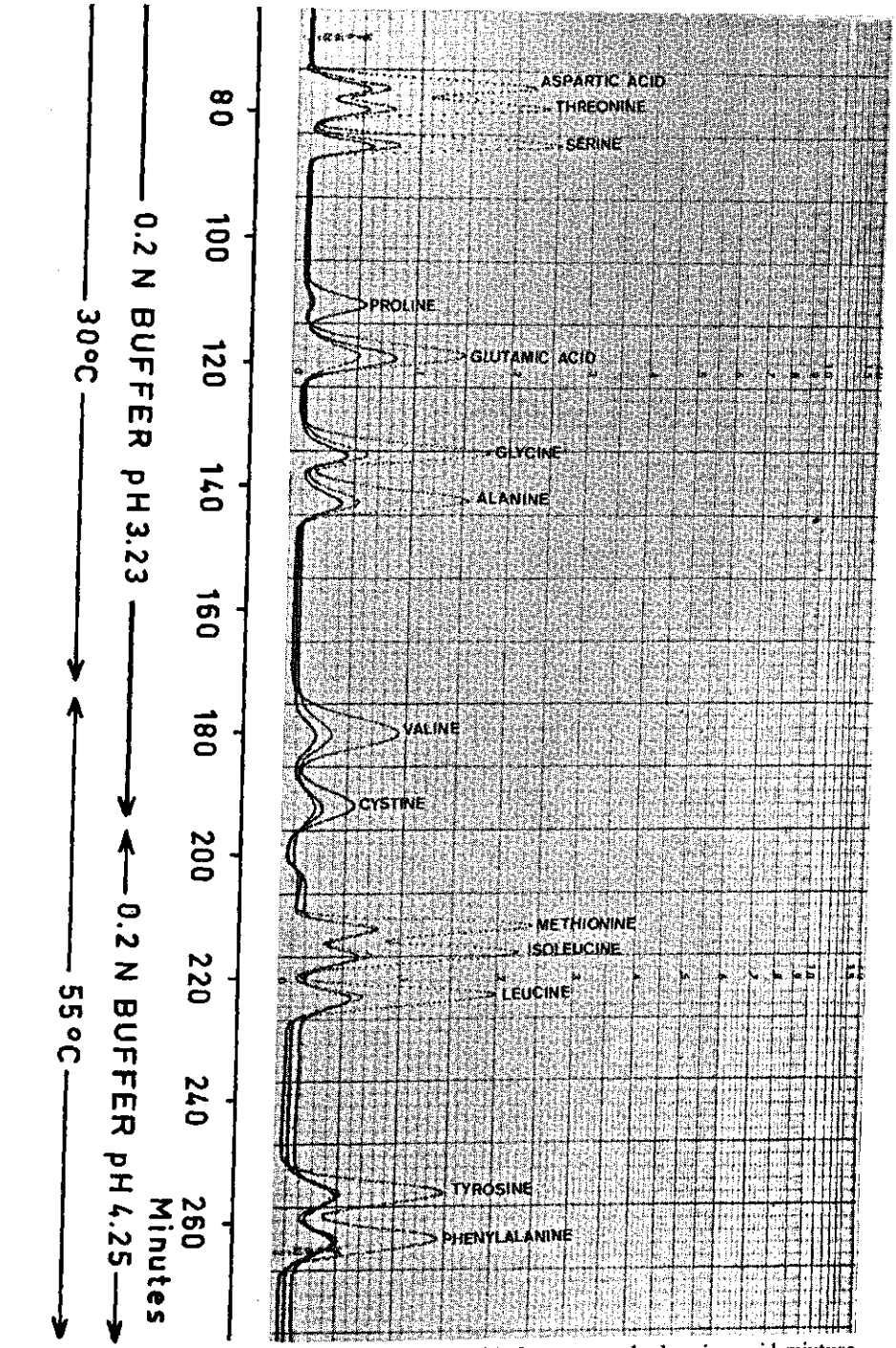


PLATE 21. Separation of acid and neutral amino acids from a standard amino acid mixture (Biocal) on a column containing 52 × 0.9 cm Biorad A6 spherical ion exchange resin.

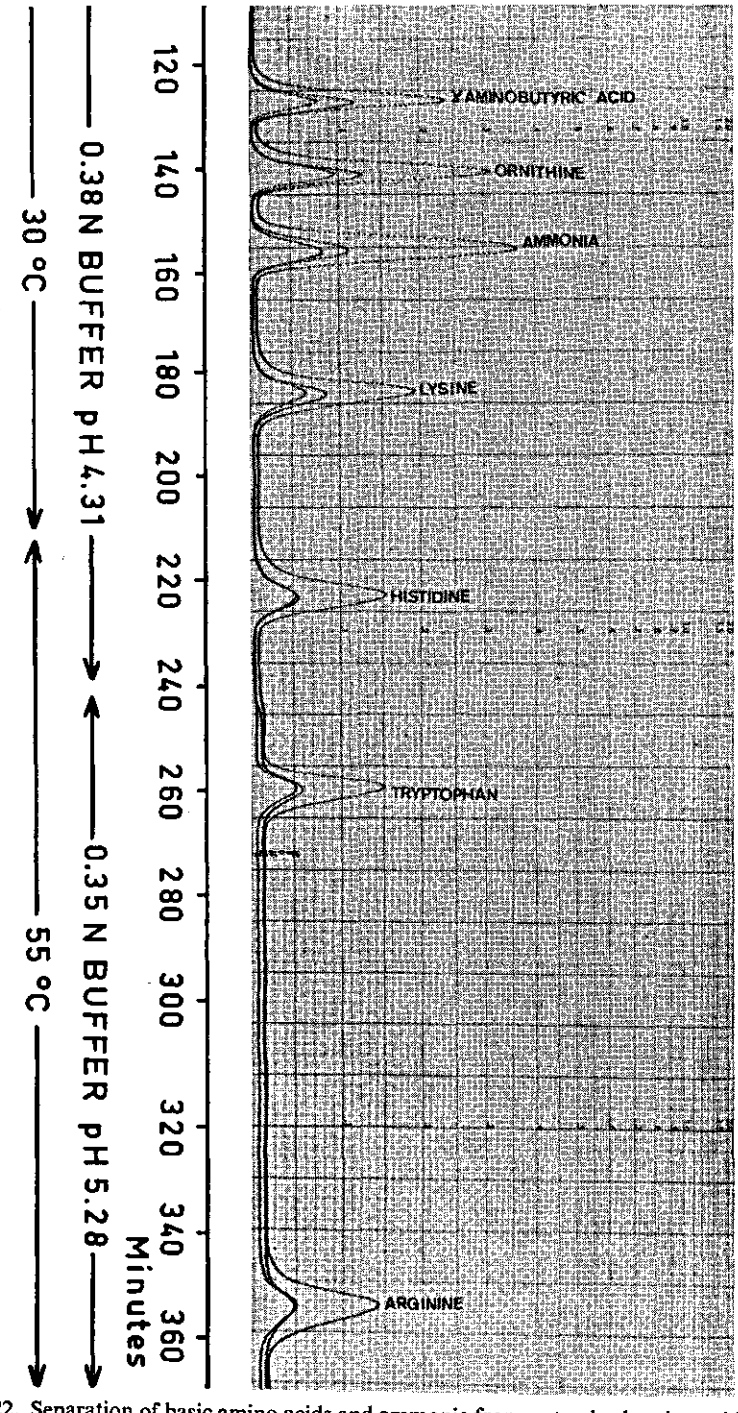


PLATE 22. Separation of basic amino acids and ammonia from a standard amino acid mixture (Biocal) on a column containing 22 × 0.9 cm Biorad A5 ion exchange resin.

TABLE 28. Buffers used in the quantitative analysis of amino acids.

Composition of buffer	pH			
	3.23 ± 0.02	4.25 ± 0.02	4.31 ± 0.02	5.28 ± 0.02
Normality	0.2	0.2	0.38	0.35
Sodium citrate.$2H_2O$ (g)	19.6	19.6	37.3	34.3
HCl, conc., 36–38% (ml)	12.3	8.4	15.3	6.5
Thiodiglycol (ml)	5.0	–	–	–
Brij-35 (ml)	2.0	2.0	2.0	2.0
Pentachlorophenol (ml)	0.1	0.1	0.1	0.1
Octanoic acid (ml) (N-caprylic acid)	0.1	0.1	0.1	0.1
Final volume (l)	1.0	1.0	1.0	1.0

used programmation (temperature and buffer changes). The citrate buffers used were prepared as indicated in Table 28. The ninhydrin solution contained: methyl Cellosolve, 750 ml, sodium acetate buffer (4N, pH 5.51 ± 0.03), 250 ml; ninhydrin, 200 g; and $SnCl_2.2H_2O$, 4 g.

The amount of each component amino acid in the sample was determined by measuring the area enclosed by its corresponding peak on the chromatogram.

9.3. RESULTS AND DISCUSSION

Tables 29 and 30 show the concentrations of different amino acids in Limburger cheese during the different ripening stages calculated as mg per 15.65 g nitrogen.

After salting the cheese, not all the amino acids could be detected in the free state. In experiment A, aspartic acid, glycine, cystine, isoleucine, tyrosine, γ-aminobutyric acid, histidine and arginine were not detected while in experiment B only γ-aminobutyric acid was absent. Early appearance of amino acids in cheese was mentioned before by HONER and TUCKY (1951), KOSIKOWISKI (1951), KIURU et al. (1953), OSWALD (1954), WILSSENS and VLEESCHAUWER (1954), FRICKER (1956), CHEBOTAREV et al. (1959) and ALI (1960).

In the samples taken subsequently, nearly all amino acids occurring in casein were found in the free state in the cheese. In the overripened cheeses (35 days after salting), when approximately 80% of the total nitrogen in cheese was in the soluble state and about 48% in the amino acids + ammonia state (cf. Table 15), the following amino acids were present in amounts of more than 2000 mg/15.65 g nitrogen: glutamic acid, valine, leucine and lysine. Amino acids present at concentrations between 1000 and 2000 mg/15.65 g nitrogen included alanine, methionine, isoleucine, phenylalanine and ornithine. Amino acids which were found in concentrations of about 500 to 1000 mg/15.65 g nitrogen were proline, glycine, γ-aminobutyric acid and histidine. The rest of the amino acids were found in concentrations lower than 500 mg/15.65 g nitrogen. Figures 19, 20, 21 and 22 show the trend of the concentration of different amino acids during the

TABLE 29. The free amino acid content of Limburger cheese of different ages, mg/15.65 g N (Exp. A).

Amino acid	After[3] salting	Age of cheese, days					
		5	9	14	20	27	35
Aspartic acid	–	18	47	53	65	117	138
Threonine	3	40	91	200	228	309	421
Serine	Present	Present	Present	Present	Present	Present	Present
Glut. + Asp.[1]	17	121	204	591	1320	1059	903
Proline	9	51	48	297	513	609	676
Glutamic acid	48	288	616	1659	2280	2787	3082
Glycine	–	29	75	315	602	910	973
Alanine	4	38	89	556	1211	1506	1723
Cystine	–	–	34	49	99	149	160
Valine	23	93	333	984	1662	2505	2757
Methionine	3	13	87	259	692	1035	1126
Isoleucine	–	21	133	429	978	1062	1395
Leucine	28	234	749	1299	2637	3239	3651
Tyrosine	–	46	88	107	243	259	283
Phenylalanine	6	100	173	389	1380	1475	1884
γ-Aminobutyric acid	–	–	31	198	435	517	535
Ornithine	8	76	186	252	879	977	1095
Ammonia	96	306	657	1041	2158	2660	3940
Lysine	8	117	299	1206	1953	2274	2385
Histidine	–	13	63	232	570	616	678
Arginine	–	3	–	23	6	16	19
A[2]	–	–	13	–	–	–	–
B[2]	–	–	8	24	–	130	91

[1] Glutamine + Asparagine
[2] Unidentified compounds
[3] Immediately after salting

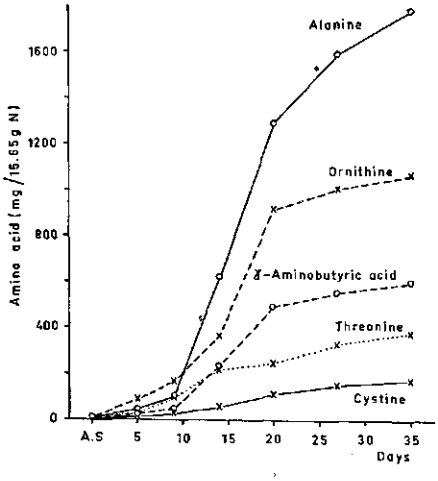


FIG. 19. Concentrations of different amino acids throughout the different stages of ripening of Limburger cheese.

TABLE 30. The free amino acid content of Limburger cheese of different ages, mg/15.65 g N (Exp. B).

Amino acid	After[3] salting	Age of cheese, days					
		5	9	14	20	27	35
Aspartic acid	6	27	43	64	70	100	147
Threonine	4	31	99	219	244	330	379
Serine	Present	Present	Present	Present	Present	Present	Present
Glut. + Asp.[1]	10	103	210	649	1360	1281	1216
Proline	13	39	49	315	537	641	696
Glutamic acid	40	240	651	1575	2323	2973	3241
Glycine	8	34	69	309	616	832	987
Alanine	11	45	102	618	1287	1587	1770
Cystine	3	12	24	55	111	155	174
Valine	18	88	301	892	1814	2565	2896
Methionine	4	19	99	289	688	974	1062
Isoleucine	3	16	135	459	993	1125	1460
Leucine	23	217	709	1218	2584	3198	3615
Tyrosine	8	61	104	124	233	286	297
Phenylalanine	5	93	180	498	1485	1494	1981
γ-Aminobutyric acid	–	16	51	235	489	553	596
Ornithine	4	84	166	367	912	1004	1059
Ammonia	105	349	608	1130	2291	2982	4133
Lysine	8	135	309	1194	2108	2352	2410
Histidine	4	31	87	309	694	706	727
Arginine	3	8	21	36	–	12	24
A[2]	–	–	–	41	19	–	12
B[2]	–	–	–	72	–	168	110

[1] Glutamine + Asparagine
[2] Unidentified compounds
[3] Immediately after salting

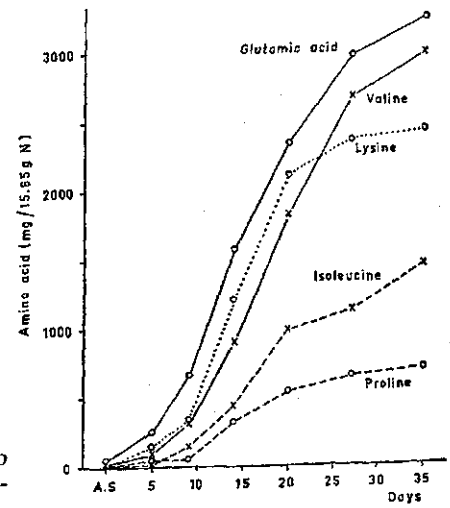


FIG. 20. Concentrations of different amino acids throughout the different stages of ripening of Limburger cheese.

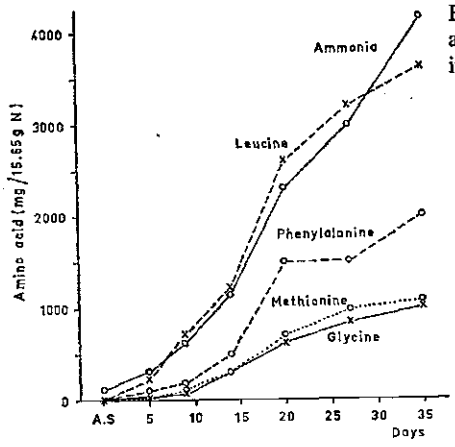


FIG. 21. Concentrations of different amino acids throughout the different stages of ripening of Limburger cheese.

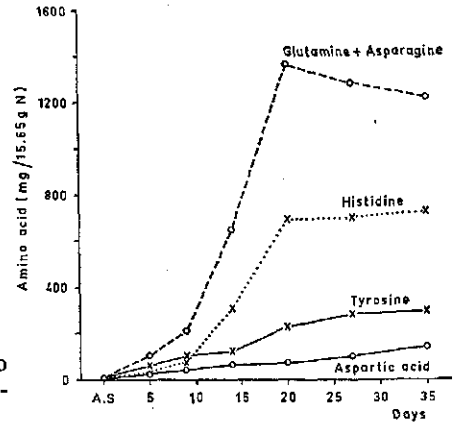


FIG. 22. Concentrations of different amino acids throughout the different stages of ripening of Limburger cheese.

ripening process. It will be seen that between the 9th and the 20th day after salting nearly all the amino acids showed their highest rate of increase. In the literature on cheese ripening, amino acids have been recorded to increase throughout the ripening as found in Cheddar by HARPER and SWANSON (1949), REIHARD and GAREY (1949), HONER and TUCKY (1951), KOSIKOWSKI (1951), DACRE (1953) and BULLOCK and IRVINE (1956) in Emmenthal by KIURI et al. (1953), HINTZ et al. (1954) and SALERNO and PAOLIS (1956), in Tilsit by OSWALD (1954) and FRICKER (1956), in Edam by ALI (1960) and in Limburger by TUCKY and SAHASRABUDHE (1957).

In the Limburger cheese studied in the present paper, all the amino acids occurring in casein were found in the free state but not necessarily relative to their percentages in casein. Figures 23 and 24 give a comparison between the free amino acids in Limburger cheese of different ages (calculated: weight as percent of total weight of amino acids) and the free amino acids in casein hy-

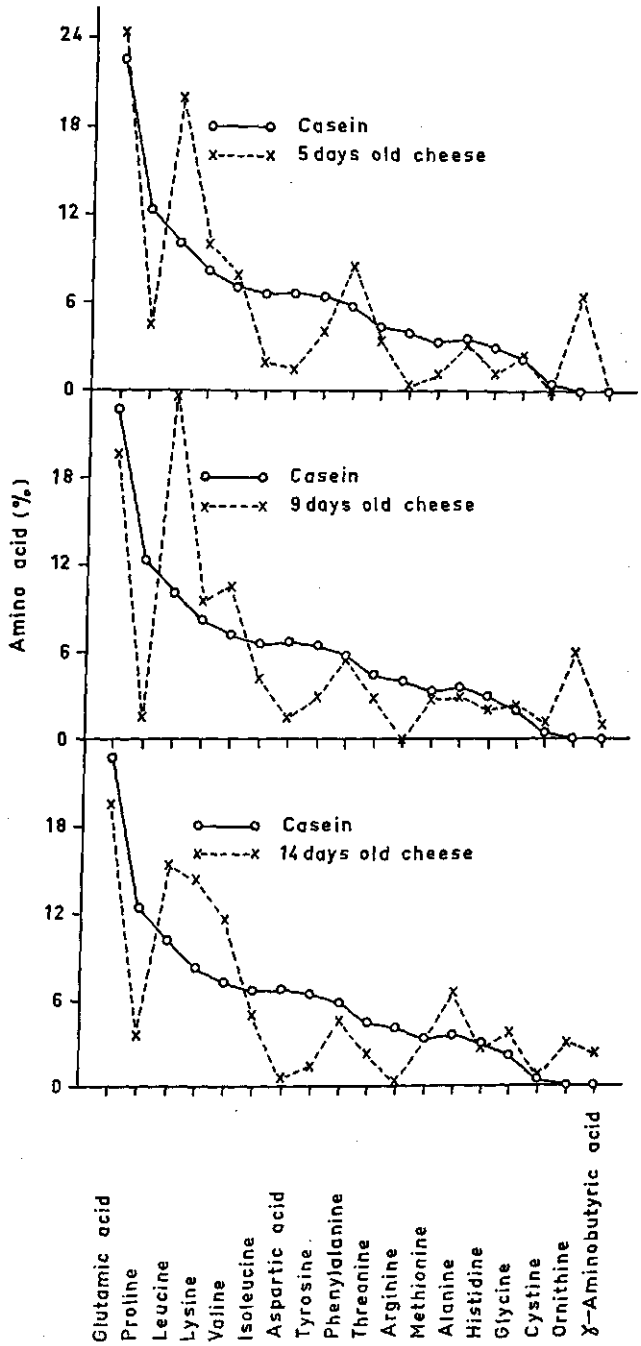


FIG. 23. Comparison between the free amino acids (% of the total) in Limburger cheese of different ages and in casein hydrolysates.

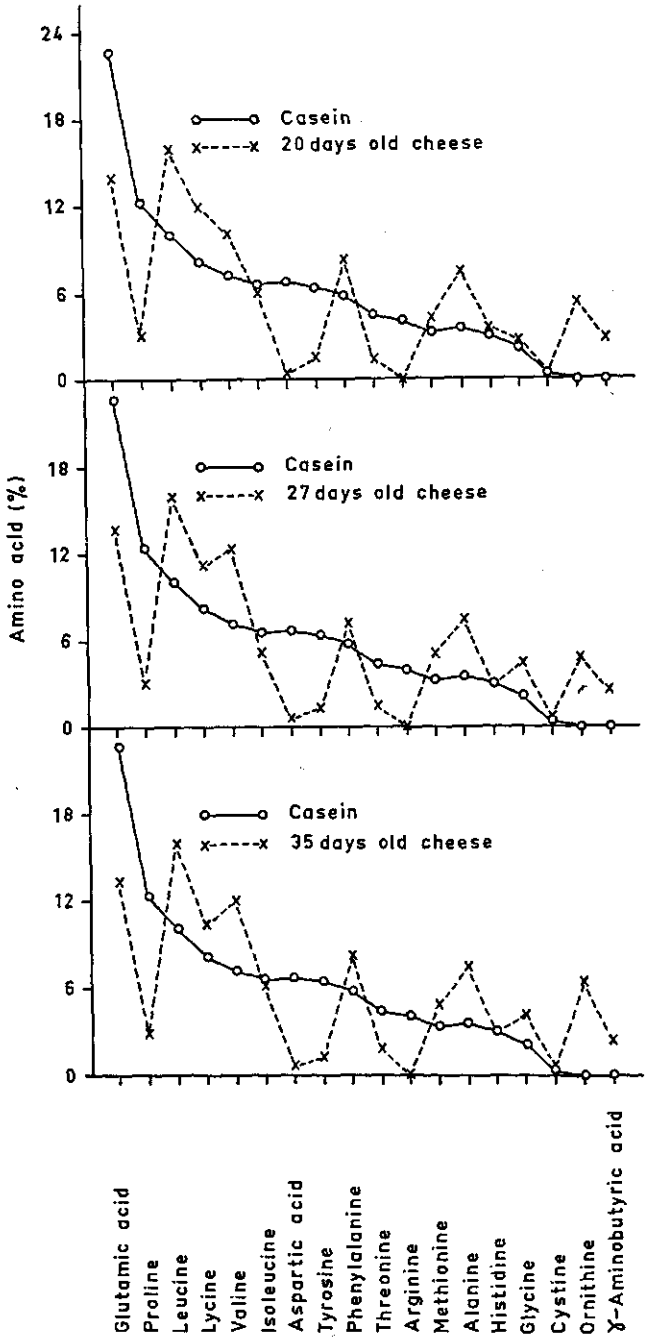


FIG. 24. Comparison between the free amino acids (% of the total) in Limburger cheese of different ages and in casein hydrolysates.

drolysate also calculated: weight as percent of total weight (average values from the literature). The presence of some amino acids in cheese in percentages considerably deviating from their percentages in casein hydrolysate was previously reported in Edam, Cheddar and Gouda cheeses (STORGARDS and LINDQVIST, 1953; HARPER and LONG, 1956 and ALI, 1960).

To gain more clear information about the fate of the different amino acids during ripening, a comparison was made of the free amino acids (calculated as mg/15.65 g nitrogen) in Limburger cheese of different ages, and the theoretical amounts of free amino acids to be found if a corresponding amount of casein would have been acid-hydrolysed (cf. Tables 31 and 32). It will be seen that all the values found in cheese samples were lower than their corresponding values in casein hydrolysate, owing to the decomposition of the amino acids during ripening. However, a pronounced difference occurred between the different amino acids as to their degree of being recovered in the soluble fraction of the ripening cheese. At all ripening stages (recorded in Tables 31 and 32), except that after 9 days, alanine was recovered for approximately 100%. Other amino acids which were recovered at a high degree were: glycine, cystine, valine and leucine (more than 70%, except in the 9 days stage). A low degree of recovery

TABLE 31. Comparison of amino acids (mg/15.65 g N) in Limburger cheese of different ages and in acid-hydrolysed casein.

Amino acid	Age, days							
	9				20			
	P[1]	T[2]	$\frac{P}{T} \times 100\%$	T-P (as mg N)	P	T	$\frac{P}{T} \times 100\%$	T-P (as mg N)
Aspartic acid	43	670	6.42	62.7	70	2290	3.06	222.0
Threonine	99	440	22.50	40.9	244	1505	16.21	151.3
Proline	49	1238	3.96	142.7	537	4234	12.68	443.8
Glutamic acid	651	2275	28.61	154.3	2323	7770	29.90	517.5
Glycine	69	215	32.09	27.3	616	735	83.81	22.3
Alanine	102	360	28.33	40.5	1287	1290	99.77	0.5
Cystine	24	40	60.00	2.4	111	136	81.62	3.7
Valine	301	720	41.81	50.3	1814	2462	73.68	77.8
Methionine	99	330	30.00	21.7	688	1130	60.88	41.5
Isoleucine	135	660	20.45	56.2	993	2260	43.94	135.6
Leucine	709	1012	70.06	32.4	2584	3460	74.68	93.7
Tyrosine	104	640	16.25	41.3	233	2188	10.65	150.5
Phenylalanine	180	580	31.03	34.0	1485	1983	74.89	42.3
Lysine	309	820	37.68	102.2	2108	2800	75.29	138.4
Histidine	87	300	29.00	57.5	694	1026	67.64	89.6
Arginine	21	400	5.25	121.3	–	1370	0	438.4

[1] P is present in the amino acid + NH_3 fraction.

[2] T is the theoretical amount to be found if a corresponding amount of casein (containing the same amount of nitrogen as present in the analysed amino acid + NH_3 fraction) would have been hydrolysed.

TABLE 32. Comparison of amino acids (mg/15.65 g N) in Limburger cheese of different ages and in acid-hydrolysed casein.

Amino acid	Age, days							
	27				35			
	P[1]	T[2]	$\frac{P}{T} \times 100\%$	T-P (as mg N)	P	T	$\frac{P}{T} \times 100\%$	T-P (as mg N)
Aspartic acid	100	2880	3.47	178.0	147	3350	4.39	320.0
Threonine	330	1890	17.46	187.2	379	2200	17.23	230.5
Proline	641	5325	12.04	562.1	696	6190	11.24	659.3
Glutamic acid	2973	9769	30.43	645.6	3241	11360	28.53	771.3
Glycine	832	924	90.04	17.2	987	1075	91.81	16.5
Alanine	1587	1648	96.30	9.6	1770	1800	98.33	0.5
Cystine	155	170	91.18	2.2	174	200	87.00	3.8
Valine	2565	3096	82.78	63.7	2896	3600	80.44	84.5
Methionine	974	1419	68.64	41.8	1062	1650	64.36	55.3
Isoleucine	1125	2840	39.61	183.5	1406	3300	44.24	196.9
Leucine	3198	4350	73.51	123.3	3615	5060	71.44	154.6
Tyrosine	286	2750	10.40	189.7	297	3200	9.28	223.5
Phenylalanine	1494	2490	60.00	84.7	1981	2900	68.31	78.1
Lysine	2352	3526	66.70	234.8	2410	4100	58.78	338.0
Histidine	706	1290	54.73	157.7	727	1500	48.47	208.7
Arginine	12	1720	0.69	546.6	24	2000	0.12	632.3

[1] P is present in the amino acid + NH_3 fraction.

[2] T is the theoretical amount to be found if a corresponding amount of casein (containing the same amount of nitrogen as present in the analysed amino acid + NH_3 fraction) would have been hydrolysed.

(below 30%) was observed for: threonine, proline, glutamic acid and tyrosine, while aspartic acid and arginine were nearly absent from the cheese samples. The rest of the amino acids were recovered in moderate amounts (between 30 and 70%).

The poor recovery of a number of the above-mentioned amino acids may be mainly described to their decomposition by enzymes excreted by the surface flora of the ripening cheese. A confirmation of this conclusion can be derived from the experiments with cheese slices and Casamino acids-containing medium, described in Chapter 8.

As ammonia is one of the main products of decomposition of amino acids, a comparison of the total amount of ammoniacal nitrogen found in the cheese samples of different ages with the total amount of nitrogen calculated from the apparent amounts of decomposed amino acids during ripening (sum of T-P in Tables 31 and 32), may decide how far the above-mentioned conclusion is correct. For the different sampling dates the following recovery values (%) were obtained: 9 days, 55; 20 days, 67; 27 days, 66; and 35 days, 80. The amounts of nitrogen not accounted for in ammonia are partly contained in those amino acids not included in the calculations of Tables 31 and 32 (viz. glutamine + asparagine, γ-aminobutyric acid and ornithine). These values accounted for

another 6% in 9 days old cheese, 20% in 20 days old cheese, 13% in 27 days old cheese and 12% in 35 days old cheese. The rest of the above-mentioned differences could have been due to losses in ammonia by evaporation, either from the cheese or during the experimental procedure, or to transformation of part of the amino acids into other compounds not detected in the analysis.

The presence of such high amounts of ammonia may indicate the contribution of this compound to the typical cheese flavour.

SUMMARY AND CONCLUSIONS

A study has been made of the ripening process of Limburger cheese, including microbiological investigations of the surface flora and chemical analyses of the cheese during the different stages of ripening. The microbial flora was studied both qualitatively and quantitatively. In addition to the morphological characters, the nutritional requirements of the main types of micro-organisms occurring on the cheese surface were studied.

A survey dealing with the micro-organisms growing on the surface of Limburger cheese and present in the brine and on different shelves, used for cutting and holding the cheese for ripening, gave the following results:

1. The main groups of micro-organisms to be found on the surface of commercial Limburger cheese included: arthrobacters, *Br. linens*, 'other coryneforms' and yeasts.
2. The percentage of colonies belonging to organisms of the *Br. linens* type was always higher when counted on media supplemented with 4% NaCl than on media without this amount of salt.
3. The brine flora consisted of the same series of micro-organisms as found on the cheese surface except for the presence of moulds which were almost absent on the cheese.
4. The organisms isolated from the scraped material of the shelves were mainly arthrobacters and yeasts with small numbers of *Br. linens* and 'other coryneforms'.

In a morphological and nutritional study of 251 strains of cheese coryneform bacteria isolated from the surface of commercial Limburger cheese, the following conclusions could be summarized:

1. All of the tested strains were found to be Gram-positive in both the coccus and the rod stages.
2. All of the strains were able to tolerate high salt concentrations. The greenish-yellow arthrobacters and the *Br. linens* organisms were able to tolerate higher concentrations of salt than were the cream + white, grey-white and red arthrobacters, and the 'other coryneforms'.
3. About 66% of the greenish-yellow arthrobacters needed light to induce pigmentation.
4. Approximately 45% of the *Br. linens* strains required light for the development of the orange pigment.
5. No light effect was observed in the case of the cream+white-coloured, grey--white and red arthrobacters and in the strains of the 'other coryneforms'.
6. All the strains tested were able to utilize both glucose or lactate as the only carbon source, which was not true for lactose and to a larger extent for sucrose.
7. All the isolated strains were catalase-positive.
8. The greenish-yellow strains were very highly proteolytic while the grey arthrobacters and the *Br. linens* strains were moderately proteolytic. The

cream + white-coloured arthrobacters were almost entirely inactive, whereas about 45% of the red arthrobacters were either moderately or slightly proteolytic.

9. The red and greenish-yellow arthrobacters, the *Br. linens* strains and the 'other coryneforms' were largely unable to utilize inorganic nitrogen and required Casamino acids either with or without vitamins. About 50% of the grey arthrobacters and 70% of the cream + white-coloured ones were able to utilize ammonium sulphate in the presence or absence of vitamins.
10. Cell-form was found to be the most important differential character in these groups of cheese coryneforms. The organisms of the *Arthrobacter* group were easily and quickly transformed from rod into coccus forms upon ageing or under poor nutritional conditions; this was a much retarded process in the group of *Br. linens* and the 'other coryneforms'. The *Arthrobacter* rods were mostly short and thick while those of the *Br. linens* group were slender, relatively long and sometimes branched. However, all the isolated strains showed the morphological implications of the coryneform bacteria.

A chemical and microbiological analysis of Limburger cheese during ripening gave the following results.

(a) Throughout the ripening process of Limburger cheese the pH values gradually increased. The values of protein breakdown increased along with ripening. A low rate of proteolysis occurred during the first week of ripening, followed by a much higher rate until the age of 20 days, after which it slightly slowed down. At the age of 35 days, the cheese was heavily proteolysed and partly liquefied.

(b) The frequent changes in the composition of the surface flora in the course of ripening may be summarized as follows:

1. Fresh cheese contained only lactic acid bacteria.
2. Immediately after salting, the surface flora resembled that of the brine.
3. At the age of 5 days the surface flora consisted of yeasts only.
4. Four days later the yeasts had decreased and were replaced by arthrobacters which predominated until the end of the ripening period.
5. Strains of *Br. linens* started to be a part of the surface flora of 14 days old cheese and increased upon ageing. They never reached numbers higher than 1/3 of the total of the surface flora, a value which was found on 35 days old cheese.

(c) The colour of the cheese surface ranged from white in fresh cheese to reddish-orange in 35 days old cheese. The following micro-organisms may be responsible for the colour of Limburger cheese:

1. Cream-coloured yeasts.
2. White, cream, grey, red and greenish-yellow arthrobacters.
3. Orange-coloured *Br. linens*.

Light effect on pigmentation was detected in the group of grey arthrobacters and the group of *Br. linens* when grown on agar media as well as on the surface of Limburger cheese. A number of grey arthrobacters developed a greenish-yellow pigment when grown in light. The same light effect was essential for

inducing the orange pigment in part of the *Br. linens* strains. It may be stated that the colour of the cheese surface is a combination of the colour of the above-mentioned organisms together with that of the cheese curd.

(d) The highest percentage (of the total) of highly proteolytic organisms occurred in the period between 9–20 days of cheese age. The grey and greenish-yellow arthrobacters were the highest proteolytic organisms followed by strains of the *Br. linens* type and the red arthrobacters. The majority of the white and cream arthrobacters had no proteolytic activity.

To study the effect of the micro-organisms of the surface of Limburger cheese on the ripening process, particularly on the transformation of protein and other nitrogenous compounds, a number of representative micro-organisms were separately grown on cheese slices placed in Petri dishes. Chemical properties of the cheese slices, including pH, degree of proteolysis, amino acid + ammonia content and growth features of the micro-organisms on the cheese slices, including colour and organoleptic ones, were recorded after different periods of incubation. For comparison, the same organisms were grown in Casamino acids-containing media, while some strains were cultivated in media with single amino acids. In all series of cultures free amino acids were estimated by paper chromatography. The results of these experiments may be summarized as follows:

a. During the first week of the incubation period, several *Arthrobacter* strains brought about a drop in the pH of the cheese slices, which was followed by a rise during the second and third weeks, resulting in ultimate pH values being between 7 and 8. In the Casamino acids-containing media the pH changes, both into the acid and the alkaline directions, were much more pronounced than those of the more strongly buffered cheese slices. The organisms belonging to the *Br. linens* group only caused an increase of pH, when growing on cheese slices as well as in Casamino acids-containing media.

b. After 3 weeks of incubation, the yeasts and most of the cream-coloured arthrobacters had not succeeded in liquefying the cheese slices. Owing to this fact, these organisms had caused only a slight increase of the soluble and amino acid nitrogen. Most of the grey, red and greenish-yellow arthrobacters and the strains of *Br. linens*, after 3 weeks of incubation, showed a moderate and often a pronounced liquefaction of the cheese slices. They had hydrolysed about 70 and sometimes even more than 80% of the protein, and in some cases had produced large amounts of free amino acids + ammonia.

c. The chromatograms obtained from the three weeks old cheese slices which had been inoculated with the weakly proteolytic cream-coloured *Arthrobacter* strains contained relatively higher amounts of aspartic acid, glutamic acid, serine and glycine, when compared with the chromatograms of a diluted mixture of Casamino acids.

Cheese slices inoculated with the moderately proteolytic strains of the grey and greenish-yellow arthrobacters and of the *Br. linens* strains gave chromatograms more or less resembling those derived from ripening Limburger cheese.

In the case of the highly proteolytic grey, red and greenish-yellow arthrobac-

ters and most of the *Br. linens* strains, the chromatograms of the liquefied cheese slices resembled those of the Casamino acids mixture with the exception of the leucines and valine which occurred in higher concentrations and serine and threonine occurring in lower concentrations.

d. When the representative micro-organisms, used in the experiment with cheese slices, had grown in Casamino acids-containing media for 10 days, the chromatograms obtained with the weakly and highly proteolytic organisms were relatively similar to those of the uninoculated blank, indicating that there was no preference to decomposing particular amino acids. The chromatograms obtained with most of the moderately proteolytic strains were completely different from those of the other cultures. Many amino acids occurred in considerably lower concentrations than in the cultures of the strongly proteolytic strains, indicating a much higher degree of decomposition of the amino acids in the former cultures.

e. The results of the experiments with cheese slices and of those with Casamino acids-containing media, are in agreement with the experiment with single amino acids. A number of highly proteolytic organisms, also used in the previous experiments, had a much poorer ability to decompose various amino acids than this was true of a number of moderately proteolytic strains.

The quantitative analysis of the free amino acids of Limburger cheese, carried out at various stages of ripening, revealed pronounced differences between various amino acids as to their occurrence in the free state. This was demonstrated in two different ways, viz. (a) by calculating the different amino acids as percentage of the total weight of amino acids, and comparing the results with a similar set of values calculated from average figures recorded in the literature for acid-hydrolysed casein. (b) A more clear picture of the fate of the liberated amino acids during the ripening of Limburger cheese was obtained by comparing the free amino acids in the cheese sample (calculated as mg/15.65 g nitrogen) with the theoretical amounts of free amino acids to be found if a corresponding amount of casein would have been acid-hydrolysed. From this comparison the following conclusions may be drawn:

1. All the values found in the cheese samples were lower than their corresponding values in casein hydrolysate, owing to the decomposition of the amino acids during ripening.
2. Of all the amino acids present in casein, alanine was recovered at the highest degree, viz. nearly 100%. Other amino acids recovered for more than 70% were glycine, cystine, valine and leucine.
3. A low recovery value (below 30%) was found for: threonine, proline, glutamic acid and tyrosine, while aspartic acid and arginine were nearly absent from the cheese samples.
4. The remaining amino acids were recovered in amounts between 30 and 70%.

The poor recovery of several amino acids was due to microbial decomposition, resulting in the liberation of most of the nitrogen as ammonia. A comparison of the total amount of ammoniacal nitrogen found in the cheese samples of different ages with the total amount of nitrogen calculated from the

amounts of apparently decomposed amino acids during ripening, gave the following recovery values (%) for the different sampling dates: 9 days after salting, 55; 20 days, 67; 27 days, 66; and 35 days, 80. The amounts of nitrogen not accounted for in ammonia are partly present in those amino acids not recorded in casein hydrolysate (viz. glutamine + asparagine; γ-aminobutyric acid and ornithine). These values accounted for another 6, 20, 13 and 12% in 9, 20, 27 and 35 days old cheeses, respectively. The rest of the above-mentioned differences may have been due to losses in ammonia by evaporation, or to transformation of part of the amino acids to other nitrogenous compounds not detected in the analysis.

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SAMENVATTING

De rijping van Limburger kaas vormde het onderwerp van het hier beschreven onderzoek. Behalve een microbiologische studie van de oppervlakteflora van de kaas, werden chemische analyses van de kaas in verschillende stadia van rijping uitgevoerd. De microflora van het kaasoppervlak werd zowel kwalitatief als kwantitatief bestudeerd. Verder werden de morfologische eigenschappen en de voedingseisen van de belangrijkste typen van micro-organismen van het kaasoppervlak bepaald.

Een microbiologische analyse van de flora van het kaasoppervlak, van de pekel waarin de kaas werd gezouten, en van de planken waarop hij werd gesneden en van die waarop hij rijpte, gaf de volgende resultaten:

1. De belangrijkste groepen van micro-organismen die op rijpende Limburger kaas, bestemd voor de handel, werden aangetroffen waren arthrobacters, stammen van *Brevibacterium linens*, 'andere corynebacteriën' en gisten.
2. Het percentage kolonies van organismen van de *Br. linens* groep was altijd hoger indien de monsters waren uitgestreken op agar media met 4% NaCl dan op media zonder deze toevoeging.
3. De microflora van de pekeloplossing bestond uit dezelfde groepen van micro-organismen als die van de kaas, met uitzondering van schimmels, die op de kaas nauwelijks voorkwamen.
4. Het schraapsel van de planken bestond hoofdzakelijk uit arthrobacters en gisten met kleine aantallen vertegenwoordigers van de *Br. linens* groep en van de 'andere corynebacteriën'.

Een onderzoek aangaande de morfologie en de voedingseisen van 251 stammen van verschillende typen van corynebacteriën, geïsoleerd van het oppervlak van Limburger kaas bestemd voor de handel, leverde de volgende resultaten:

1. Alle stammen waren Gram-positief, zowel in het coccen- als in het staafstadium.
2. Alle stammen konden hoge zoutconcentraties verdragen. De groen-gele arthrobacters en de tot *Br. linens* behorende stammen verdroegen hogere zoutconcentraties dan de groep van de roomkleurige + witte, de grijs-witte en de rode arthrobacters, en de 'andere corynebacteriën'.
3. Ongeveer 2/3 van de groen-gele arthrobacters had licht nodig voor de ontwikkeling van hun kleurstof.
4. Van de tot *Br. linens* behorende corynebacteriën had ongeveer 45% van de stammen licht nodig voor de vorming van het oranje pigment.
5. Bij de roomkleurige en witte, de grijs-witte, en de rode *Arthrobacter*-stammen werd geen lichteffect waargenomen.
6. Alle stammen konden glucose en lactaat als enige koolstofbron gebruiken. Dit was in mindere mate het geval met lactose en vooral saccharose.
7. Alle geïsoleerde stammen waren katalase-positief.
8. De groen-gele *Arthrobacter*-stammen waren sterk proteolytisch; de grijze arthrobacters en de stammen van *Br. linens* waren matig sterk proteolytisch,

terwijl de roomkleurige + witte arthrobacters niet proteolytisch, en de rode stammen voor 45% matig of licht proteolytisch waren.

9. De rode en groen-gele arthrobacters, de *Br. linens* stammen en de 'andere corynebacteriën' waren niet in staat anorganische stikstof te assimileren; behalve Casaminozuren hadden ze soms vitaminen nodig. Ongeveer 50% van de grijze arthrobacters en 70% van de roomkleurige en witte stammen konden ammoniumsulfaat, al of niet met vitaminen, benutten.
10. De morfologische kenmerken waren de belangrijkste om de verschillende groepen van corynebacteriën te onderscheiden. De arthrobacters gingen snel van de staaf- in de coccusvorm over bij ouder worden en bij een slechte voedselvoorziening. Bij *Br. linens* en de 'andere corynebacteriën' was dit een zeer vertraagd proces. De *Arthrobacter*-staven waren meestal kort en dik, in tegenstelling tot de slanke, betrekkelijk lange en soms vertakte staven van *Br. linens*. Alle 251 stammen vertoonden de typische eigenschappen van de corynebacteriën.

Een chemische en microbiologische analyse van Limburger kaas tijdens de rijping gaf de volgende resultaten:

(a) Tijdens de rijping steeg de pH van de kaas regelmatig. De eiwitafbraak nam toe met de voortschrijdende rijping. Een geringe proteolyse trad op tijdens de eerste week van de rijping, gevolgd door een sterk verhoogde proteolyse tot aan de 20ste dag van de rijping, waarna de eiwitsplitsing langzaam verminderde. Bij 35 dagen oude kaas was deze grotendeels afgebroken en gedeeltelijk vervloeid.

(b) De volgende veranderingen in microbiologische samenstelling deden zich tijdens de rijping voor:

1. Verse kaas bevatte alleen melkzuurbacteriën.
2. Direct na het zouten leek de oppervlakteflora van de kaas op die van de pekel.
3. Vijf dagen na het zouten bestond de oppervlakteflora uit alleen gisten.
4. Vier dagen later waren de gisten gedaald in aantal en gedeeltelijk vervangen door arthrobacters die tot aan het eind van de rijping de dominerende groep van micro-organismen waren.
5. Stammen van *Br. linens* begonnen te verschijnen op 14 dagen oude kaas en met toenemende ouderdom van de kaas namen ze toe in aantal. Ze bereikten nooit een groter aantal dan 1/3 van het totale aantal micro-organismen, een waarde die op 35 dagen oude kaas werd gevonden.

(c) De kleur van het kaasoppervlak variëerde van wit (verse kaas) tot rood-oranje bij 35 dagen oude kaas. De volgende micro-organismen kunnen verantwoordelijk zijn voor de kleur van de Limburger kaas:

1. Roomkleurige gisten.
2. Witte, roomkleurige, grijze, rode en groen-gele arthrobacters.
3. Oranjekleurige *Br. linens*.

Een invloed van licht op de pigmentvorming werd gevonden bij de groep van de grijze arthrobacters en die van *Br. linens*, zowel bij de groei op agar-media als op Limburger kaas. Een aantal grijze arthrobacters vormde een groen-geel

pigment wanneer deze bacteriën in het licht groeiden. Hetzelfde lichteffect werd bij een aantal stammen van *Br. linens* waargenomen op de vorming van de oranje kleur. De kleur van het kaasoppervlak is een combinatie van de kleur van de genoemde micro-organismen en die van de wrongel.

(d) Het percentage sterk proteolytische micro-organismen was het hoogst tussen de 9de en 20ste dag na het zouten van de kaas. De grijze en groen-gele arthrobacters vertoonden de sterkste proteolytische activiteit, gevolgd door de tot *Br. linens* behorende stammen en de rode arthrobacters. De meerderheid van de witte en roomkleurige arthrobacters, die verreweg de grootste groep vormden, vertoonde geen proteolytische activiteit.

Om de werking van de op Limburger kaas voorkomende bacteriën op het rijpingsproces na te gaan, in het bijzonder die op de omzetting van eiwit en andere stikstofhoudende verbindingen, werd een aantal van het kaasoppervlak geïsoleerde representatieve bacteriën gekweekt op kaasschijven in Petrischalen. Chemische eigenschappen van de kaasschijven (intensiteit van de proteolyse en het gehalte aan aminozuren + ammoniak) en de groei van de micro-organismen op deze schijven, de kleurstofvorming en de organoleptische kwaliteiten werden na verschillende incubatietijden bepaald.

Ter vergelijking werd dezelfde groep van geselecteerde micro-organismen gekweekt in voedingsoplossingen met Casaminozuren, terwijl enkele stammen bovendien werden gekweekt in media waaraan afzonderlijke aminozuren waren toegevoegd. In al deze cultures werden na verschillende incubatietijden de vrije aminozuren met behulp van papierchromatografie bepaald. De resultaten van deze bepalingen waren de volgende:

(a) Gedurende de eerste week van de incubatieperiode veroorzaakten verscheidene *Arthrobacter*-stammen een daling van de pH van de kaasschijven, die gedurende de tweede en derde week gevolgd werd door een stijging van de pH tot waarden tussen 7 en 8. In de voedingsoplossingen met Casaminozuren waren de pH-veranderingen, zowel in zure als in alkalische richting, aanzienlijk groter dan die in de sterker gebufferde kaasschijven.

De tot de groep van *Br. linens* behorende organismen veroorzaakten alleen de pH-stijging, zowel op kaas als in voedingsoplossingen met Casaminozuren.

(b) Na een incubatieperiode van drie weken, waren de gisten en de meeste roomkleurige arthrobacters er niet in geslaagd de kaasschijven te doen vervloeien. Als gevolg hiervan hadden deze organismen het gehalte aan oplosbare stikstof en dat aan aminozuurstikstof slechts weinig doen stijgen.

De meeste grijze, rode en groen-gele arthrobacters, en de stammen van *Br. linens* vertoonden na een incubatietijd van 3 weken een matige, en vaak een sterke, vervloeiing van de kaasschijven. Zij hadden ongeveer 70 en soms zelfs meer dan 80% van het eiwit gehydrolyseerd en hadden grote hoeveelheden vrije aminozuren en ammoniak gevormd.

(c) De chromatogrammen van de drie weken oude kaasschijven, geïncubeerd met de zwakproteolytische room-kleurige *Arthrobacter*-stammen, bevatten relatief grotere hoeveelheden asparaginezuur, glutaminezuur, serine en glycine

in vergelijking met de chromatogrammen van een verdund mengsel van Casaminozuren.

Kaasschijven geïncubeerd met matig proteolytische stammen van de grijze en groen-gele arthrobacters, en van *Br. linens* gaven chromatogrammen die leken op die verkregen van rijpende Limburger kaas.

De chromatogrammen verkregen van de vloeibaar geworden kaasschijven, die geïncubeerd waren geweest met de sterk proteolytische grijze, rode en groen-gele arthrobacters, en die van de *Br. linens* groep, leken op die van een Casaminozuur mengsel met uitzondering van leucine en valine, die in hogere concentratie voorkwamen, en serine en threonine die in lagere concentratie voorkwamen.

(d) Wanneer de representatieve micro-organismen, die in de proef met kaasschijven waren gebruikt, gedurende 10 dagen in de voedingsoplossingen met Casaminozuren waren gekweekt, vertoonden de chromatogrammen van de cultures met zwak en sterk proteolytisch actieve bacteriën relatief veel overeenkomst met die van het niet geënte blanco mengsel van Casaminozuren. Dit wijst niet op een voorkeur voor een bepaald aminozuur bij de afbraak van de aminozuren door deze bacteriën. De chromatogrammen verkregen na incubatie van de Casaminozurenbevattende voedingsoplossingen met matig proteolytisch actieve bacteriën waren geheel anders. Vele aminozuren kwamen in aanzienlijk lagere concentratie voor dan in de cultures met sterk proteolytisch actieve stammen, hetgeen wijst op een veel sterkere afbraak van de aminozuren in de eerstgenoemde cultures.

(e) De resultaten van de proeven met kaasschijven en van die met de Casaminozuren-bevattende media zijn in overeenstemming met de uitkomsten van de proeven met afzonderlijke aminozuren. Een aantal sterk proteolytisch actieve micro-organismen, die ook in de voorgaande proeven waren gebruikt, had een veel geringer vermogen verschillende aminozuren af te breken dan het geval was bij de matig proteolytische stammen.

De kwantitatieve analyse van de vrije aminozuren in Limburger kaas, uitgevoerd in verschillende stadia van de rijping, demonstreerde het bestaan van grote verschillen tussen de verschillende aminozuren voor zover dit hun voorkomen in vrije toestand betreft. Dit werd op twee verschillende manieren aangetoond nl. (a) door de verschillende aminozuren te berekenen in % van het totale gewicht van de aanwezige aminozuren, en de resultaten te vergelijken met een zelfde serie procent waarden, verkregen van met zuur gehydrolyseerde caseïne (berekend uit gemiddelde cijfers vermeld in de literatuur voor met zuur gehydrolyseerde caseïne). (b) Een duidelijker beeld van het lot van de tijdens de rijping vrijkomende aminozuren werd verkregen door de vrije aminozuren van de kaasmonsters (berekend per 15.65 g N) te vergelijken met de theoretische hoeveelheden aminozuren die vrij zouden komen als een overeenkomstige hoeveelheid caseïne (met dezelfde hoeveelheid stikstof als aanwezig in de geanalyseerde aminozuur + NH_3 fractie) met behulp van zuur zou worden gehydrolyseerd. Uit deze vergelijking werden de volgende conclusies getrokken:

1. Alle waarden in de kaasmonsters waren lager dan de corresponderende

waarden in het caseïne-hydrolysaat. Dit was blijkbaar een gevolg van de afbraak van de aminozuren gedurende de rijping van de kaas.

2. Van alle in caseïne aanwezige aminozuren, werd alanine in de grootste hoeveelheid (bijna 100%) in vrije vorm in de kaasmonsters aangetroffen. Andere aminozuren die in relatief grote hoeveelheden (>70% van de in caseïne aanwezige hoeveelheden) voorkwamen, waren glycine, cystine, valine en leucine.
3. Zeer lage waarden (vrije aminozuren <30% van die in caseïne) werden gevonden voor: threonine, proline, glutaminezuur en tyrosine, terwijl asparaginezuur en arginine in vrije vorm vrijwel afwezig waren in de kaasmonsters.
4. De overblijvende aminozuren kwamen in de kaasmonsters voor in hoeveelheden variërend van 30 tot 70% van die van de waarden in caseïne.

De lage gehalten aan verscheidene vrije aminozuren in de kaasmonsters waren een gevolg van de microbiologische afbraak van de vrije aminozuren tijdens de rijping. Het grootste deel van de stikstof van deze aminozuren kwam vrij als ammoniak. Een vergelijking van de totale hoeveelheid ammoniak-stikstof, gevonden in de kaasmonsters van verschillende leeftijd, met de totale hoeveelheid stikstof berekend uit de hoeveelheden vermoedelijk afgebroken aminozuren leverde de volgende cijfers op voor de verschillende stadia van rijping: 9 dagen na zouten, 55; 20 dagen, 67; 27 dagen, 66; en 35 dagen, 80%. De hoeveelheden stikstof die niet als ammonia werden teruggevonden, waren gedeeltelijk aanwezig in die aminozuren welke niet in caseïne-hydrolysaat voorkomen (nl. glutamine + asparagine, γ-aminoboterzuur en ornithine). Deze waarden bedroegen respectievelijk 6, 20, 13 en 12% in de 9, 20, 27 en 35 dagen oude kaasmonsters. De rest van de bovengenoemde verschillen kan een gevolg zijn geweest van ammoniakverliezen (door verdamping) of van de omzetting van een gedeelte van de aminozuren in andere stikstofhoudende verbindingen die bij de analyse niet werden bepaald.

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