Commission of the European Communities

INFORMATION ON AGRICULTURE

Objectivation of the bacteriological and organoleptic quality of milk for consumption

No. 21
September 1976

OBJECTIVATION OF THE BACTERIOLOGICAL AND ORGANOLEPTIC QUALITY OF MILK FOR CONSUMPTION

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The presence of non-pathogenic microorganisms and the organoleptic characteristics determine the length of time over which milk may be kept fresh and be enjoyed as a beverage by the consumer.

Community legislation on drinking milk calls for the definition of the criteria and methods for determining the bacteriological and organoleptic characteristics of milk at the various stages, production (raw milk), before and after heat treatment (pasteurization etc.) and on consumption after various periods of conservation. The criteria and methods of analysis applied by the various Member States differ considerably. The work reported here is intended to contribute towards the perfection of objective scientific tests which are rapid and inexpensive for determining the bacteriological and organoleptic properties of milk for human consumption at the various stages of its production, processing and conservation, until the time when it is consumed.

To this end, the potential offered by determination of the pyruvic acid content in particular has been examined.

This study is published only in English.

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Manuscript finished in May 1976

COMMISSION OF THE EUROPEAN COMMUNITIES
DIRECTORATE-GENERAL FOR AGRICULTURE
Directorate: Agricultural Economics – Division: "Balance-sheets, Studies, Statistical Information"

Foreword

The present study has been carried out within the framework of the study-programme of the Directorate General for Agriculture by

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The Division "Balance-sheets, Studies, Statistical Information", "Milk products" and "Harmonization of Laws, regulations and administrative provisions relating to veterinary matters and zootechnics" of the Directorate general for Agriculture have cooperated in this project.

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Contents

	Page
1. Introduction (MABBIT)	9
1.1 Assessment of the bacteriological quality of milk in the past and present	9
1.2 Present problems	10
1.3 Subjects of investigations	10
2. Critical evaluation of tests for the bacteriolgical quality of refrigerated milk (MABBIT)	10
2.1 Total bacterial counts	10
2.2 Tests for bacterial activity	11
2.3 Economic aspects	12
3. Pyruvate, the analoguous parameter for the bacteriological quality of milk (TOLLE and HEESCHEN)	13
3.1 Microbial catabolism	13
3.1.1 Glycolysis	
3.1.2 Proteolysis	
3.1.3 Lipolysis	
3.2 Pyruvate as the central metabolic parameter for the measurement of bacterial activity under psychrotrophic conditions	19
3.2.1 A model scheme for the extracellular accumulation of pyruvate	
3.2.2 Analysis	
3.2.2.1 Fluorometry	
3.2.2.2 Colorimetry	
3.2.2.2.1 Auto Analyzer System	
3.2.2.2.2 Manual determination	
3.3 Results of experimental assays	34
3.3.1 Specifity of the enzymatic determination of pyruvate	
3.3.2 Sampling	
3.3.3 Preservation of samples	
3.3.4 Secretory background value	
3.3.5 Pyruvate and pasteurization	
3.3.6 Pyruvate and bacterial activity	
3.3.6.1 Pyruvate as parameter for bacterial activity in relation to the number of microbes as the quantitative morphologic value	
3.3.6.2 Time series investigations for the measurement of catabolic activity in pure cultures	
3.3.6.3 Ability to form pyruvate from germ-groups, isolated from milk	
3.4 Results of assays under field conditions	58

Page

4. The suitability of the pyruvate-pool for the estimation of the keeping quality of milk (TOLLE and HEESCHEN) 68

4.1 The concept of keeping quality 68

4.1.1 The flavour quality of milk and influencing factors
4.1.2 Microbiological methods for the estimation of keeping quality

4.2 Pyruvate and keeping quality 73

4.2.1 Material and methods
4.2.2 Results
4.2.2.1 Pyruvate-difference-value and keeping quality of milk
4.2.2.2 Microbial processes in raw milk and the keeping quality of pasteurized milk
4.2.2.3 Pyruvate-threshold-value and spoilage of pasteurized milk
4.2.2.4 Reduction-test, pyruvate and metabolites of microbial catabolism
4.2.2.5 Alcohol-test, pyruvate and metabolites of bacterial catabolism
4.2.2.6 Clot-on-boiling-test, pyruvate and metabolites of bacterial catabolism

5. Discussion and summary of chapters 3. and 4. (TOLLE and HEESCHEN) 109

6. Collaborative experiments between Institut für Hygiene der Bundesanstalt für Milchforschung, Kiel, and the National Institute for Research in Dairying, Shinfield (MABBIT) 114

6.1 Introduction 114

6.1.1 Raw milks
6.1.2 Pasteurized milks

6.2 Experimental methods 115

6.2.1 Milk samples and pre-test storage
6.2.1.1 Raw milks
6.2.1.2 Pasteurized milks

6.2.2 Bacteriological analyses (Ministry of Agriculture, Fisheries & Food, 1968)
6.2.2.1 Total Count (TC)
6.2.2.2 Coli-aerogenes (Coliform) Count (CAO)
6.2.2.3 Psychrotrophic Count (PC)
6.2.2.4 Thermoduric Count (LPC)
6.2.2.5 Total Aerobic Spore Count (TS)
6.2.2.6 Streptococcal Count (SSM) NIRD Report 1971-72)
6.2.2.7 Resazurin Test (Hygiene Test for England and Wales)
6.2.2.8 Methylene Blue (MB) Test (Statutory test for consumer milk in England and Wales)
6.2.2.9 Alcohol Precipitation Test (APT)

Page

6.2.3 Somatic cell counts
6.2.4 Examination of microfloras
6.2.5 Biochemical analyses
6.2.5.1 Pyruvate
6.2.5.1.1 NIRD colorimetric manual method (NIRD)
6.2.5.1.2 Kiel colorimetric automated method (Kc)
6.2.5.1.3 Kiel and NIRD fluorimetric automated method (Kf and NIRDf)

6.2.5.2 Lactate
6.2.5.2.1 Kiel fluorimetric automated method
Details of these methods are given in Appendix

6.3 Results and discussion 119

6.3.1 Raw milks
6.3.1.1 Bacteriological results
6.3.1.1.1 Initial values
6.3.1.1.1.1 Total counts (TC)
6.3.1.1.1.2 Streptococcal counts (SSM counts)
6.3.1.1.1.3 Psychrotroph counts (PC)
6.3.1.1.1.4 Coliform counts (CAO)
6.3.1.1.1.5 Thermoduric counts (LPC)
6.3.1.1.1.6 Resazurin test (RT)

6.3.1.1.2 After storage at 5° for 4 and 5 days
6.3.1.1.2.1 Total and psychrotroph counts (TC and PC)
6.3.1.1.2.2 Streptococcal counts (SSM)
6.3.1.1.2.3 Coliform counts (CAO)
6.3.1.1.2.4 Thermoduric counts (LPC)
6.3.1.1.2.5 Resazurin Test (RT)

6.3.1.1.3 After incubation at 20° for 17h
6.3.1.1.3.1 Total count and psychrotroph count (TC & PC)
6.3.1.1.3.2 Streptococcal counts (SSM)
6.3.1.1.3.3 Coliform counts (CAO)
6.3.1.1.3.4 Thermoduric counts (LPC)
6.3.1.1.3.5 Resazurin Test (RT)

6.3.1.1.4 Microfloras of the milks after storage at 5° and at 20°
6.3.1.1.5 The error of the plate count

6.3.1.2 Somatic Cell Counts (cells)
6.3.1.3 Biochemical Results
6.3.1.3.1 Pyruvate
6.3.1.3.1.1 Relationship of initial pyruvate to initial status of the milks
6.3.1.3.1.2 Relationship of pyruvate (values) to total and psychrotroph colony counts after storage at 5° for 4 and 5 days
6.3.1.3.1.3 Relationship of pyruvate to total and psychrotroph colony counts after storage at 20° for 17h
6.3.1.3.1.4 Pyruvate values equivalent to one colony forming unit (CFU)

Page

6.3.2 Pasteurized milks
6.3.2.1 Initial biochemical and bacteriological status
6.3.2.2 After incubation at 10° for 2 days
6.3.2.3 Comparison of Kiel and NIRD pyruvate results

6.4 Conclusion 148

6.4.1 Raw milks
6.4.2 Pasteurized milks

6.5 References 151

6.6 APPENDIX 152

7. Final statements 158

1. INTRODUCTION (MABBIT)

1.1 Assessment of the bacteriological quality of milk in the past and present

The main objectives in determining the bacteriological quality of raw milk are

1) to check whether the milk has been produced according to recognised or statutory standards of hygiene

2) to indicate whether the milk is suitable for processing or will be suitable after the inevitable storage period before processing.

Grading schemes depending on test results are based on tests for bacteriological quality and usually involve financial penalties or bonuses.

The principles of bacteriological grading of milk were discussed and enumerated by Wilson (1937) and remain basically unchanged. They apply to both non-refrigerated and refrigerated raw milk and those relevant to this report may be summarised as follows:

a) The variability of the bacterial content of a milk supply and the variation between replicate samples necessitate regular and frequent sampling throughout the year.

b) Because of this variability no attempt should be made to grade milk into more than 3 or 4 classes.

c) Preferably the test method should be one that can be used by relatively unskilled operators and should have as small an experimental error as is consistent with this.

Testing pasteurized or other forms of heat treated milk is undertaken to ensure that for consumer protection post heat treatment contamination is minimal and that the milk has an acceptable keeping quality. Failure of the appropriate milk test, usually a statutory test, may result in withdrawal of a licence to process milk or some other penalty on the processor rather than grading of the milk for sale at different prices.

Interstate shipment of both raw and pasteurized milk may require the milk to meet agreed bacteriological standards.

1.2 Present problems

Although the objectives and principles of testing milk have not changed over the years the rapid increase of refrigeration both at the point of production and in the home have necessitated the application of different test methods and standards. Without refrigeration the rate of deterioration of both raw and processed milk is particularly dependent on hygienic production and processing. Standards based on plate counts or dye reduction tests for milk which has been stored at atmospheric temperatures, e.g. on the farm before collection are reasonably satisfactory although correlation between these two types of tests is not good.

However with refrigerated milks correlation of such tests with production conditions and with the keeping quality of the milk at, say, 5^{o} is very poor. Pre-incubation at temperatures ranging from 12-18^{o}C for up to 24 h before applying these tests has unpredictable results and does not sufficiently improve the correlations particularly as far as dye reduction tests are concerned. Reduction tests satisfy Wilson's requirements for suitable grading tests but most psychrotrophic bacteria (i.e. bacteria capable of growth at 5-7^{o}C) are poor dye reducers. Thus a new test, preferably sensitive to multiplication of psychrotrophs, which can be applied to refrigerated milk is required.

1.3 Subjects of investigations

Present investigations aim to develop a suitable test to replace bacterial {plate} counts or dye reduction tests and if necessary some means of preserving samples which would not invalidate test results.

2. Critical evaluation of tests for the bacteriological quality of refrigerated milk (MABBIT)

2.1 Total bacterial counts

Methods for assessing the bacteriological quality of cooled bulk milk have recently been reviewed and critically assessed (International Dairy Federation, 1974). Conclusions relevant to bacterial counts are as follows.

Milk produced under very good hygienic conditions should have a total count of <10,000 per ml as determined by the standard plate count method. With efficient cooling, the total count of the milk should not appreciably increase after storage below $4^{o}C$ for two days. Under practical conditions, for various reasons, the production of milk of this quality may not be achieved but in any case, the bacterial count should not greatly exceed this figure. Consequently the number of bacteria in such milk at the time of collection is a direct and reliable indicator of the hygiene of production if this is taken to mean udder health and the effectiveness with which bacteria are excluded from the milk during production.

Tests for the suitability of the milks for processing are more difficult to consider. Milk destined for the liquid market should preferably have a low count of bacterial spores and be free from objectionable taints. Present methods for the specific counting of spores are unsuitable for routine testing but bacterial taints could be effectively excluded by a bacterial count. Even those organisms which are particularly objectionable because of their taint producing capacity must be present in numbers above 10^{6}/ml before they cause trouble.

The disadvantages of counting methods are their innate inaccuracy (Wilson, 1937) and the time and expense involved. A new test which would more accurately assess bacterial numbers and which is rapid and cheap to operate is therefore required.

2.2 Tests for bacterial activity

Dye reduction tests have already been discussed. Reduction of nitrate, the catalase test and determination of dissolved oxygen have been shown to be unsatisfactory for refrigerated milk. Production of pyruvate, lactate free, fatty acids and ammonia have all been investigated and of these the pyruvate level in milk appears to be the most sensitive determinant of bacterial numbers and activity in milk. Determination of pyruvate is suited to centralized automated testing.

For refrigerated raw milk further investigations are needed to

provide information on the value of or necessity for pre-incubation before testing for pyruvate, and the most suitable means of preserving samples, which would also permit determination of lactate, free fatty acids and ammonia if required. Pyruvate and lactate values in relation to the bacterial flora and numbers would need to be assessed.

Factors other than bacterial activity affect the acceptability of pasteurized milk to consumers. However, as with refrigerated raw milk, a rapid test such as that for pyruvate capable of assessing post-pasteurization deterioration brought about by psychrotrophic bacteria in pasteurized milk would undoubtedly contribute greatly to improving its keeping quality.

2.3 Economic aspects

Centralized automated testing, e.g. for pyruvate, would not only permit frequent and regular testing of both raw and pasteurized milk but would be less costly than local testing. If at the same time somatic cell counts could be performed on the raw milk samples testing costs would be further reduced.

3. Pyruvate, the analoguous parameter for the bacteriological quality of milk (TOLLE and HEESCHEN)

3.1 Microbial catabolism

Microbial activity proceeds at the expense of the nutrients contained in the medium. Metabolism is the series of chemical reactions proceeding in proper sequence in the living cell. In this process, anabolic reactions serve to build up the organism and catabolic reactions to break it down. Sources of energy are nutrients taken up from the environment. Large molecules are split by exoenzymes and pass into the cell by means of various vehicles in the cell, they are metabolized by means of numerous enzymic reactions and through various pathways of glycolysis, proteolysis and lipolysis. Adenosintriphosphoric acid serves as a universal carrier of energy. It is commonly understood that glycolysis results in acidification or fermentation, the result of lipolysis is rancidity and that of proteolysis is putrefaction.

3.1.1 Glycolysis

The main sources of energy for most of the bacterial species are sugars, hexoses in particular. Polysaccharides are broken down differently outside the cell from monosaccharides (hexoses) by the various microorganisms. From these hexoses several pathways lead to the C_3 substance pyruvic acid (pyruvate) which thus takes a central place in metabolism (see Fig. 2).

The most common process of degradation proceeds via fructose-1,6-diphosphate and is defined as FDP-pathway or EMBDEN-MEYERHOF-PARNAS (EMP)-pathway. The most important steps of the reaction are: convertion of glucose into 2 molecules of triose phosphate, dehydration of phosphoglycerate and conversion of pyruvate.

Outcome: 1 Mole glucose + 2 NAD + 2 ADP $\longrightarrow$ 2 pyruvate + 2 $NADH_2$ + 2 ATP

Another pathway of degradation of hexoses is that of pentose-phosphate (PP) or the DICKENS-HORECKER scheme. Degradation starts with direct glucose oxidation, where the hydrogen split off is transferred to NADP and gluconolactone-phosphate is produced which spontaneously hydrolyzes to 6-P-gluconic acid. Further dehydration and decarboxylation reaction results in the formation of ribulose-5-phosphate via 3-keto-6-phosphogluconic acid. In the following reactions of transketolase-transaldolase, the hexoses are converted into pentoses.

Outcome: 1 Mole glucose + 1 ATP + 2 NADP $\longrightarrow$ 1 Mole pentose + 1 ADP + 2 $NADPH_2$ + 1 CO_2

On the KDPG pathway or ENTNER-DOUDOROFF pathway, 6-P-gluconic acid develops again at first as on the PP-pathway which, by splitting off of water, is converted into 2-keto-3-desoxy-6-phosphogluconic acid (KDPG). By a specific aldolase, KDPG is split into pyruvic acid and glycerin aldehyde.

Outcome: 1 Mole glucose + 1 NADP + 1 NAD + 2 ADP $\longrightarrow$ 2 pyruvate + 1 $NADPH_2$ + 1 $NADH_2$ + 1 ATP

The microbial genera are, to various degrees, oriented towards the pathways of degradation described. The enzymes of FDP and PP pathways belong to the basic stock of most of the cells, while the KDPG pathway is followed mainly by pseudomonads.

Temperature also has an influence on the pathway of degradation. In the case of Pseudomonas fluorescens we were able to show that the percentage share of KDPG in glucose degradation decreased, the more the temperature dropped below the optimum, while the percentage share of FDP in it increased.

Because of its central situation, pyruvic acid may be introduced into various metabolic pathways. The most important are the oxidative decarboxylation to acetyl CoA and dehydration to lactic

acid. For further degradation, for making available the precursors of synthesis and for energy production acetyl CoA is introduced into the citric acid cycle and the respiration chain.

In lactic acid fermentation the $NADH_2$ produced on the FDP pathway is regenerated by transfer of hydrogen to pyruvic acid. The transfer of split-off hydrogen to organic H acceptors is the only means of energy production for many microorganisms (obligatory fermenters). The lactic acid formers are divided into homofermentative and heterofermentative species according to their ability to metabolize glucose either to lactic acid only or also to other fermentation products and carbon dioxide.

Homofermentative species produce lactic acid only; they include:

Streptococcus lactis, S.cremoris, Lactobacillus casei, L.acidophilus, L.plantarum, L.helveticus, L.bulgaricus.
Heterofermentative species produce lactic and acetic acid, ethanol, and CO_2, for instance:

Lactobacillus brevis, L.buchneri, L.fermenti, L.thermophilus, Leuconostoc citrovorum, Lc.mesenteroides, Microbacterium lacticum, Micrococcus luteus, M.varians, M.freudenreichii.

Their temperature optimum is around 25° C - 30° C in most cases.

The distinction between homo- and heterofermentative species is in principal dependant on the different ways of degradation of hexoses. The heterofermentative lactic acid bacteria do not produce enzymes of the FDP pathway. The first steps of glucose degradation are made over the PP pathway.

Whether L-, D- and/or DL-lactic acid is produced, depends on the stereospecificity of lactate dehydrogenase and on the presence of a lactate racemase. The stereospecificity of lactic acid is characteristic for the different lactic acid formers.

It may be deduced from the nature of hexose degradation that lactic acid and pyruvic acid may be used as parameters of glycolytic processes.

3.1.2 Proteolysis

The proteins are split up into permeable fragments by exoenzymes. The fragments taken up by the cell are degraded by the peptidases to amino acids. These are either used in protein biosynthesis, or are passed to final degradation over various pathways of the intermediary metabolism.

Amino acids are fermented under anaerobic condition over special metabolic pathways.

Temperatures of 85° C up to 150° C are required for heat inactivation of extracellular proteolytic enzymes of psychrotrophic organisms.

Typical proteolytes are: Bacilli, Pseudomonads, Clostridia, Proteus, Streptococcus faecalis var.liquefaciens, Micrococcus caseolyticus. They are putrefactants as far as they ferment the proteins anaerobically to hydrogen sulphide, mercaptans, indol and fatty acids.

Among the numerous possibilities for microbial spoilage, protein decomposition appears to be the most complicated process and has not been cleared up in detail so far.

It is characterized by the development of a putrefactive smell and changes in consistency of a foodstuff.

Utilization of amino acids, the source of energy, is mostly initiated by the reaction of decarboxylation or desamination. The reaction products of decarboxylation are carbon dioxide and primary or biogenic amines. Ammonia is split off in the case of desamination. Transamination, in which the amino group of an amino acid is transferred to an α-keto-acid, serves to synthesize amino acids

and also to degrade some of them. Further degradation of the carbon structure differs with the different amino acids. Some desamination products are intermediary products of central ways of degradation (pyruvic acid, α-keto-glutaric acid, hydroxy-pyruvic acid, succinic acid, fumaric acid, oxalacetic acid). Other carbon hydrogen structures are introduced into the pathways of intermediary metabolism over special ways of degradation. Fig. 3.1 gives a scheme of the ways of degradation of amino acids.

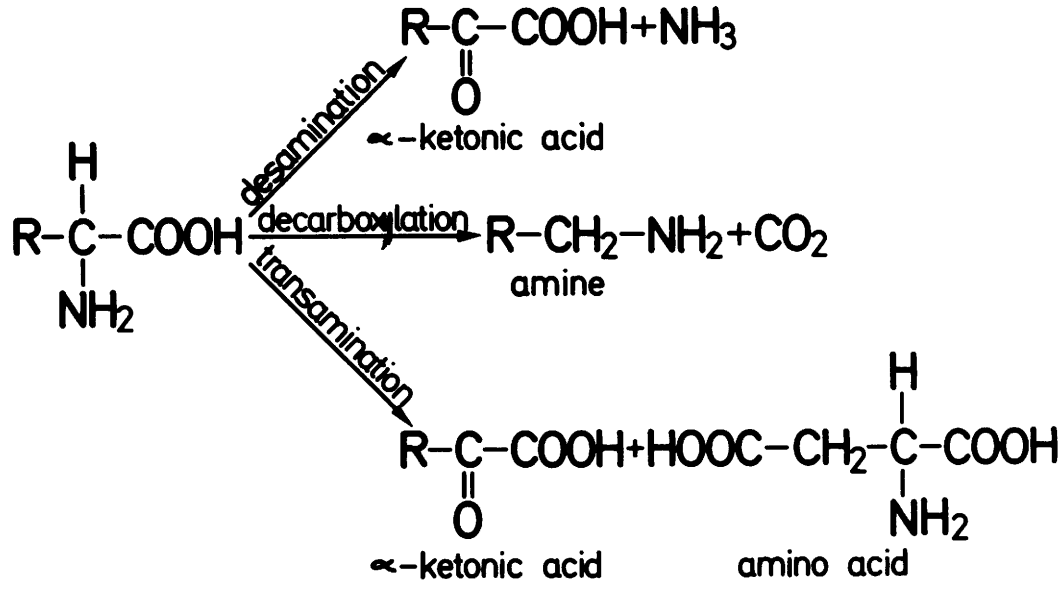


Fig. 3.1

Several microorganisms such as Escherichia coli and Proteus vulgaris desaminate tryptophane so that indole, and later-on pyruvate and ammonia are produced.

Ammonia may be considered a parameter of proteolysis, and the central metabolite pyruvate may be seen as a desamination or transamination product of some amino acids.

3.1.3 Lipolysis

Most of the microbial lipases studied so far behave very much like pancreas lipase since they preferably attack the 1,3-positions of the triglycerides present in dietary fat. Then the fat is decomposed into its two major components - glycerol and fatty acids. Furthermore, this hydrolysis involves 1,2-diglycerides or 2-monoglycerides as intermediary products. The 2-monoglycerides are easily converted into 1-monoglycerides by the migration of alkyls. At the beginning of lipolysis, particularly short-chain fatty acids are split off.

The splitting products of fats pass over various metabolic ways: glycerol is closely related to carbohydrates. It may either be used for the build-up of fructose or glucose or may be further metabolized after oxidation and phosphorylation to dehydroxy acetone phosphate over the EMBDEN-MEYERHOF pathway.

According to the principle of ß-oxidation, the free fatty acids are decomposed into C_2 units (activated acetic acid) which are either used in biosyntheses or are further metabolized in the citric acid cycle.

The limited information obtained from the detection of free fatty acids may be seen in the fact that the fatty acids are further stepwise degraded through the ß-oxidation.

Typical lipolytes are: Pseudomonades, alcaligenes aeromonades, flavobacteria, achromobacter, micrococci, coryne bacteria, Escherichia, Klebsiella and bacilli. Of special importance are psychrotrophic bacteria which are able to grow massively during prolonged storage even in refrigerated food. The lipolytic enzymes are either extracellular and/or intracellular. Many of the extracellular enzymes are extremely heat-resistant. Temperatures of up to 150° C are required for their inactivation. Though the microorganisms themselves are destroyed by pasteurization, their lipolytic enzymes are hardly or only slightly inactivated.

Due to their biochemical activity, fat-splitting microorganisms may, in this way, also affect the quality of pasteurized products. The organoleptic alterations are provoked particularly by even-numbered fatty acids with 4 to 12 C-atoms. Kind and quantity of the liberated fatty acids vary from strain to strain and depend on substrate conditions.

Lipolysis is detected through the senses by rancidity, soapiness and discolorations.

Appendix:

Apart from microbial lipases, lipolysis may also be produced by original lipases and by autoxidation (catalyzed by copper and iron ions and released by short-wave light and high storage temperatures).

Originally milk contains several lipolytic enzymes. We distinguish between plasma and membrane lipases, between lipoprotein lipases and acid lipases. As regards the activity of lipases there is a difference between cows; moreover some dependence on feeding, stage of lactation, season and cell counts has been proved to exist. The content of free fatty acids in milk is influenced by the milking plant, due to turbulence and foam formation. No influence is exerted by breed, milking method (manual or mechanical) and daily milk or fat yield. These lipolytic enzymes are sensitive to heat and are generally inactivated by pasteurization.

Lipolytic processes taking place in milk are of great economic importance, since the fatty acids liberated during fat hydrolysis as well as mono- and diglycerides confer a rancid or bitter taste upon milk and milk products.

3.2 Pyruvate as the central metabolic parameter for the measurements of bacterial activity under psychrotrophic conditions

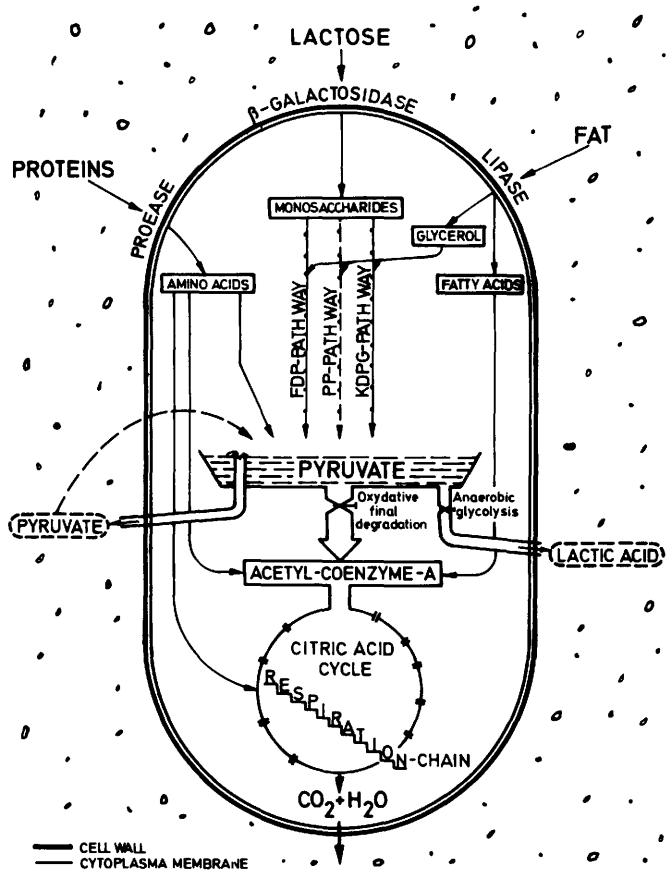
The special difficulties associated with representative sampling and efficient sample preservation have encouraged a working group of our Institute to search for a bacterial metabolite which

- would extensively describe the bacterial breakdown in milk even under psychrotrophic conditions and would not be affected by other factors,
- could be determined precisely and with an automated procedure if possible,
- would be a soluble substance and as such allow representative sampling and reliable sample preservation, and
- as a chemical substance would not be affected by pasteurization and thus provide an additional picture of substrate changes.

Due to its central position in molecular metabolism, pyruvate (pyruvic acid) appeared especially suitable for this purpose. Preceeding papers have reported on the analytical as well as the enzymatic pyruvate determination for the measurement of the bacterial biochemical status of milk; the essential results of our previous work together with the latest state of knowledge and practical experience will be reported in the following.

3.2.1 A model scheme for the extracellular accumulation of pyruvate

Pyruvate is a central substance (pool in the scheme, see Fig.3.2) of metabolism and bacterial catabolism which is of special interest from the point of view of food hygiene. Its further fate is highly dependent on the kind of microorganisms and their environmental conditions: the end products of anaerobic glycolysis are lactic acid and other fermentation products; in the process of oxidative degradation and after decarboxylation pyruvate is introduced as activated acetic acid into the citric acid cycle which for its part is closely linked up with the chain of respiration.

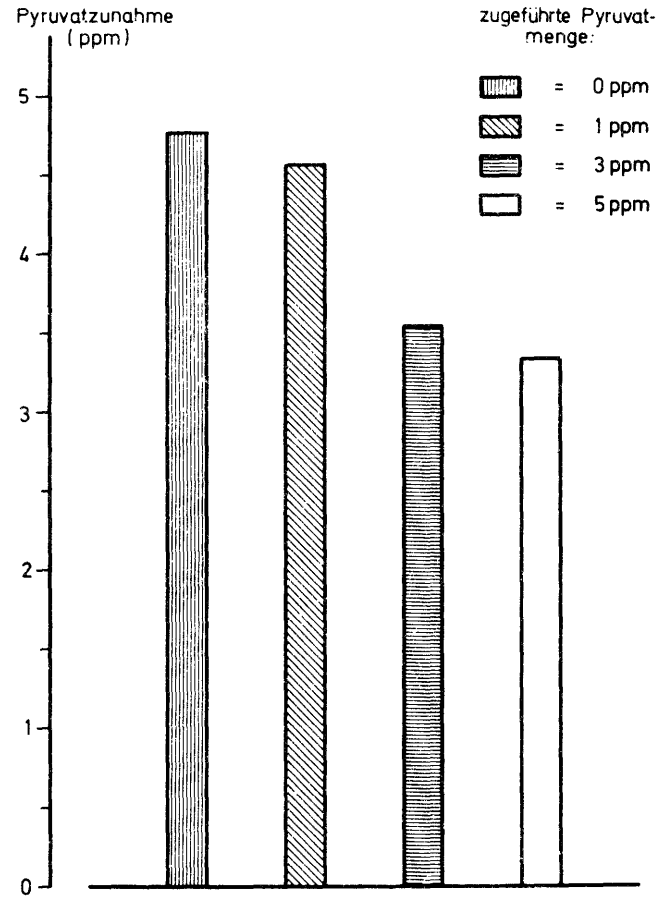


MODEL OF PYRUVATE-FORMATION WITHIN THE SYSTEM CELL - SUBSTRATE (MILK)

Fig. 3.2

The first enzymatic steps leading to pyruvate evidently proceed more quickly than the following steps, since we have observed a systematic accumulation of pyruvate dependant on bacterial growth. In this process, the amount of pyruvate released is apparently related to the extracellular concentration of pyruvate. To milk samples with equal bacterial counts different concentrations of pyruvate were added experimentally; if the extracellular concentration of pyruvate is low, more pyruvate is released from the cell than in the case of higher pyruvate concentrations, as may be seen from the following Fig. 3.3.

Increase of pyruvate



Pyruvate added

INCREASE OF PYRUVATE IN MILK
AFTER EXPERIMENTAL ADDITION
(INCUBATION: 72 h/12°C) (n=11)

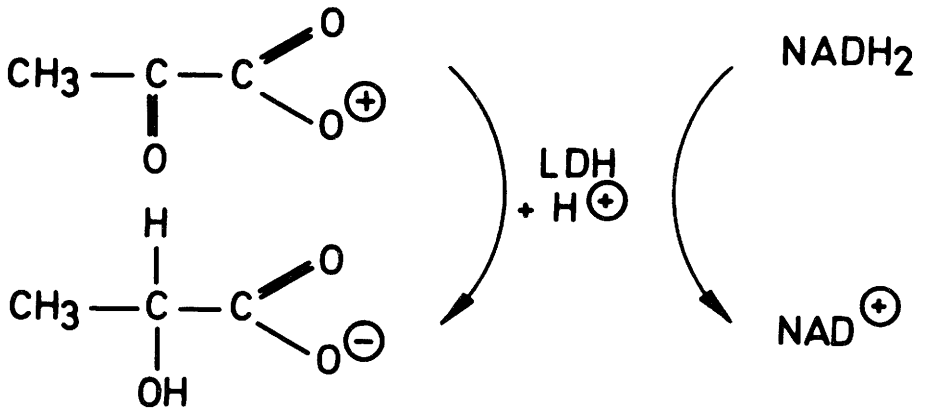
Fig. 3.3

The four columns show the pyruvate difference values which were measured after incubation for 72 hours at 12° C. The first column is that of a milk sample to which pyruvate was not added prior to incubation. The other three columns show the difference values of equal samples as before but with additions of 1 ppm, 3 ppm and 5 ppm of pyruvate respectively; the additions were made prior to incubation. It may clearly be seen that the pyruvate difference value decreases with increasing extracellular pyruvate concentration.

This means that pyruvate measurements are more sensitive in samples with low bacterial counts or low extracellular pyruvate concentration than in higher bacterial ranges. This observation is supported by studies on bacterial count equivalents attributed to the respective pyruvate values (see Chapter 3.3.6). It appears that a diffusion equlibrium develops in the cell system and surrounding nutritive medium.

3.2.2 Analysis

Pyruvate is reduced to lactic acid in the presence of lactate dehydrogenase (LDH) together with simultaneous oxidation of reduced nicotinic acid amide-adenin-dinucleotid ($NADH_2$), which is illustrated in the following scheme (Fig. 3.4):



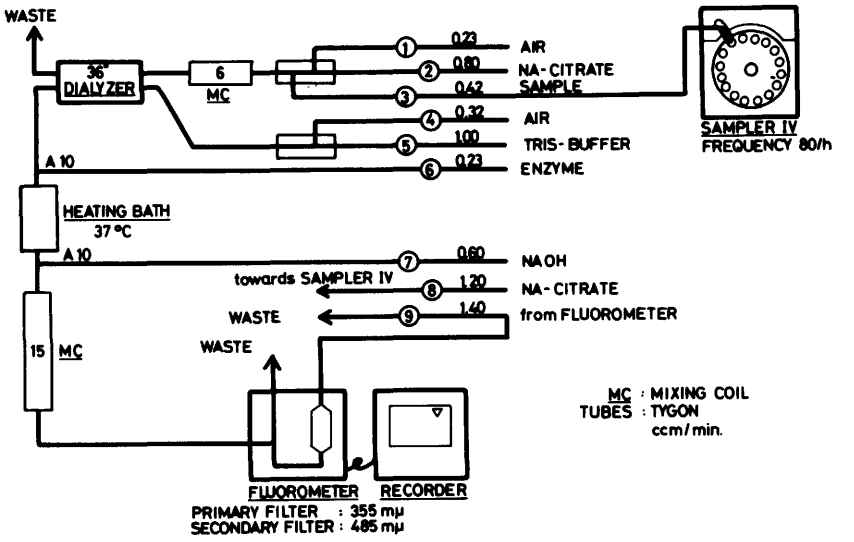
PRINCIPLE OF ENZYMATIC PYRUVATE DETERMINATION 74/037 Wi

Fig. 3.4

The amount of $NADH_2$ consumed in this process is equivalent to the quantity of pyruvate. The decrease of $NADH_2$ is measured fluorometrically as well as colorimetrically.

3.2.2.1 Fluorometry

The reaction will be complete only at a ph value around 7.5, since only then it proceeds quantitatively from pyruvate to lactate. Continuous flow analysis in the Auto Analyzer allows samples to be analyzed at a rate of 80 - 100 per hour. The following Figure shows a flow diagram of enzymatic pyruvate determination by means of a fluorometer.



ENZYMATIC PYRUVATE DETERMINATION IN THE AUTO-ANALYZER SYSTEM II

Fig. 3.5

0.42 ml of the milk sample is diluted with 0.80 ml sodium citrate solution (5 %) and segmented with 0.23 ml air. Dialysis is made against air-segmented tris-buffer (pH = 7.5; 0.3 M). 0.23 ml LDH-$NADH_2$ mixture is added to the dialysate (1.00 ml tris-buffer + 0.32 ml air). The reaction proceeds in a heating bath at 37° C. The addition of 0.60 ml 1 M NaOH and subsequent mixing will stabilize the base line.

If standards are carried along with the flow of samples and if the reaction mixture is constant, the reproducibility obtained is nearly absolute: the correlation coefficient between independent preparations is found to be r = 0.99. (Methodical details see reference 21). By reducing the quantity of $NADH_2$ to about 5 ml of the stock solution and by extending the amplitude in the diagram we have recently succeeded in making the analysis more sensitive and in fixing the accuracy of readings to 0.1 ppm.

Reagents (use volumetric flasks, make up to volume)

Sodium citrate soln.

Sodium citrate		50.0	gms
Distilled water	up to	1000.0	ml

Tris-buffer

Tris		36.3	gms
Distilled water		900.0	ml
adjust pH to 7.6 with conc. hydrochloric acid make up to volume (1000 ml)			

Sodium hydroxide soln. (1 N)

Sodium hydroxide (pellets)		40.0	gms
Distilled water	up to	1000.0	ml
0.1 M Titriplex III soln.		30.0	ml

$NADH_2$ - stock soln.

NADH + H^+ (sodium salt, MERCK)		0.06	gms
Tris-buffer (pH 9.6)	up to	100.0	ml
(12.1 gms Tris in 1000.0 ml distilled water)			
- store in refrigerator -			

Enzyme-working soln.

$NADH_2$ - stock soln.		5.0	ml
LDH (rabbit muscle, 5.0 mgms/ml)		0.5	ml
Tris-buffer (pH 9.6)	up to	100.0	ml
- prepare fresh daily -			

Pyruvate - stock soln. (1000 ppm)

Pyruvic acid (sodium salt)		0.128	gms
Distilled water	up to	1000.0	ml

- prepare fresh weekly -

Working standards for pyruvate determinations

Stock soln. (ml)		Pyruvate-concentration in ppm
1.0	Make up to	1.0
2.5	100 ml with	2.5
5.0	distilled	5.0
7.5	water	7.5
10.0		10.0

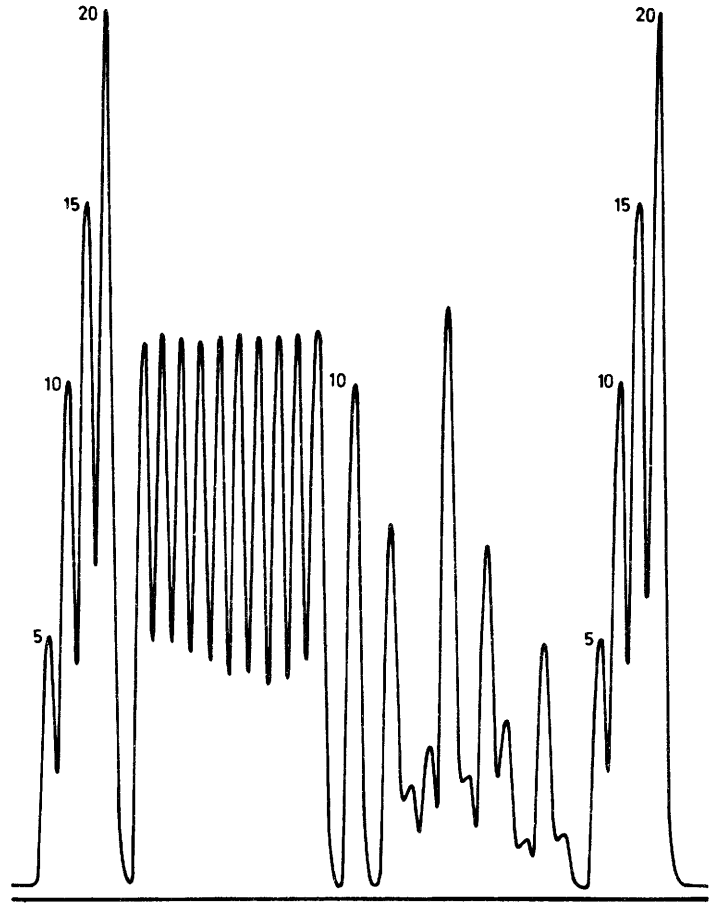
Precision of the method

PRECISION OF PYRUVATE DETERMINATION
(fluorometrically)
Standard deviations and coefficients of variation
in relation to pyruvate level -samples in duplicate

Pyruvate (ppm)	n	$\bar{X}_A$	s_a	CV
< 1,0	54	0,74	0,048	7,75
1,01 - 1,5	85	1,31	0,046	3,42
1,51 - 2,0	127	1,77	0,063	3,52
2,01 - 2,5	73	2,26	0,057	2,45
2,51 - 3,0	39	2,73	0,070	2,51
3,01 - 5,0	41	3,80	0,076	1,89
> 5,0	57	7,99	0,26	3,17
Total/average	476	2,64	0,082	3,55

75/447 La

Table 3.0 a



Pyruvat-Chromatogramm (Frequenz 80/Std.)
(4 Arbeitsstandards (ppm), Wiederholungsreihe einer Milchprobe, 10 unterschiedliche Milchproben)

PYRUVATE CHROMATOGRAM (RATE 80/h)
(4 WORKING STANDARDS (ppm),
REPITITION OF ONE SAMPLE,
10 DIFFERENT SAMPLES)

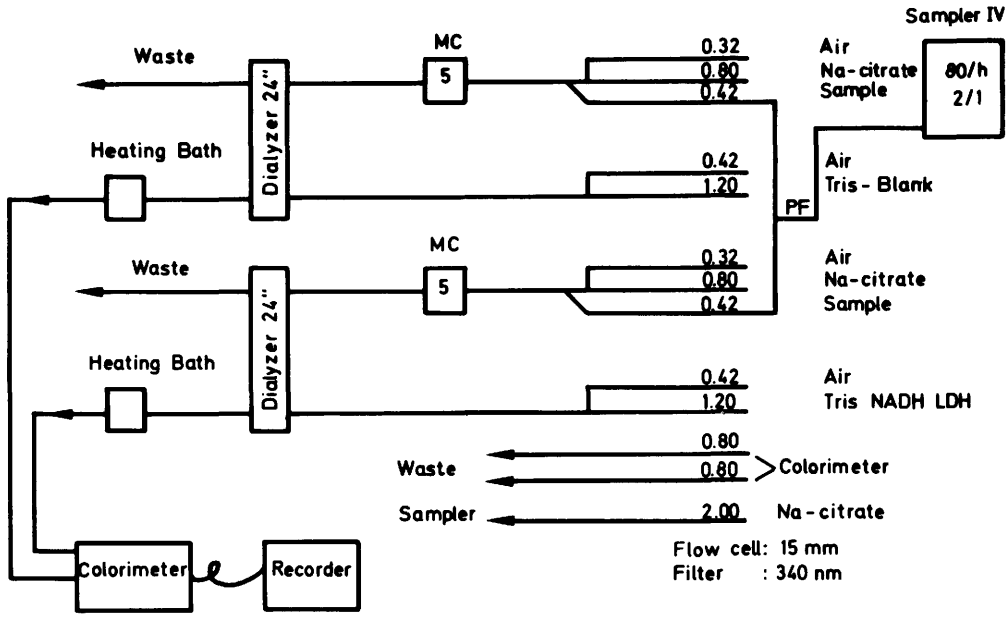
Fig. 3.6

3.2.2.2 Colorimetry

3.2.2.2.1 Auto Analyzer System

Colorimetric pyruvate determination in the Auto-Analyzer System proceeds according to the same analytical principles as in fluorometry (Fig. 3.4). The essential difference consists in the fact that in the colorimetric pyruvate determination with blank the flow of samples and of references passes through the same system, however, no enzyme being added to the flow of references. This way will

automatically correct eventual unspecific reactions. The flow diagram is represented in Fig. 3.6 a.



Colorimetric pyruvate determination with blank in the Auto-Analyzer-System II

Fig. 3.6 a

0.42 ml of the sample are diluted with 0.80 ml sodium citrate and segmented with 0.32 ml air. Dialysis is made in the "analytical" canal against 1.20 ml tris-buffer-$NADH_2$-LDH solution which is segmented with 0.42 ml air. No LDH is added to the receptor stream of the reference canal. The reaction proceeds in a heating bath at 37^{o} C. The measurements are made in a 15 mm flow cuvette at 340 nm.

Reagents

Sodium citrate soln.

Sodium citrate		50.0	gms
Distilled water		1000.0	ml

Tris-buffer

Tris		72.6	gms
Distilled water		1800.0	ml
Adjust pH to 7.6 with conc. HCl			
Distilled water	up to	2000.0	ml

$NADH_2$ stock soln.

$NADH_2$ (sodium salt)	60	mg
Tris-buffer pH 9.6 (12.1 gms tris in 1000 ml distilled water)	100.0	ml

Sample and reference reagent

Tris-buffer pH 7.6	2000.0	ml
$NADH_2$ stock soln.	30.0	ml

After careful mixing divide into 2 x 1 litre. 0.6 ml LDH (from rabbit muscle 5.0 mg/ml) is added to the sample reagent (1000.0 ml)

Pyruvate stock soln.

Pyruvic acid (sodium salt)	0.128	gms
Distilled water	1000.0	ml

Working standards

Stock soln.		Pyruvate concn. (ppm)
1.0		1.0
2.5		2.5
5.0	up to 100 ml	5.0
7.5	distilled water	7.5
10.0		10.0

Precision of the method

For the colorimetric pyruvate determination with blanks Table 3.0 b shows standard deviations and coefficients of variation in dependence on various pyruvate contents.

Distilled water is used instead of the filtrate in order to determine the blank. The extinction difference measured hereby must be subtracted from the extinction difference obtained with the filtrate.

Calculation

The pyruvate values are calculated in accordance with the following equation:

$$C = \frac{\Delta E \cdot V \cdot MG \cdot 10^6}{\Sigma \cdot d \cdot V} \cdot F \text{ (ppm)}$$

where:

C = concentration

Δ E = extinction difference

V = test volume (ml)

v = sample volume (ml)

MG = molecular weight of the substance (g) (pyruvate 88.1 gms) to be determined

Σ = extinction coefficient of $NADH_2$
(366 nm: $3.3 \cdot 10^6$ nm^2/Mole
340 nm: $6.22 \cdot 10^6$ nm^2/Mole
334 nm: $6.0 \cdot 10^6$ nm^2/Mole

d = thickness of layer

10^6 = conversion factor into ppm

F = dilution factor

There are the following conversion factors:

366 nm : C = E . 88.9 (ppm)
340 nm : C = E . 47.2 (ppm)
334 nm : C = E . 48.9 (ppm)

Calibration curve

The pyruvate values may also be determined by means of a calibration curve. In this case, aqueous pyruvate solutions of known concentrations are treated like the samples and the extinction differences are recorded in dependence on the concentration.

PRECISION OF PYRUVATE DETERMINATION
(colorimetrically with blank)
Standard deviations and coefficients of variation
in relation to pyruvate level-samples in duplicate

Pyruvate (ppm)	n	$\bar{X}_A$	s_a	CV
< 1,0	19	0,76	0,036	5,36
1,01 - 1,5	50	1,32	0,038	2,83
1,51 - 2,0	87	1,82	0,038	1,93
2,01 - 2,5	93	2,26	0,080	3,44
2,51 - 3,0	80	2,76	0,086	3,09
3,01 - 5,0	71	3,69	0,134	3,55
> 5,0	63	8,05	0,322	3,88
Total/average	463	3,11	0,108	3,18

75/448La

Table 3.0 b

3.2.2.2.2 Manual determination

Preparation of samples

For deproteinizing 4 ml milk and 4 ml ice-cold 1 M perchloric acid are mixed and centrifugated for 10 minutes at 3000 r.p.m. 4.0 ml of the supernatant are mixed with 2.0 ml of a 0.7 M tripotassium-phosphate buffer, allowed to stand in an ice/water mixture for about 15 minutes and then filtered. The filtrate is warmed up to 25° C.

Determination

Measurement is made in a glass cuvette of 1 cm thickness of layer at 25° C against air and at wave lengths of 366, 340 or 334 nm. 2.00 ml of the filtrate and 0.20 ml $NADH_2$ are pipetted into the cuvette and mixed by a plastic spatula. The extincture E_1 is then measured. Subsequently, 0.02 ml LDH is added. It is again mixed by a plastic spatula and the standstill of the reaction, after about 5 minutes, is awaited; E_2 is then measured. E_1 minus E_2 will give the extinction difference (ΔE).

Reagents

1 M Perchloric acid

Perchloric acid 70 %		86.0	ml
Distilled water	up to	1000.0	ml

0.7 M Tripotassium-phosphate buffer

tri-potassiumphosphate-7-hydrate	236.9	gms
Distilled water	1000.0	ml

$NADH_2$-solution

$NADH_2$ (sodium salt)	60	mg
Tris-buffer pH 9.6	100.0	ml

(12.1 gms of tris in 1000.0 ml distilled water)
Keep in refrigerator

LDH-solution

LDH from rabbit muscle (5.0 mg/ml)	0.4	ml
Distilled water	0.6	ml

Pyruvate solution

Pyruvate (Na-salt)	0.128	gms
Distilled water up to	1000.0	ml

Working standards

Pyruvate soln (ml)		Pyruvate concn (ppm)
0		0
2.5	up to 100 ml	2.5
5.0	distilled water	5.0
7.5		7.5

Precision of the method

Table 3.0 c shows deviations and coefficients of variation involved in manual pyruvate determination.

PRECISION OF PYRUVATE DETERMINATION (manually)
Standard deviations and coefficients of variation in relation to pyruvate level
- samples in duplicate -

	Calculated values				Calibration curve			
Pyruvate (ppm)	n	$\bar{X}_A$	s_a	CV	n	$\bar{X}_A$	s_a	CV
< 1,0	0	-	-	-	0	-	-	-
1,01 - 2,0	5	1,60	0,14	8,7	5	1,64	0,11	6,9
2,01 - 3,0	16	2,49	0,11	4,4	10	2,45	0,16	6,5
3,01 - 4,0	6	3,39	0,20	6,1	10	3,26	0,15	4,5
4,01 - 5,0	6	4,48	0,20	4,6	10	4,30	0,23	5,9
> 5,0	7	6,26	0,35	5,4	5	6,24	0,33	5,0
Total/average	40	3,47	0,18	5,4	40	3,52	0,19	5,8

75/449 La

Table 3.0 c

3.3 Results of experimental assays

3.3.1 Specifity of the enzymtic determination of pyruvate

The check for the specifity of the method was carried out with coreactants under identical conditions to that of pyruvate. The coreactants occuring with LDH are:

1. ß-hydroxy-pyruvate
2. glyoxalate
3. a-ketobutyrate
4. a-keto-n-valerate
5. oxalacetate

Under these substances ß-hydroxy-pyruvate and glyoxalate reacted with a rate of the same order as that of pyruvate. The remaining three substances reacted essentially more slowly. The a-keto-analogues of valine, leucine, isoleucine, phenylalanine, tyrosine, glutamic acid and aspartic acid practically do not react.

The investigations in the AutoAnalyzer proved, that the cross-activity of LDH against ß-hydroxy-pyruvate was about 50 %, The enzyme reaction on the four other substrates was so poor, that it could be neglected in the assays under study. To elucidate a possible interference of ß-hydroxy-pyruvate, the pyruvate and ß-hydroxy-pyruvate concentrations in 100 milk-samples were determined and compared with another.

This was done by merely changing the enzymes in the continuous flow analysis. For the determination of ß-hydroxy -pyruvate glycerate-dehydrogenase from spinach (BOEHRINGER) was used in a concentration of 5 mg/ml in the working solution. This enzyme also reacts with glyoxalate with a rate, four to five times lower than with ß-hydroxy-pyruvate. Pyruvate itself is not affected.

The pyruvate concentrations in milk ranged between 1 and 20 ppm. The maximum ß-hydroxy -pyruvate content was 2.5 ppm. This shows that only small quantities of this substance are present in milk and will practically cause no interference with pyruvate. The simultaneous determination of ß-hydroxy -pyruvate will not restrict the specifity of these methods, as this compound is a break-down product from serine and as such is fed into the pathway of glycolysis. Here it is metabolized to pyruvate too.

3.3.2 Sampling

Pyruvic acid occurs in milk in a dissolved state; this facilitates representative sampling. Associated with reliable preservation by formalin in equal concentration to that required in electronic cell counting, this circumstance appears to be an inestimable advantage for practical dairy hygiene.

The reliability of sampling is depicted in Figures 3.7 and 3.8. they compare pyruvate values and total microbial counts in cold-stored milk. The samples were taken from the lower, the medium, and the upper third of a 40 kg milk can. This can filled with milk was allowed to stand in a cold-store for 2 hours and was then transported over a distance of 15 km. Another two hours later samples were taken from the three thirds for determination of pyruvate and total bacterial count. The remaining milk was then stirred 15 times and samples taken again. This procedure was repeated after 48 and 96 hours.

From first sampling of uncooled milk we obtained bacterial counts of

around	2 100	from the lower third of the can
around	105 000	from the medium third
around	7 200	from the upper third

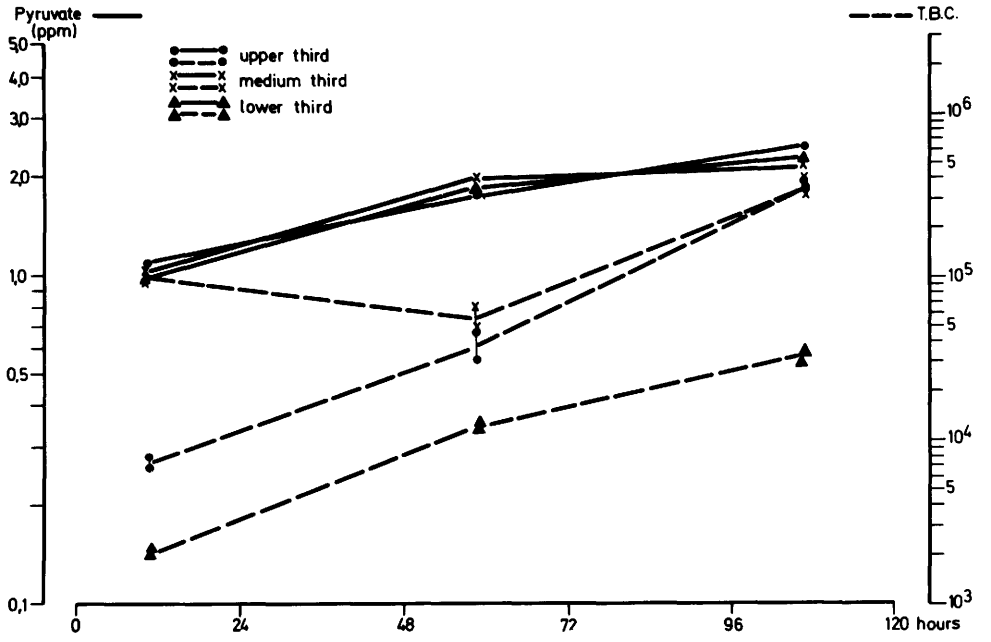
The pyruvate values were found to be 1.0, 1.0 and 1.1 ppm respectively.

After stirring we found bacterial counts of

around	9 900	from the lower third of the can
around	27 300	from the medium third
around	7 200	from the upper third

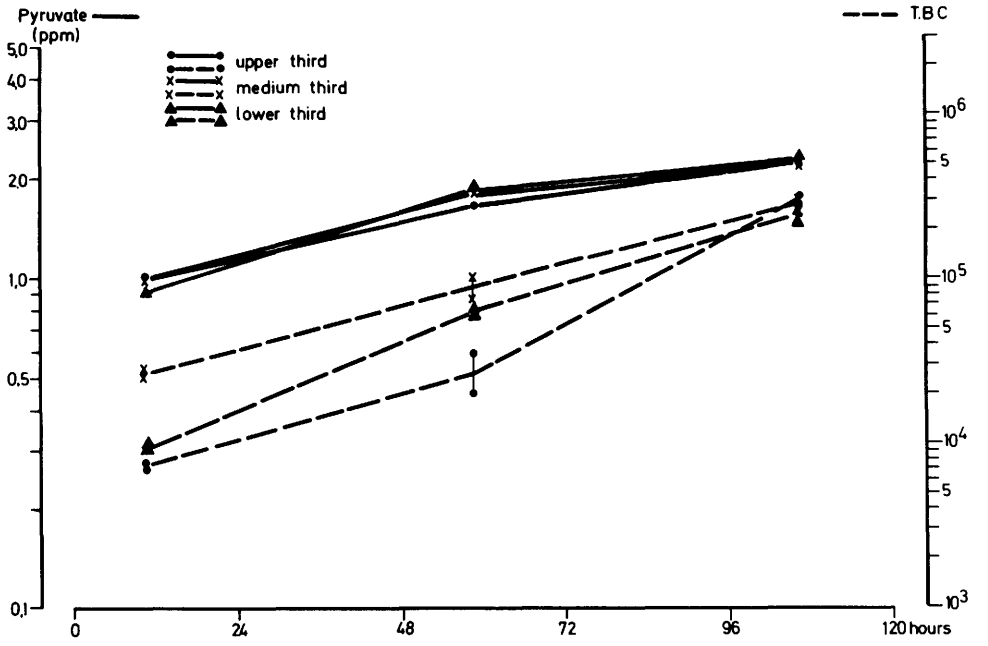
The pyruvate values were found to be 0.9, 1.0 and 1.0 ppm respectively.

The conditions were similar on the following days, but there was a greater variation of total bacterial count in several samples, while pyruvate was rather uniformly distributed in the samples from all three thirds of the can, irrespective of whether milk was stirred or unstirred, the variation being not more than 0.3 ppm. This margin appears to be due to the errors of mixing and reading.



INFLUENCE OF SAMPLING ON PYRUVATE CONTENT AND BACTERIAL COUNT (T.B.C.) IN COLD-STORED MILK IN RELATION TO SAMPLING LEVEL AND MIXING CONDITIONS (unstirred)

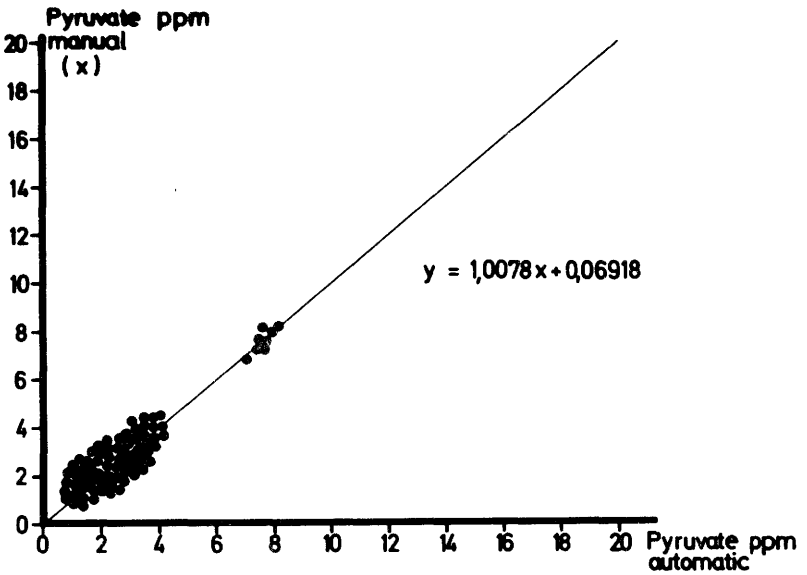
Fig. 3.7



INFLUENCE OF SAMPLING ON PYRUVATE CONTENT AND BACTERIAL COUNT (T.B.C.) IN COLD-STORED MILK IN RELATION TO SAMPLING LEVEL AND MIXING CONDITIONS (stirred 15 times)

Fig. 3.8

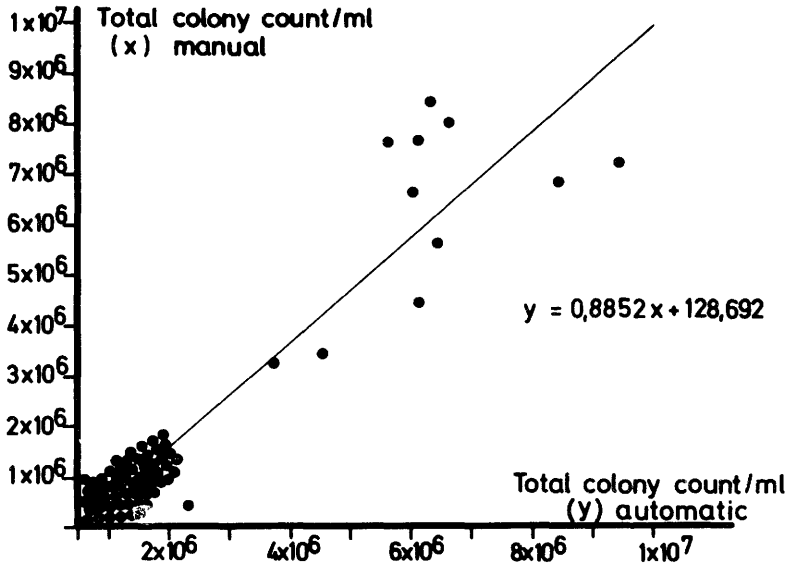
The comparative superiority of pyruvate measurement is also shown when automatic sampling is compared with manual sampling. In this case, samples for pyruvate determination and total bacterial counts were drawn manually and automatically by means of the JANSKY system. The principle of this automated sampling system consists in the fact that a sampling tube with narrow diameter is run in parallel line to the long milk tube. This makes it possible that the sampling tube is carfully flushed and contamination is minimized. It is clear from Fig. 3.8 a that deviating values may not be expected from manual and automatic sampling for pyruvate determinations.



Comparison of automatic and manual sampling.
Test: Sampling of milk with low pyruvate content after previous sampling of milk with high pyruvate content.
(Jansky system)

Fig. 3.8 a

Great deviation of values may be expected from manual and automatic sampling for total bacterial counts (Fig. 3.8 b).



Comparison of automatic and manual sampling.
Test: Sampling of milk with low total colony count after previous sampling of milk with high total colony count.
(Jansky system)

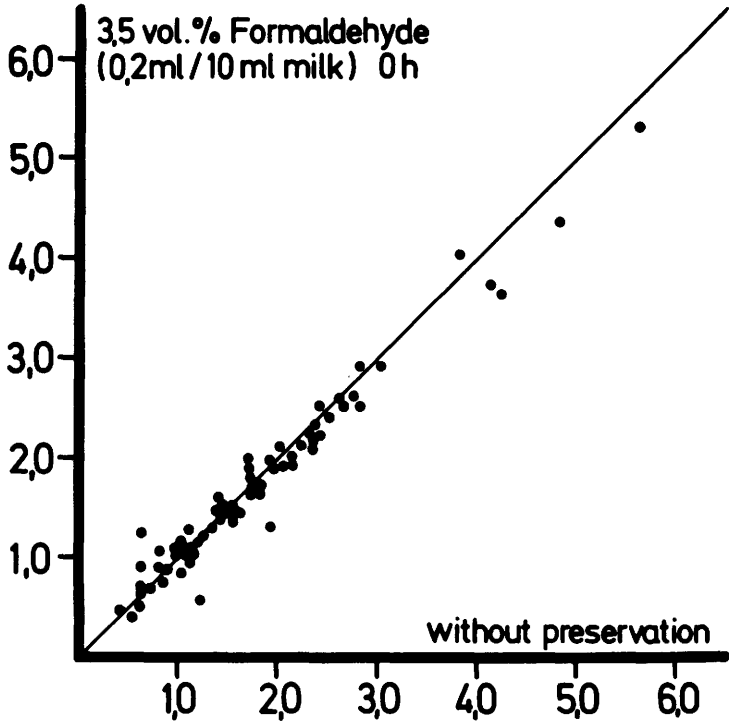
Fig. 3.8 b

3.3.3 Preservation of samples

For fixation of the pyruvate pool, milk samples may principally be preserved by cooling down to 0° C, by heating up to for instance 70° - 80° C or by the addition of chemicals. Sample fixation by cooling is subject to relatively severe uncertainties under practical conditions. This procedure, however, makes it possible to divide the samples after they have arrived at the laboratory or dairy and thus to use it for various kinds of analyses (for somatic cells, for antibiotic inhibitors, and others). Sample fixation by deep-cooling is limited to a period of time of only few hours.

Heat-treatment of samples for fixation of the pyruvate pool may involve some difficulties under practical conditions, since such treatment is required immediately or within shortest time after milk sampling.

Amongst numerous chemicals tested, formalin - in the same concentration as required in somatic cell counting - proved to be suitable for sample preservation for a period of time of 24 hours at maximum. Fig. 3.9 depicts the pyruvate values prior to and immediately after preservation with formalin. Formalin treatment does not involve any systematic displacement of values.



Preservation of milk samples for pyruvate determination (fluorometrically)

Without preservation/ 3,5 vol.% Formaldehyde (0,2ml/10ml milk) 0 h

75/369 Wi

Fig. 3.9

After storage at room temperature for 24 hours, a slight increase of the pyruvate values is generally observed in formaldehyde preservation (Fig. 3.9 a).

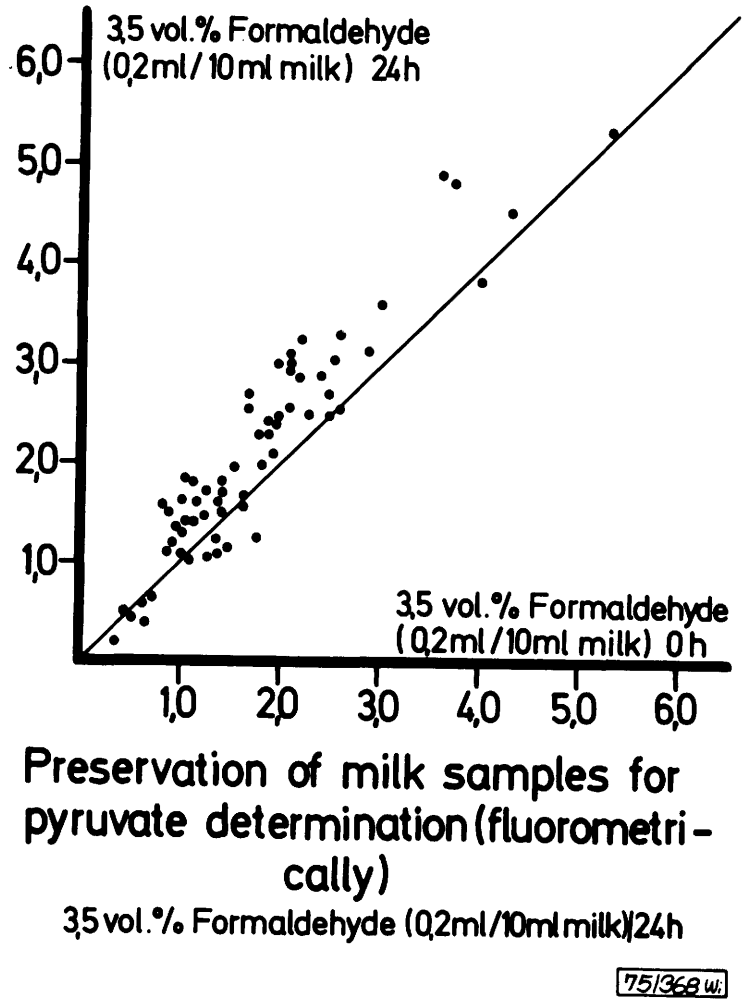


Fig. 3.9 a

Therefore, the time of preservation with formaldehyde should not exceed 24 hours. If this time is prolonged, the pyruvate values measured may also considerably drop.

Milk samples may very well be preserved for up to 6 days by treating them with 10 % trichloroacetic acid. Immediately after the addition of trichloroacetic acid the pyruvate values remain practically constant (Fig. 3.9 b).

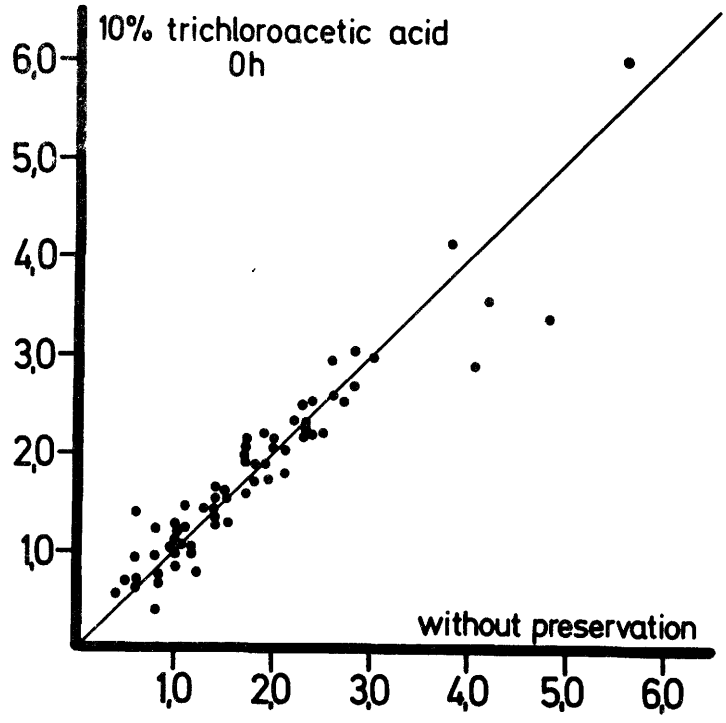


Fig. 3.9 b

After storage of 24 hours, preservation of samples is still very good and does not involve any systematic displacement of values (Fig. 3.9 c).

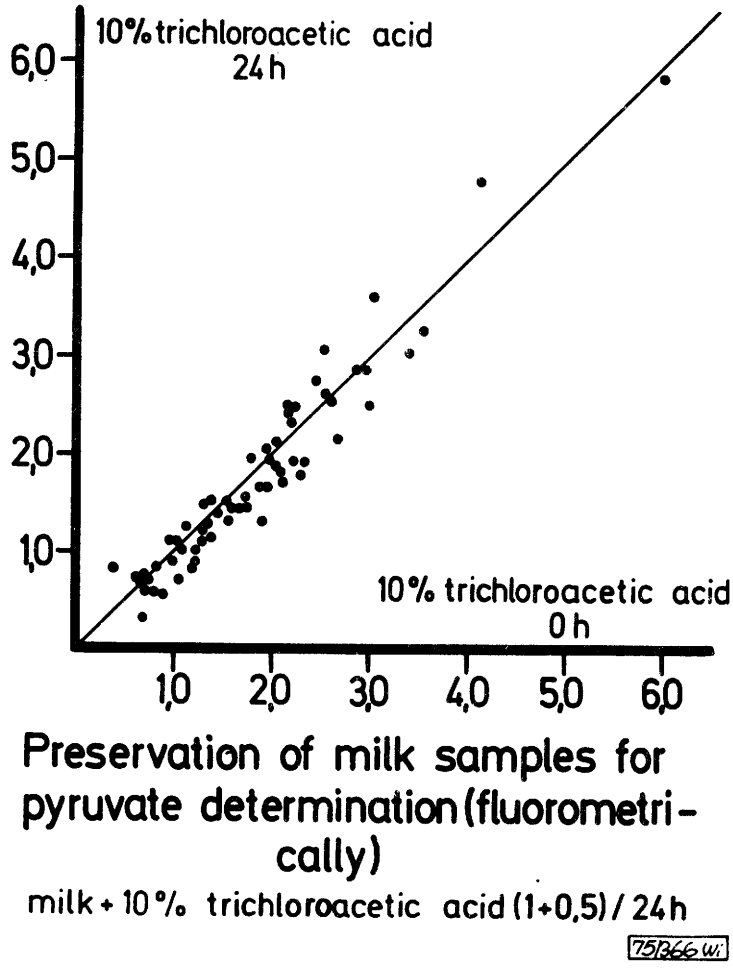


Fig. 3.9 c

Even after storage of 6 days (144 hours), pyruvate determination may still be performed in samples preserved by this way, though the deviation of values will be somewhat greater. Systematic displacement of values, however, will not be recognized (Fig. 3.9 d).

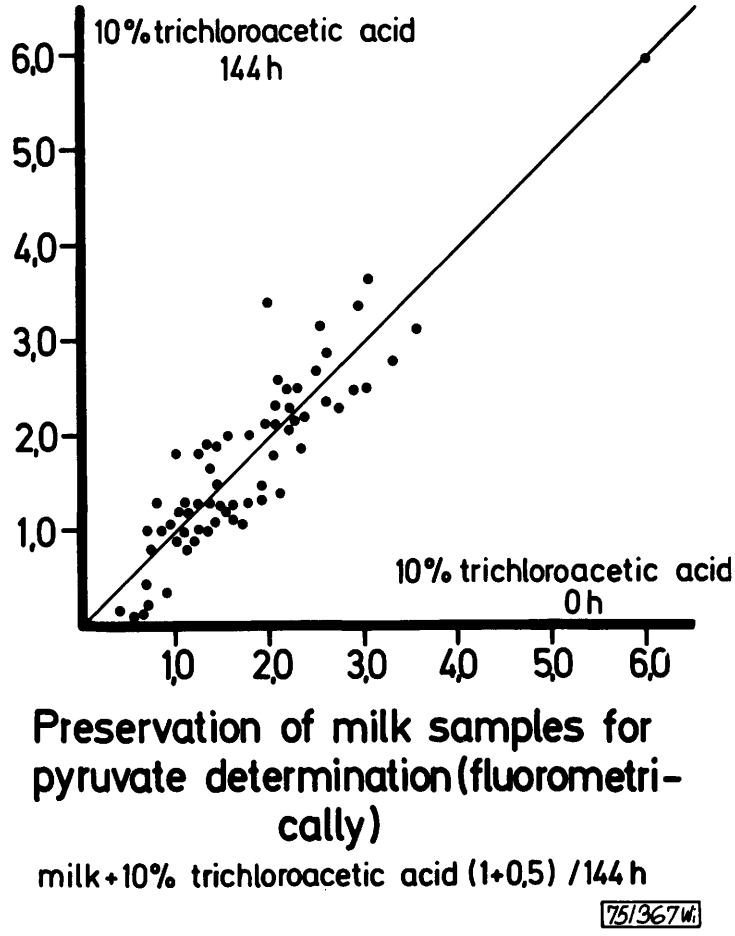


Fig. 3.9 d

For manual pyruvate determination after the colorimetric principle, protein precipitation and preservation of samples by 1 M perchloric acid is used. This will yield good preservation of milk samples for up to 6 days. The advantage involved in sample preservation by trichloracetic acid or perchloric acid may primarily be seen in that the analysis of the samples may be adapted to the actual working conditions and requirements without the samples becoming unsuitable for analysis in the course of several days storage.

3.3.4 Secretory background value

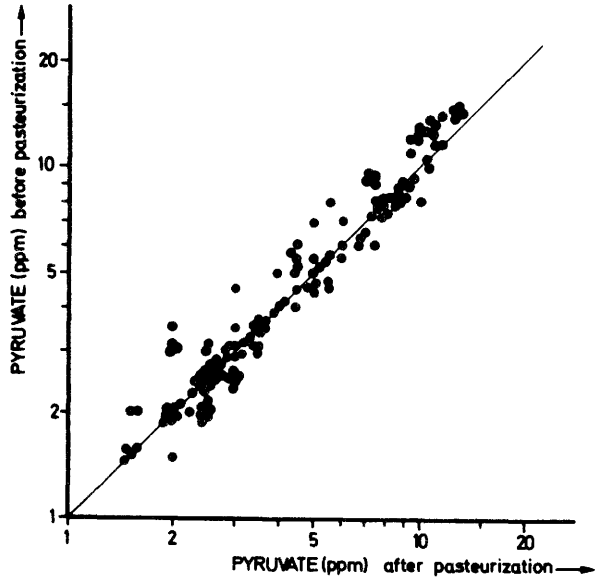
42 quarter samples of the first **strips from an experimental** herd with a cell count of up to 1 million/ml showed a geometric mean of 0.24 ppm pyruvate. With the inclusion of normal and double standard deviation, we obtained a pyruvate base value from this material of 0.26 and 0.29 ppm, respectively. These values were obtained after improvement of the technique of preparation to give a reading accuracy of 0.1 ppm.

50 % of another herd with a total of 120 quarters showed pyruvate values of up to 0.2 ppm, 90 % of up to 0.4 ppm.

For healthy black-pied lowland cattle we have actually assumed a secretory base value of 0.5 ppm pyruvate. There are indications that this value is lower in cattle breeds of higher regions. From recent studies made in England it is seen that bacterial counts in herd bulk milks exceeding 10 000 cfU/ml are frequently due to mastitis streptococci.

3.3.5 Pyruvate and pasteurization

Pyruvate endures pasteurization without being affected by it (Fig. 3.10). For the first time, this opens up the possibility of measuring the additive effect of bacterial activity in every stage between production and consumption.



PYRUVATE CONCENT OF MILK BEFORE AND AFTER PASTEURIZATION (71°C/40sec) (n = 151)

Fig. 3.10

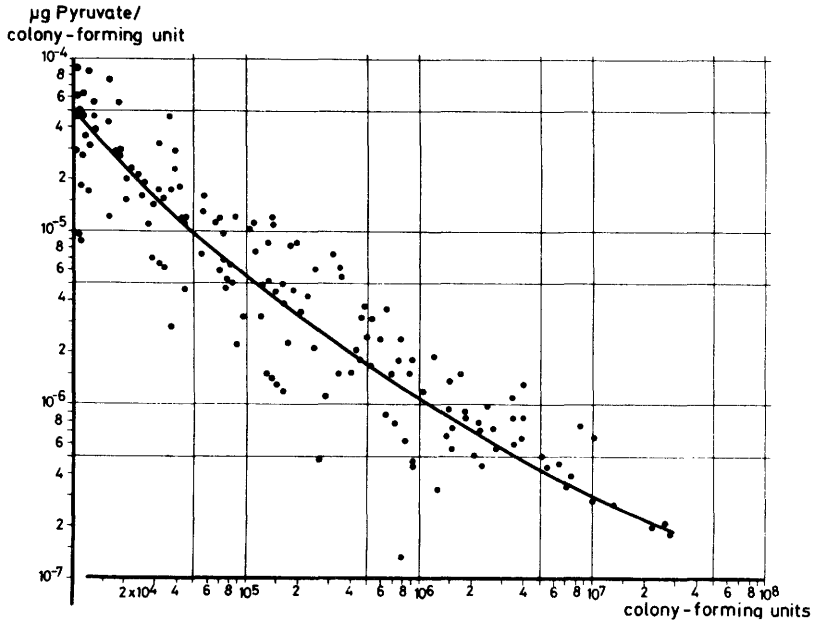
The pyruvate values found before and after short-time pasteurization are grouped closely around the angle of 45°.

3.3.6 Pyruvate and bacterial activity

3.3.6.1 Pyruvate as parameter for bacterial activity in relation to the number of microbes as the quantitative morphologic value

If the base value of 0.5 ppm pyruvate is exceeded, any increase is exclusively due to bacterial activity. Because of varying metabolic activities which depend on bacterial species and their adaptation under practical dairy conditions, we may expect an equivalence with the number of organisms only with considerable limitations, since we are comparing two different biological quantities: metabolic activity on the one hand, and morphological units on the other.

The quantitative relations between colony-forming units from a composite flora of bulk milk and the respective pyruvate values may be seen from Fig. 3.11.



PYRUVATE INCREASE PER COLONY-FORMING UNIT IN MILK IN RELATION TO BACTERIAL COUNT LEVEL
(Koch's plate method/presubstraction of 0.5 ppm of pyruvate for phys.base value)

Fig. 3.11

In the interpretation of the pyruvate and bacterial count equivalent it should be realised that the values obtained from single determinations with the KOCH plate method may considerably differ from the true values, as reported by several authors. Even multiple determinations from one dilution series may give values which differ from the mean value by two- to threefold. Consequently, the pyruvate value equivalent to a colony-forming unit must vary; the pyruvate value itself may be recorded with an accuracy of 0.1 ppm.

As may be seen from Fig. 3.11, 100 000 colony-forming units produce pyruvate in amounts varying between 3×10^{-6} to 1.5×10^{-5} per unit with a mean of 5×10^{-6} µg, i.e. 0.1 ppm pyruvate is equivalent to 20 000 colony-forming units $\pm$ 65 % within this range. 1 million colonies produce an amount of pyruvate varying between 5×10^{-7} and 2.5×10^{-6} per unit with a mean of 1×10^{-6} µg, or 0.1 ppm pyruvate is equivalent to approx. 100 000 colony-forming units $\pm$ 65 % within this range.

These results show the desired differentiation to be more pronounced in the lower than in the higher range.

Corresponding conditions were found in individual direct bacterial counts after BREED, where the quantitative response of a single-cell is lower by one power of ten than that of a colony.

A number between 50 000 - 100 000 colony-forming units/ml of milk may actually be considered the lowest limit of sensitivity of the method at which bacterial activity makes the pyruvate content increase and exceed the base value of 0.5 ppm.

3.3.6.2 Time series investigations for the measurement of catabolic activity in pure cultures

The assays are used for the determination of the direction and intensity of the bacterial catabolism of saprophytes in milk. The isolated observation of pure cultures is of an experimental character. However it is of fundamental importance for the understanding of the processes operating in mixed cultures.

For an easier comparison of analytical data and comprehension of results, the following figures are given. They illustrate the tendencies based on mean-values together with the deviation in the order of activity of specified microbes. The mean plate count ($\overline{X}_G$) is plotted against the geometric means of pyruvate within the investigated groups of microbes as a function of incubation-time and -temperature (values for pyruvate in mgms%). The plate counts are plotted logarithmically because of the exponential reproduction of microorganisms.

As even members of a single species show differences in their biochemical performances, the investigation of single strains is only an indication of the milk hygienic importance of this species. Even replicate investigations of the same tribe can show different biochemical performances. This is illustrated with Pseudomonas fragi in the following Figs. 3.12 and 3.13.

The interpretation of plate counts is restricted by the well known objections. The extraordinary variability of biochemical parameters within the strains under given conditions, so far at present regarded as standardized, shows the problems, associated with the standardization of the following items: Composition of substrate, incubation temperature, incubation interval and preparation of the inocula.

These problems can only be resolved with an excessive expenditure.

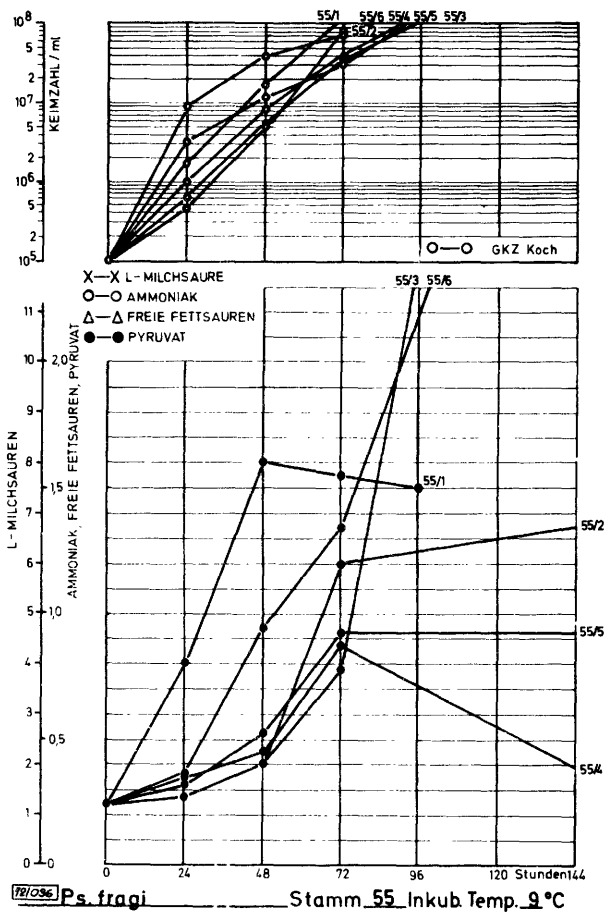


Fig. 3.12

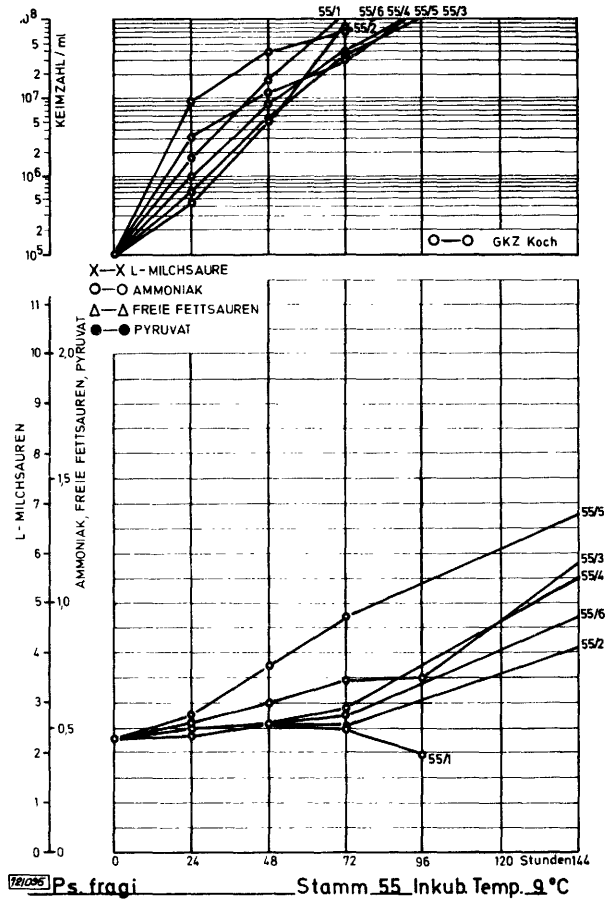


Fig. 3.13

Both illustrations show the variability of quantitative bacteriological examinations with cultures and corresponding biochemical investigations. The variability is caused by unstandardisable growth conditions, occurring in a count range of no practical relevance.

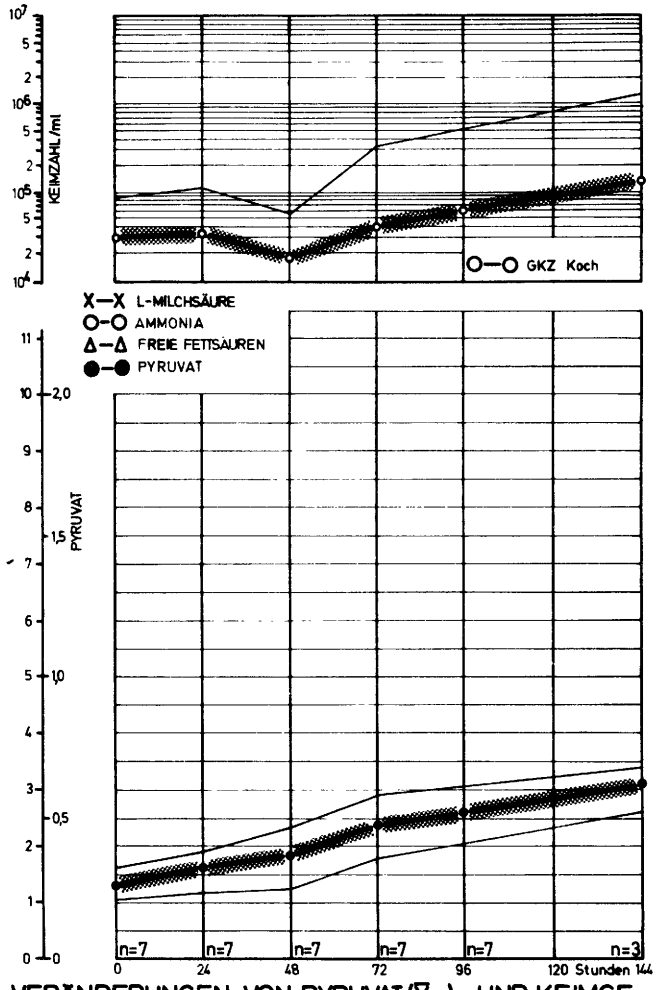
Aerobic spore-formers

The metabolic behaviour of aerobic spore-formers was only investigated at 9° C.

The reproduction rate is very low and even negative (beginning of the assays) at this temperature.

An increase of lactic-acid concentration and free fatty acids could not be measured. With slightly increasing numbers of microbes, gradual rises of ammonia and pyruvate can be detected (see Fig. 3.14).

Total count



CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES BACILLI - INCUBATION TEMPERATURE 9° C

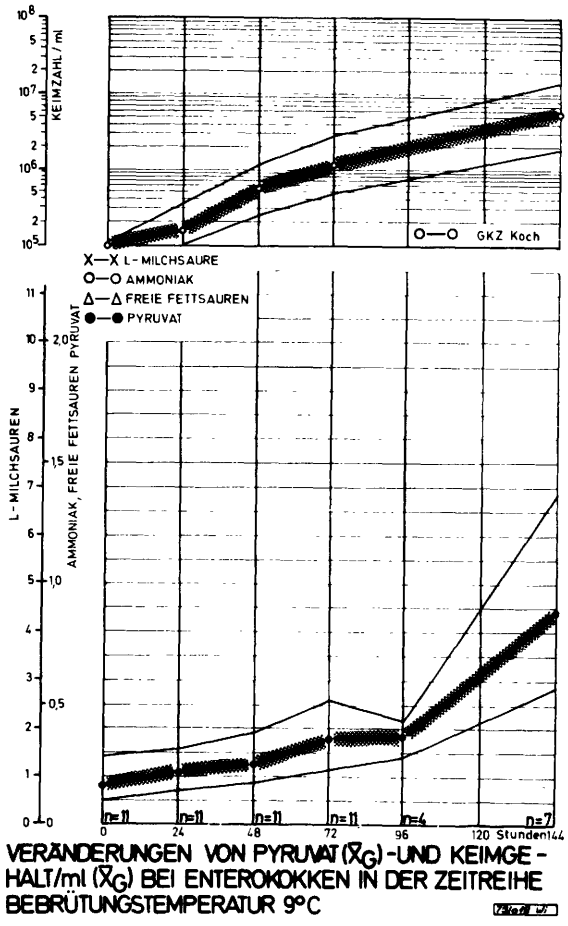
Fig. 3.14

Enterococci

Species of enterococci show the poorest metabolic activity of all groups under study.

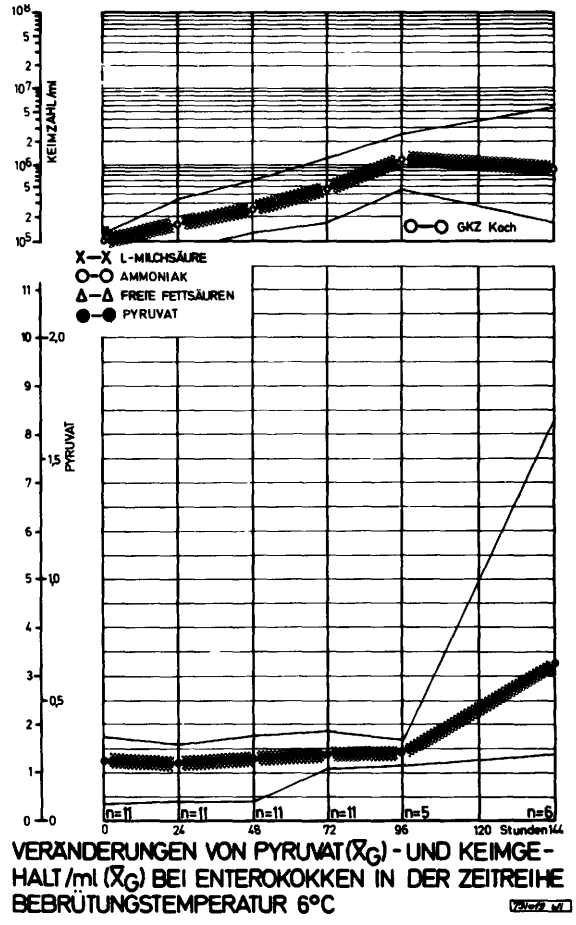
They are characterized by a glycolytic metabolic pattern at 9° C. The lactic acid concentration increases moderately after a 72-hours-incubation at 6° C. Lipolytic activities could not be detected. Deaminating enzymes have little activity, especially at 6° C. The pyruvate concentration follows the gradual increase of plate counts only slowly (see fig. 3.15 and 3.16).

Apart from their ability to form lactic acid, the enterococci contribute only relatively insignificantly to the metabolic processes in the substrate milk under psychrotrophic conditions.



CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES ENTEROCOCCI-INCUBATION TEMPERATURE 9°C

Fig. 3.15



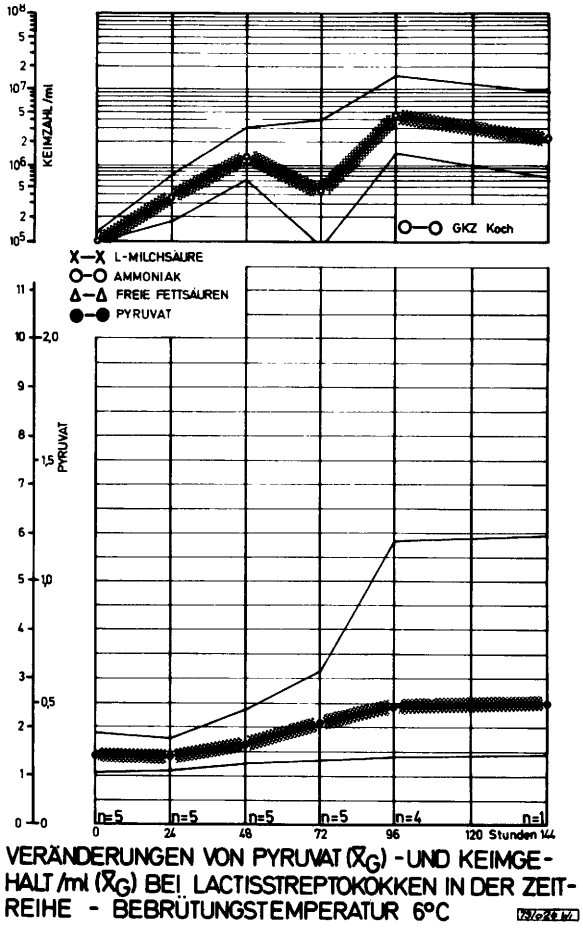
CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES ENTEROCOCCI-INCUBATION TEMPERATURE 6°C

Fig. 3.16

Lacti c-Streptococci

Glycolytic and proteolytic properties dominate in the metabolic pattern of lactic-streptococci. Already at an incubation temperature of 6° C a considerable production of lactic acid occurs within a plate count range of hygienic relevance. Not only at 6° C but also at 9° C the concentration of ammonia increases. The pyruvate content rises at 9° C more than at 6° C, parallel to the faster reproduction rate at 9° C (see fig. 3.17 and 3.18).

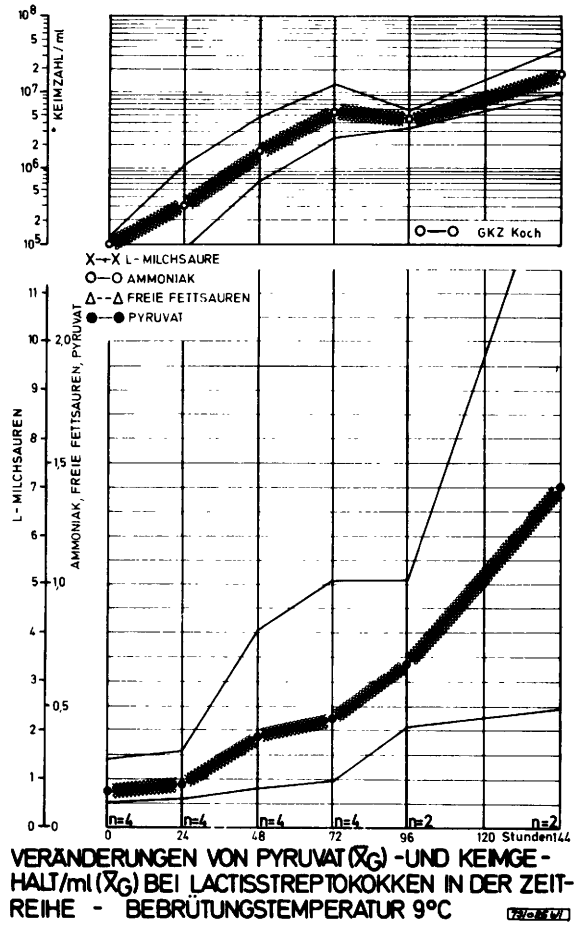
According to the higher germ count the pyruvate-content increases more due to the lactic-streptococci, than to the enterococci.



VERÄNDERUNGEN VON PYRUVAT ($\bar{X}_G$) -UND KEIMGEHALT/ml ($\bar{X}_G$) BEI LACTISSTREPTOKOKKEN IN DER ZEITREIHE - BEBRÜTUNGSTEMPERATUR 6°C

CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES LACTIC STREPTOCOCCI- INCUBATION TEMPERATURE 6° C

Fig. 3.17



VERÄNDERUNGEN VON PYRUVAT ($\bar{X}_G$) -UND KEIMGEHALT/ml ($\bar{X}_G$) BEI LACTISSTREPTOKOKKEN IN DER ZEITREIHE - BEBRÜTUNGSTEMPERATUR 9°C

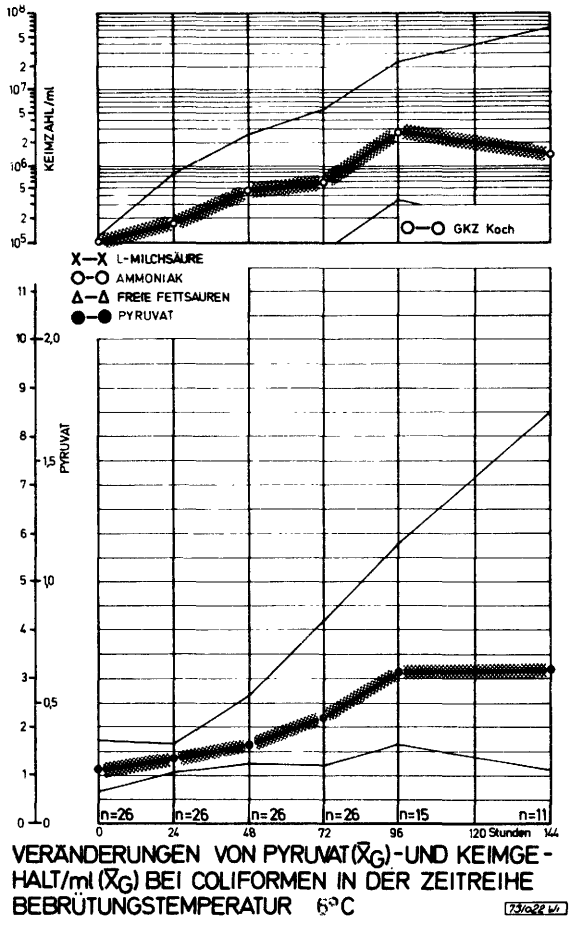
CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES LACTIC STREPTOCOCCI INCUBATION TEMPERATURE 9° C

Fig. 3.18

Coliforms

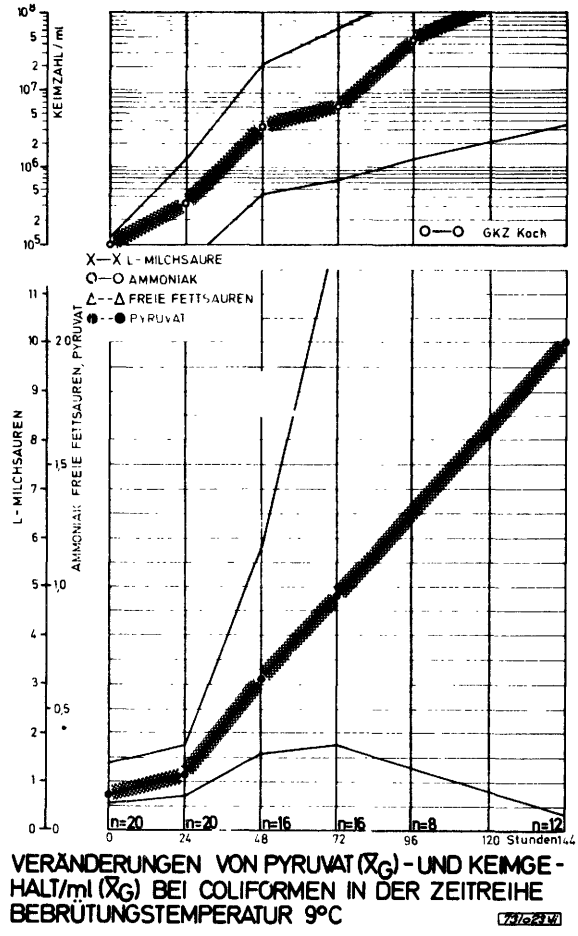
The coliforms have considerable reproduction rates and related metabolic activity under psychrotrophic growth conditions. Production of lactic acid could not be detected. The contents of free fatty acids and ammonia rise after a 4 day incubation, the latter gradually from the very beginning. At 9° C more ammonia is produced than at 6° C. The pyruvate concentration shows parallel behaviour to the plate count at 9° C (see fig. 3.19 and 3.20).

Within the heterogeneous group of coliforms the tendencies can only be shown rather coarse by as the variation of significant parameters is proportionately high.



CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES COLIFORMS-INCUBATION TEMPERATURE 6°C

Fig. 3.19



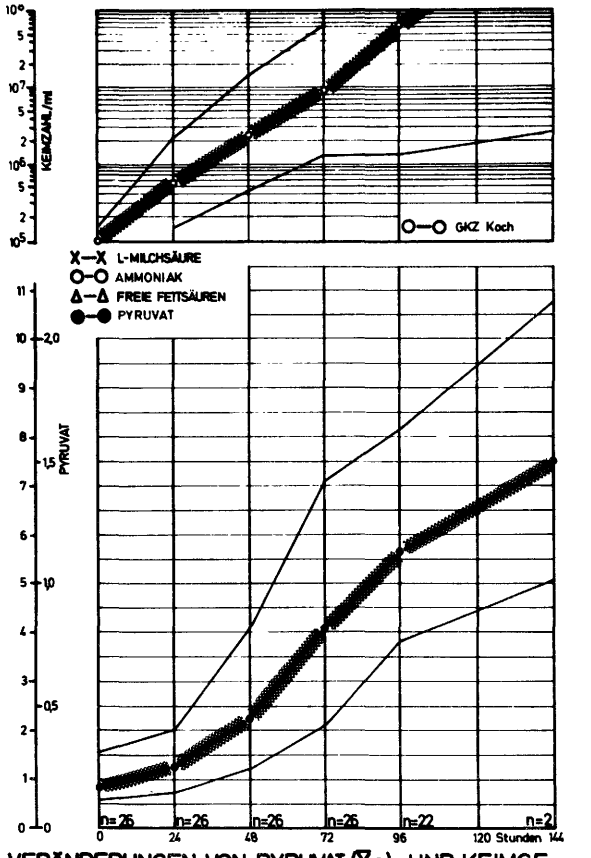
CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES COLIFORMS-INCUBATION TEMPERATURE 9°C

Fig. 3.20

Pseudomonads

Pseudomonads are very active under the applied conditions of incubation. As already observed for the coliforms, the pseudomonads also show no production of lactic acid as could be expected. The content of free fatty acids increases after a 72-hours and 96-hours-incubation respectively. The deamination-reactions are more distinct at 9° C than at 6° C. Pyruvate and plate count show significant progression (see fig. 3.21 and 3.22).

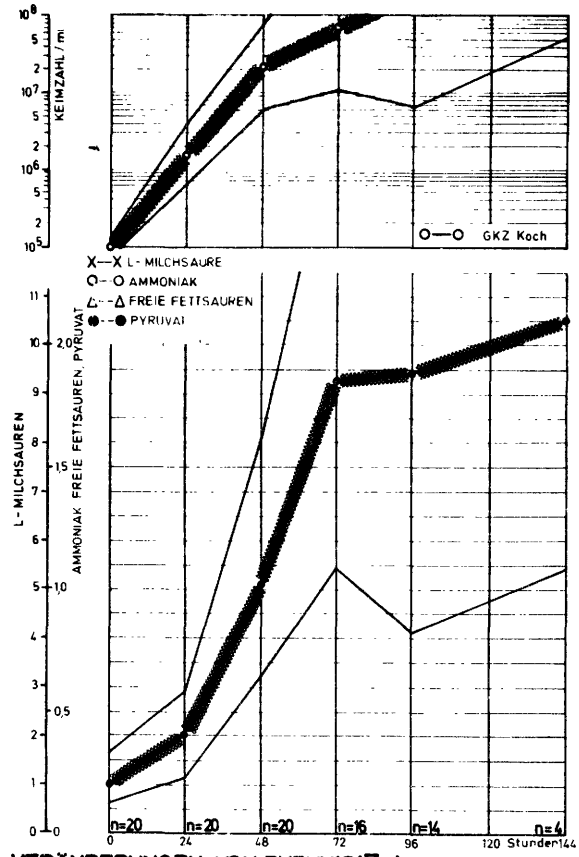
As already mentioned, also in the group of the coliforms the parameters under study show a wide spread in the ranges of higher concentrations. However it may be stated as a basic tendency, that catabolic activity is higher than that of coliforms and gram-positive cocci at an incubation-temperature of 6° C.



VERÄNDERUNGEN VON PYRUVAT ($\bar{X}_G$) - UND KEIMGEHALT/ml ($\bar{X}_G$) BEI PSEUDOMONADEN IN DER ZEITREIHE BEBRÜTUNGSTEMPERATUR 6°C

CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES PSEUDOMONADS-INCUBATION TEMPERATURE 6°C

Fig. 3.21



VERÄNDERUNGEN VON PYRUVAT ($\bar{X}_G$) - UND KEIMGEHALT/ml ($\bar{X}_G$) BEI PSEUDOMONADEN IN DER ZEITREIHE BEBRÜTUNGSTEMPERATUR 9°C

CHANGES OF PYRUVATE ($\bar{X}_G$) AND TOTAL COUNT IN TIME SERIES PSEUDOMONADS-INCUBATION TEMPERATURE 9°C

Fig. 3.22

3.3.6.3 Ability to form pyruvate from germ-groups, isolated from milk

With the exception of one strain of Klebsiella, all species and groups of microorganisms in milk, as far as they have been investigated, produce pyruvate; the 457 groups and species studied are represented in the following Table 3.1:

SPECIES, GENUS AND NUMBER OF BACTERIA STUDIED

Enterobacteriaceae	31	Bacilli	48
Escherichia	2	Bac. subtilis	1
E. coli	2	Bac. megatherium	1
E. liquefaciens	1	Bac. cereus var. mycoides	1
Citrobacter	5	Bac. cereus	1
Klebsiella	1		
Serratia	2	Micrococci	43
		Staph. epidermidis	1
Pseudomonades	87	Mc. candidus	3
Ps. taetrolens	6		
Ps. fragi	6	Others	
Ps. putida	4	Aeromonas hydrophilia	2
Ps. fluorescens	2	Aeromonas punctata var. caviae	1
		Alcaligenes marshallii	2
Streptococci	187	Alcaligenes	1
Sc. faecalis var. faecalis	3	Proteus	2
Sc. faecalis var. zymogenes	2	Sarcina flava	1
Sc. durans	2		
Sc. bovis	2		
Sc. lactis	3		
Sc. saccharolactis	2		

74/236 Wi

Table 3.1

The increase in pyruvate appears to depend exclusively on bacterial activity.

Number of microorganisms

Fig. 3.23 shows the results obtained from plate counts in raw milk free from microorganisms; each of these samples was inoculated with Pseudomonads, coliforms, enterococci and lactic streptococci and incubated at 6° C. The respective pyruvate values are also given.

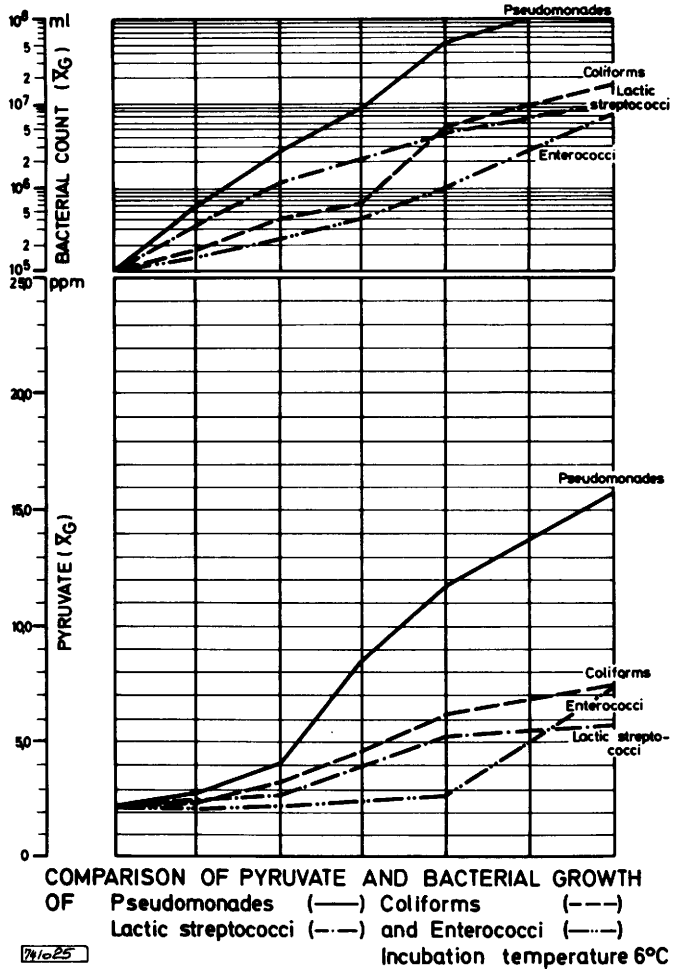


Fig. 3.23

Those groups of microorganisms which are highest in number at this temperature also produce the highest amount of pyruvate. Number of organisms and pyruvate are in correlate well under these conditions.

3.4 Results of assays under field conditions

The Institute for Hygiene has made an investigation in 9 milk collection areas between Kiel and Munich, in order to determine the bacteriological quality of milk at all stages - farm bulk milk, in the road tanker, in the dairy's balance tank before pasteurization, in the bulk tank after pasteurization, in despatch and in retail containers. A total of 5682 samples was examined by means of the enzymatic pyruvate determination and 4388 samples by means of the KOCH plate method with the international standard reference medium as well as plate counting in a computer image anaylzer. In two districts it was impossible to determine the bacterial number to be attributed to the respective pyruvate value: the outside temperature was too high and the transport distance too long to guarantee sufficient preservation by means of orthoboric acid - potassium sorbate. Therefore, Fig. 3.24 compares bacterial counts and pyruvate values of only 7 collection areas. One of the two remaining areas is represented in Fig. 3.29 in order to give evidence of systemic pyruvate accumulation, where the same milk is followed up directly from production to retail trade in packages. The other remaining area exhibits the lowest pyruvate values and is compared in Fig. 3.28 to that with the highest pyruvate pools.

Fig. 3.24 gives the results obtained from the entire sample material originating from 7 collection areas in 6 Federal Länder, divided into the different stages between production and consumption. Pyruvate values and bacterial counts were obtained from a cross-section through the various stages; however, it should be considered that milk samples between the stages may not necessarily be identical. This also explains the slight decrease of pyruvate values after pasteurization. This decrease is not due to any adverse effect of heat-treatment (see Fig. 3.10), but to the direct introduction of freshly supplied raw milk into the pasteurizer without being pre-stored in the dairy factory.

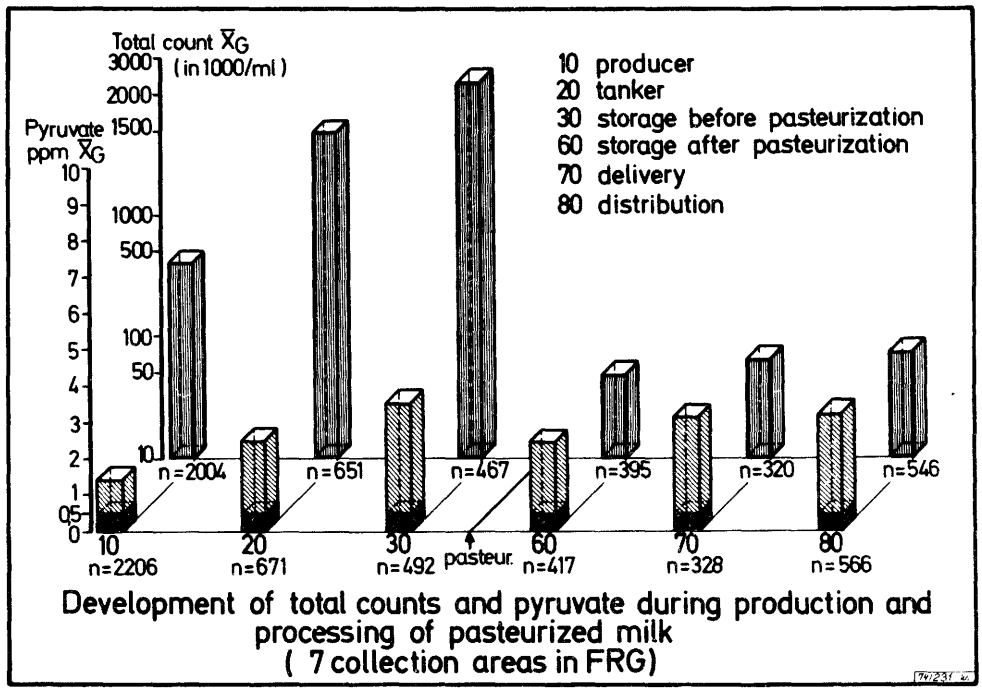
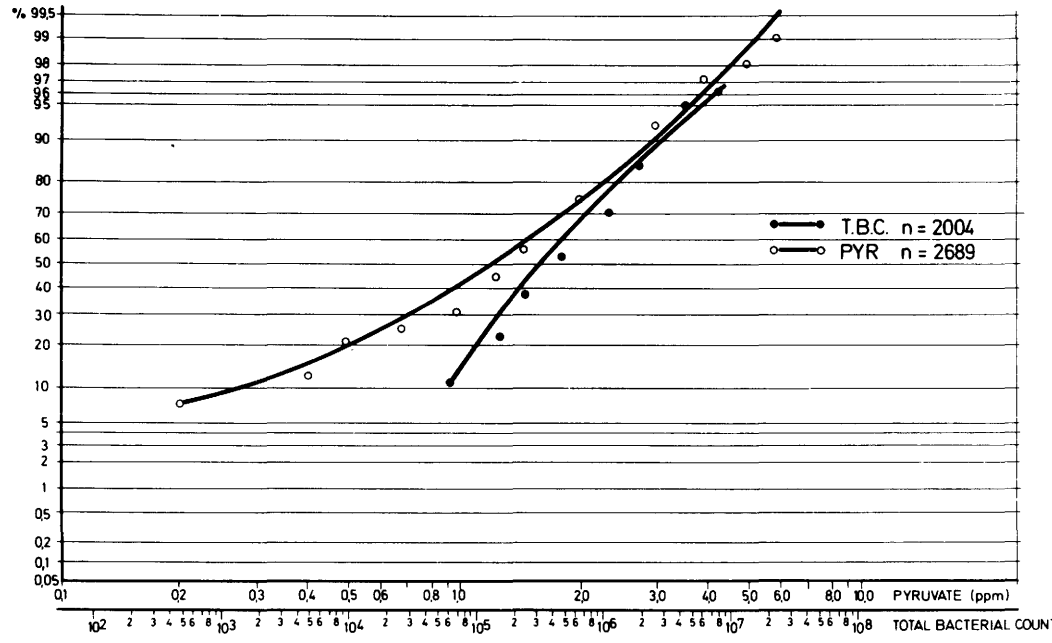


Fig. 3.24

Fig. 3.24 discloses a clear accumulation of pyruvate starting with 1.4 ppm in production up to 3.5 ppm prior to pasteurization. Market milk contains a geometric mean of 3.1 ppm pyruvate. The columns shown in the background give the respective bacterial counts: 410 000 cfU/ml during production and handling on the farm, 2 320 000 cfU/ml before pasteurization 49 000 cfU/ml after pasteurization, and 78 000 cfU/ml when milk reaches the consumer. It goes without saying, that the catabolic processes having taken place in raw milk are not considered when viewing the bacterial counts.

The high increase in bacteria in raw milk during transport and pre-storage in the dairy factory is striking. It is at this point that we should start our efforts to improve the bacterial situation of milk. The efforts, however, should include the milk producer, the more so as proper hygienic measures immediately after milking and during delivery of refrigerated milk could lead to bacterial counts of 10^4 cfU/ml and 10^5 cfU/ml respectively - and this without difficulties if all modern technical possibilities are used.

The following picture is obtained from raw milk in cumulative percentage distribution of pyruvate values and bacterial counts:

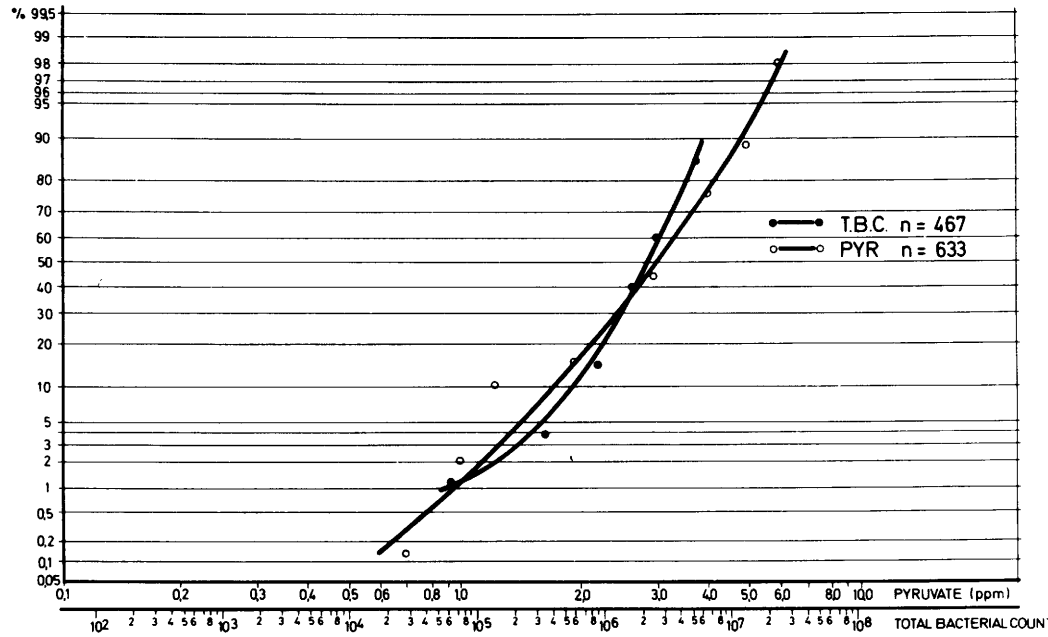


CUMULATIVE PERCENTAGE DISTRIBUTION OF TOTAL BACTERIAL COUNT (T.B.C.) AND PYRUVATE CONTENT DURING PRODUCTION AND HANDLING ON FARM
(9 sample collection areas in 7 Federal Länder)

Fig. 3.25

Raw milk from 90 % of all producers had pyruvate values below 2.7 ppm and bacterial counts below 2.7 millions/ml. All intermediate stages may also be seen from Fig. 3.25.

90 % of the samples taken from the balance tank before pasteurization had pyruvate values below 5.2 ppm and bacterial counts lower than 6.2 millions/ml as may be seen from Fig. 3.26.



CUMULATIVE PERCENTAGE DISTRIBUTION OF TOTAL BACTERIAL COUNT (T.B.C.) AND PYRUVATE CONTENT IN DAIRY FACTORY (balance tank before pasteur.)
(9 sample collection areas in 7 Federal Länder)

Fig. 3.26

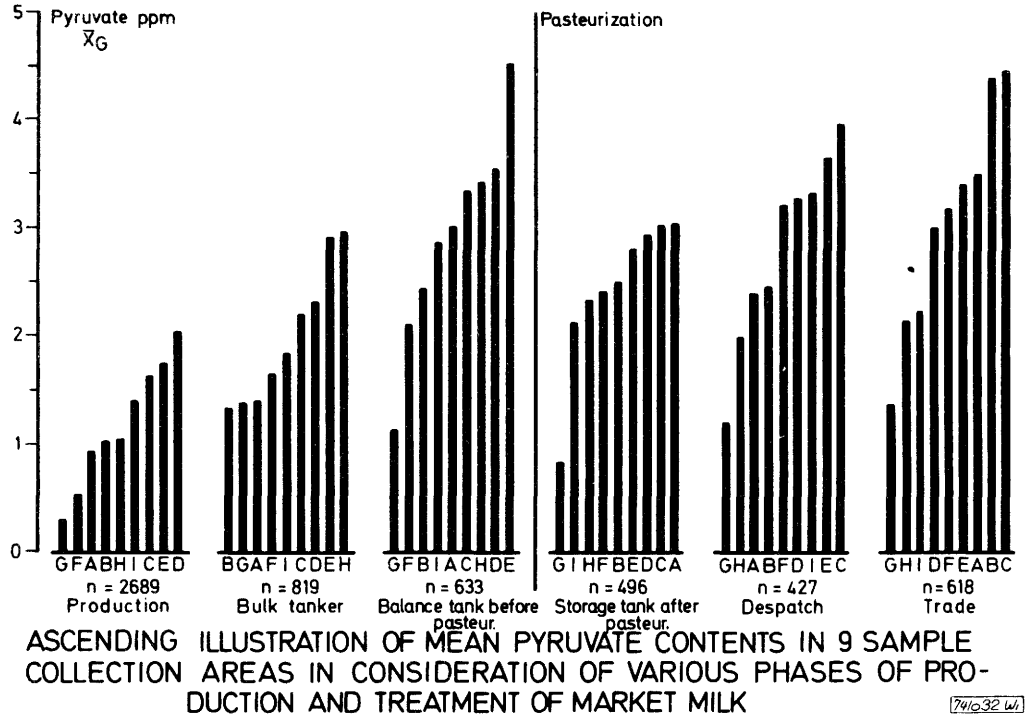
Pasteurization clearly reduces the number of living organisms, the reduction obtained from all dairy factories being around 97.8 %, and from seven selected dairies between 93.4 % and 99.5 % (see Table 3.2).

EFFECT OF PASTEURIZATION IN 7 DAIRIES

	Dairy							Total
	1	2	3	4	5	8	9	
Total counts in balance tank before pasteurization ($\bar{X}_G$ in 1000)	913	2062	3451	2308	2556	4083	1393	2321
n	16	14	12	36	121	128	140	467
Total counts in bulk tank after pasteurization ($\bar{X}_G$ in 1000)	60	40	29	11	60	100	41	52
n	15	14	12	36	73	117	128	395
Effect of pasteurization (%)	93,4	98,1	99,2	99,5	97,7	97,6	97,1	97,8

73/135 Wi

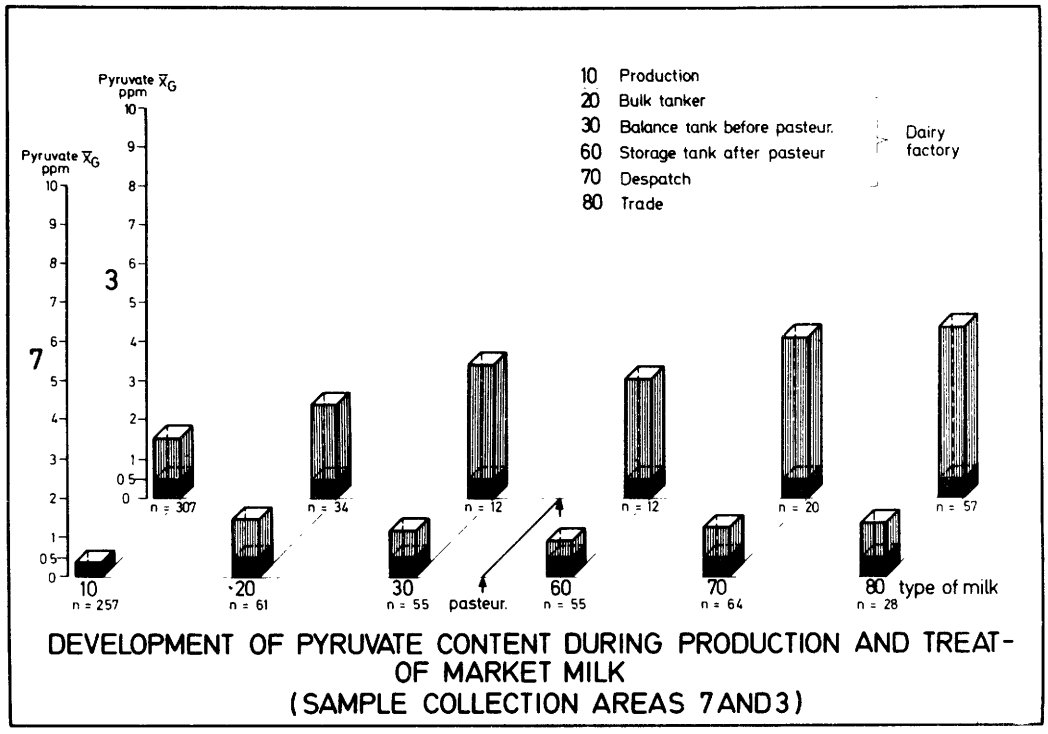
The following diagram sets out pyruvate values in relation to the different stages of production, handling and treatment of milk, the dairy factories being classified and each of them given a letter of the alphabet.



ASCENDING ILLUSTRATION OF MEAN PYRUVATE CONTENTS IN 9 SAMPLE COLLECTION AREAS IN CONSIDERATION OF VARIOUS PHASES OF PRODUCTION AND TREATMENT OF MARKET MILK 7410/32 W1

Fig. 3.27

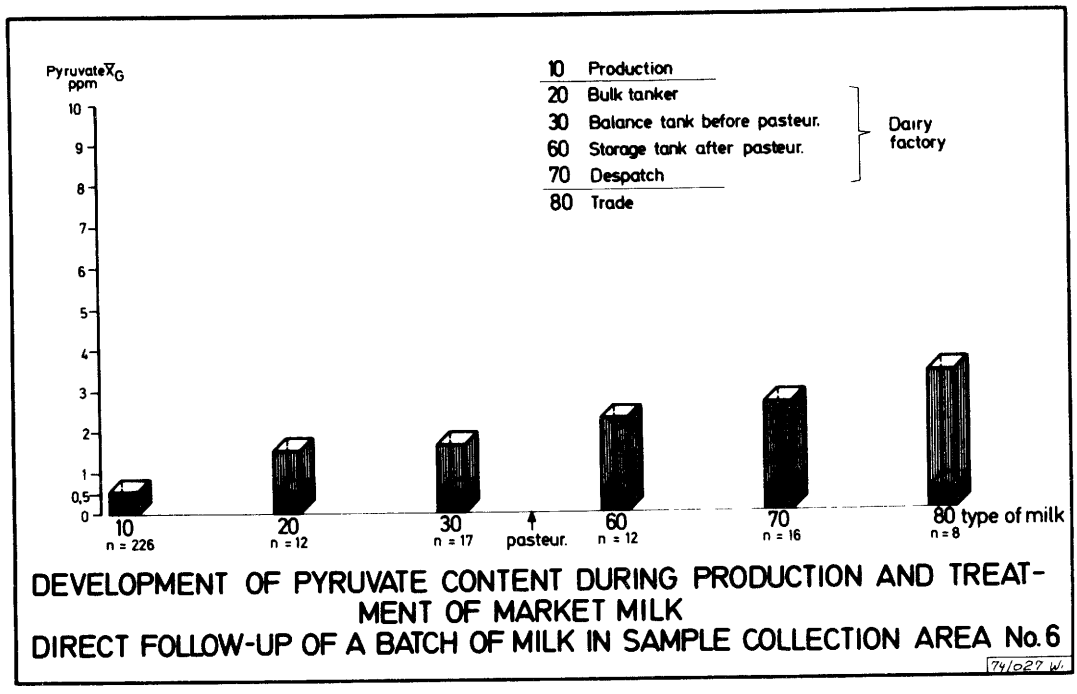
The diagram clearly shows a varying bacteriological quality of milk in the different areas, but there is also a certain variation in the changes between the different stages of treatment and handling of milk within the dairy factories. The range of pyruvate values from the most favourable to the most unfavourable was 1 : 7 within the stage of milk production on the farm, 1 : 4 before pasteurization, 1 : 3 at the retail stage. Corresponding relations were obtained for the colony counts. The differences obtained between the different areas of distribution are particularly impressive and may be seen from the following diagram:



DEVELOPMENT OF PYRUVATE CONTENT DURING PRODUCTION AND TREAT-
OF MARKET MILK
(SAMPLE COLLECTION AREAS 7AND3)

Fig. 3.28

The milk available from one collection area was followed up directly over all stages from production to pasteurization, i.e. without the intervening admixture of freshly supplied raw milk at the pasteurizer.



DEVELOPMENT OF PYRUVATE CONTENT DURING PRODUCTION AND TREAT-
MENT OF MARKET MILK
DIRECT FOLLOW-UP OF A BATCH OF MILK IN SAMPLE COLLECTION AREA No. 6

Fig. 3.29

The results obtained from pyruvate determination confirm the experience gained during the previous enquiry: the efforts made to reduce saprophytic contamination of milk are of varying efficiency in the individual areas of distribution.

As far as milk production is concerned this appears to be due to the fact that the common system for grading and payment of milk does not encourage the farmer to reduce the bacterial count of areas appears to be due to some isolated initiative of individual dairy factories or advisory services. Approximately 10 % of the milk producers contribute, to an unreasonably great extent, to an impairment of the bacteriological quality of milk which may be seen from the following 2 Tables where the pyruvate contents as well as total bacterial counts are given.

CHARACTERISTICS OF CUMULATIVE PERCENTAGE DISTRIBUTION OF PYRUVATE CONCENTRATIONS (ppm) IN RAW MILK (DELIVERED TO 9 DAIRIES)

Cumulative percentage	1	2	3	4	5	6	7	8	9
10	0,5	0,5	1,1	1,3	1,2	0,2	0,2	0,3	0,7
20	0,5	0,5	1,1	1,5	1,4	0,3	0,2	0,7	1,1
30	0,6	1,1	1,4	1,8	1,5	0,4	0,2	0,9	1,2
40	1,1	1,1	1,6	2,0	1,7	0,5	0,2	1,2	1,4
50	1,1	1,1	1,6	2,1	1,8	0,6	0,3	1,4	1,6
60	1,1	1,1	1,9	2,3	2,1	0,7	0,3	1,6	1,7
70	1,4	1,6	2,1	2,6	2,2	0,9	0,4	1,8	1,9
80	1,6	2,1	2,4	3,1	2,4	1,2	0,6	2,1	2,2
90	1,9	2,6	3,1	3,6	2,7	1,5	0,8	2,8	2,6
99	5,5	8,5	7,5	5,8	>10,0	2,9	2,3	4,7	5,5
$\bar{x}_G$	0,9	1,0	1,6	2,0	1,8	0,6	0,3	1,1	1,4

74/039 W

Table 3.3

CHARACTERISTICS OF CUMULATIVE PERCENTAGE DISTRIBUTION OF TOTAL BACTERIAL COUNT (in 1000) IN RAW MILK (DELIVERED TO 9 DAIRIES)

Cumulative percentage	1	2	3	4	5	6	7	8	9
10	62	62	62	124	62	--	--	124	124
20	62	124	124	248	124	--	--	248	124
30	124	124	248	310	124	--	--	434	248
40	124	186	434	434	248	--	--	620	310
50	186	310	620	744	372	--	--	930	496
60	248	434	992	1.178	558	--	--	1.240	620
70	310	682	1.550	1.612	806	--	--	1.488	930
80	434	1.116	2.480	2.418	1.178	--	--	1.984	1.302
90	1.054	2.046	6.200	3.906	2.046	--	--	2.790	2.108
99	>6.200	>6.200	>6.200	>6.200	>6.200	--	--	>6.200	>6.200
$\bar{x}_G$	162	277	551	630	288			731	402

74/041 Wi

Table 3.4

Corresponding conditions also apply to the lactic acid content which gives more exact results in insufficiently cooled or uncooled milk than reduction tests. Table 3.5 shows that acidity values may well be differentiated when only slight variations occur.

LACTIC ACID CONTENT WITHIN THE VARIOUS REDUCTION (GRADES)

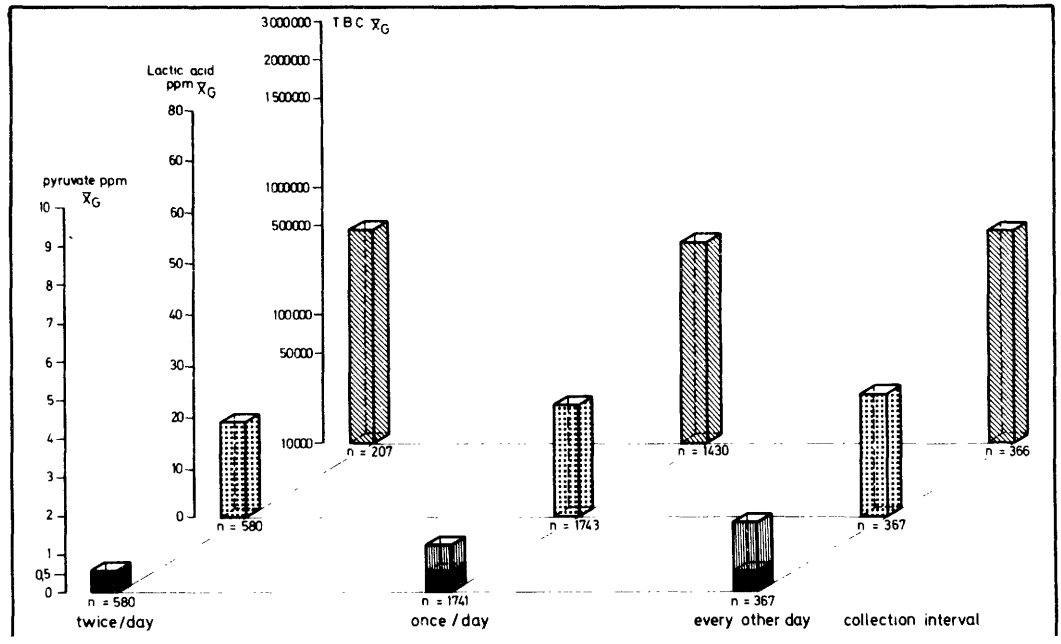
Reduction phase	n	$\bar{x}_G$	s_g
1	1837	33,6	1,46
2	165	59,3	1,79
3	43	106,5	2,08

70/040 Wi

Table 3.5

As long as milk is insufficiently refrigerated, it should be examined for acidity, particularly as its analytical determination is based on the same principles as that for pyruvate determination (see Fig. 3.5).

On the other hand, sufficient cooling is required if the bulk raw milk is collected once a day or every other day. While bacterial count and acidity hardly increase at these collection intervals, pyruvate accumulation is clearly observed at collection intervals of twice a day, once a day, and every other day. For the respective collection intervals, the pyruvate content ranges from 0.6 ppm through 1.2 ppm to 1.8 ppm (see Fig. 3.30).



VARIATIONS IN PYRUVATE CONCENTRATION, LACTIC ACID CONTENT AND TOTAL BACTERIAL COUNT IN RELATION TO COLLECTION INTERVAL (9 SAMPLE COLLECTION AREAS IN 7 FEDERAL LÄNDER)

Fig. 3.30

The unchanged bacterial counts appear to be due to the fact that, at all collection intervals, the plates had been incubated for 72 hours at 30° C and, thus, the psychrotrophic flora was not selectively determined.

Seen generally the bacterial counts increase considerably in the bulk road tanker and in the prestorage tank at the dairy factory. An improvement in organizational and technical conditions is likely to reduce most effectively the microbial contamination of milk in road tankers and prestorage tanks.

The effect of pasteurization on the bacterial count indicates that milk is heated rather similarly in the different dairy factories, though one of them appears to operate at the upper, and the other at the lower limit of the peritted time/temperature conditions.

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4. The suitability of the pyruvate-pool for the estimation of the keeping quality of milk (TOLLE and HEESCHEN)

The problem of the interrelations between pyruvate content and the keeping quality of milk is of direct practical interest. The uncertainties in this question involve the definition of freshness, spoilage and keeping quality. These aspects, seen bacteriologically include species, number, growth rate, and the appearance of metabolic products of the contaminating microorganisms. Microbial and chemical processes, occurring in milk, show significant influence on the organoleptic quality of milk. Provided that there are no pathogens, this is the most important criterion for quality. For the consumer, milk can be kept until changes can be detected by the senses. Organoleptic criteria of a food differ from man to man and country to country, and are formed by education, tradition, environmental influences and habits.

The following is a short survey of the factors, which influence the organoleptic properties of milk and its keeping quality. Describing keeping quality and organoleptic characteristics the term "flavour" will be used. The word flavour describes most sensory impressions, evident in the oral cavity. It is therefore the combined impression of taste and smell.

The flavour of fresh milk is difficult to describe as there is a low level of smell and taste. The slight sweet and salty character is produced by lactose and chlorides. Trace compounds of low molecular weight form the characteristic flavour. Many compounds in milk, when present in higher amounts, produce an undesired off-flavour.

4.1 The concept of keeping quality

4.1.1 The flavour quality of milk and influencing factors

Apart from microbial factors, milk is exposed to a number of other environmental influences from milking to consumption. The following table shows the non-microbial effects on the flavour of milk.

NON-MICROBIAL INFLUENCES ON THE MILK-FLAVOUR

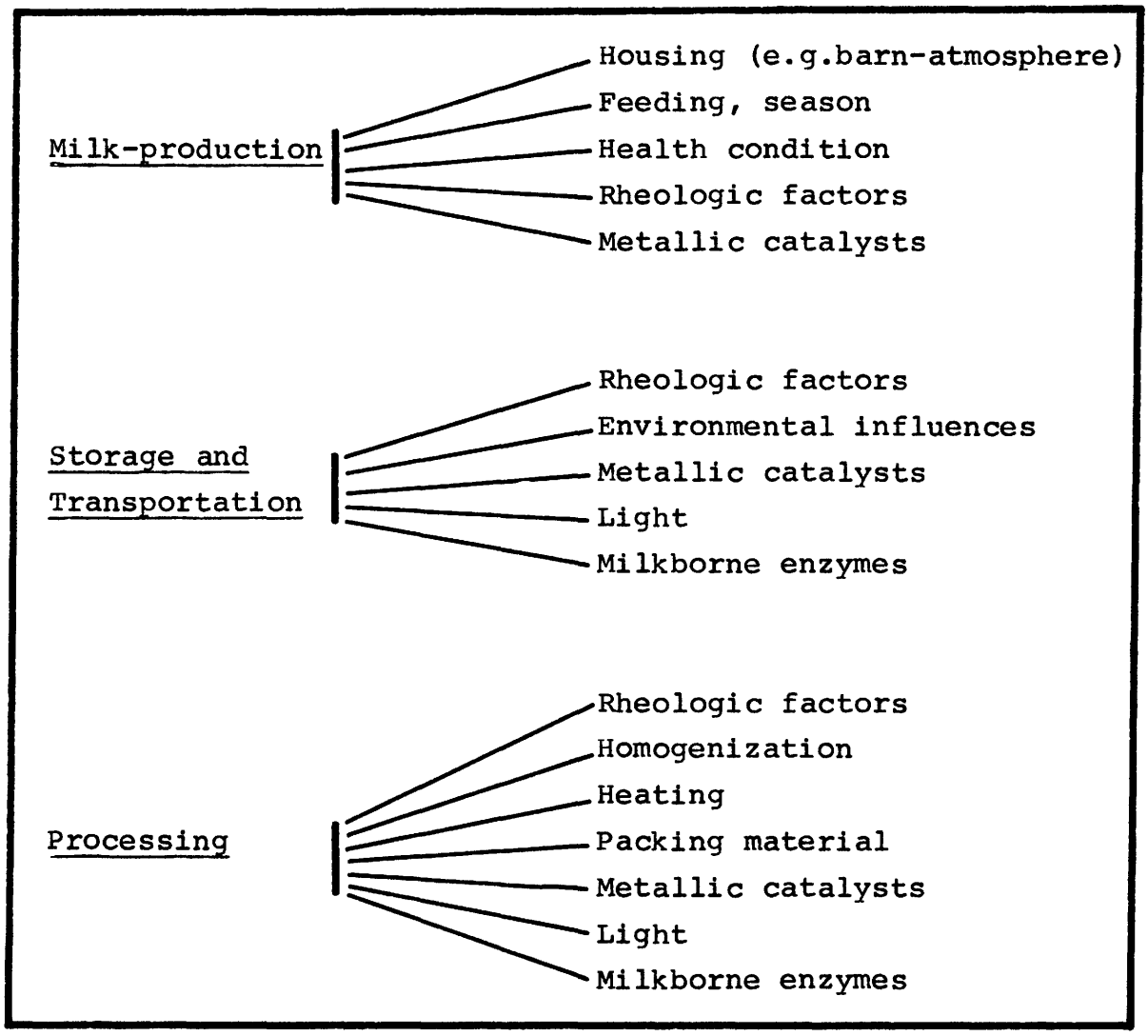


Table 4.1

The influences come from feeding, season, health condition of the cow and the exposure of the milk to the barn-atmosphere. Further changes in the flavour are caused by metals as catalysts, mechanical treatment and milkborne enzymes, especially the lipolytic enzymes. These factors, difficult to standardize and control, are excluded in the following. Nevertheless they show the difficulties, naturally associated with the search for a control parameter for microbial changes of milk.

The most important contribution to the microbial factors are species and number of contaminating microorganisms.

The question of the influence of bacterial processes in raw milk on the keeping quality of pasteurized milk is contradictory. Pasteurized milk, produced from severely contaminated milk shows despite a relatively low-recontamination with microbes, a bitter off-flavour within a short time. Poor, contaminated raw milks usually produce an off-flavour, characterized as "oxidation-taste", commonly caused by non-microbial effects.

Without any doubt, the contamination of milk after pasteurization is the main source of microbe-produced off-flavours. Next in order of importance are thermoduric organisms. In addition, products from microbial metabolism remain after pasteurization and may produce off-flavours themselves or act as precursors for a further break-down by the recontaminating flora. Last but not least heat-stable microbial enzymes, especially lipases and proteases of the psychrotrophic flora may remain active after pasteurization. Even parts of heat deactivated and digested bacteria may produce defects in flavour.

Clear flavour differences correlating with the germ population or with distinct species of bacteria, are observed for relatively high numbers of microbes. A malty flavour, produced by Streptococcus lactis var. maltigenes, can be observed at 10^7 - 10^8 microorganisms/ml. A fruity flavour, produced by Pseudomonas fragi can be smelt at 5 x 10^7 microorganisms/ml. The following two comprehensive tables show the microbial influences on the milk-flavours.

MICROBIAL INFLUENCES ON THE MILK-FLAVOUR

species	plate count/ml	flavour defects
Psychrotrophic microbes	$5 - 20 \times 10^6$	polluted, bitter, rancid, fruity
Sc.lactis var. maltigenes	$10^7 - 10^8$	malty
Ps.fragi	5×10^8	fruity
Alcaligenes viscolactis	5×10^6	viscous

Table 4.2

OFF-FLAVOURS IN MILK FROM BACTERIAL ORIGIN

Compounds produced	Off-flavour	M i c r o b e s
Free fatty acids	rancid, soapy	psychrotrophic microbes
Lactic acid	sour	Streptococci, Lactobacillae Enterobacteriaceae
Formic acid	penetrating	Enterobacteriaceae
Alcohols		Lactis-Streptococci, Ps. fragi Enterobacteriaceae, Clostridiae, Pediococcus
Aldehydes		Lactis-streptococci, Lb.casei, Achromobacter, Enterobacteriaceae, Pediococcus
Esters	fruity, aroma of cream or fungi	Lactis streptococci Ps.fragi
Ketones		Lactis streptococci
N-containing compounds	bitter, spoiled, mouldy, penetrating, caustic, sweet, sour	psychrotrophic microbes
S.containing compounds	mouldy, malty, like garlic	Enterobacteriaceae, Propionic acid bacteria anaerobic proteolytics
Lactones		yeasts, fungi, Sarcina lutea, Saccharomyces

Table 4.3

The tables make evident, that many microbial metabolic products can alter the milk flavour.

4.1.2 Microbiological methods for the estimation of keeping quality

For the estimation of keeping quality of pasteurized milk different microbiological methods have been described and used in practice (see Table 4.4).

MICROBIOLOGICAL METHODS FOR THE QUALIFICATION OF THE KEEPING QUALITY OF MILK

method	statement	correlation to keeping quality
Total plate count, number of psychrotrophic germs	quantification of the number of contaminating microbes without respect to species, and activity	indistinct
Coliform microbes	quantification of species and number of contaminating microorganisms without respect to their activity	indistinct
Selective quantification of gram-negative germs, modified catalase-test	quantification of germs, active under cooling-conditions without respect to the actual activity	distinct between the oxidase-positive germs and the off-flavour "old" and "polluted"
MOSELEY-keeping-quality-test	quantification of the dynamics of microbe-reproduction by a 5day preincubation at 7.2° C	relatively distinct
Clot-on-boiling-test, alcohol-test	quantification of alterations, caused by lactic-acid-production	positive results only at severe organoleptic changes

Table 4.4

From all procedures, based on a single determination such as the total germ count or the number of psychrotrophic or coliform microorganisms, only a poor correlation with the keeping quality could be derived. Even the selective count of gram-negative microorganisms correlated only with the production of distinct off-flavours. All these results only show the number of contaminating microbes without permitting any conclusion as to their actual activity.

The clot-on-boiling-test and the alcohol-test primarily show changes due to the production of lactic acid. Normally these tests show positive results under psychrotrophic conditions, when organoleptic alterations are evident.

The MOSELEY-keeping-quality-test is based on the determination of total microbe-numbers before and after a five-day-incubation at 7.2° C. In that space of time a reproduction of the psychrotrophic recontamination flora is possible. Correlations with keeping quality are reported to be relatively close.

Viewing the different methods makes it clear, that only the procedures based on the dynamics of the reproduction of microbes give reliability.

4.2 Pyruvate and keeping quality

Within the objectives of this investigation the following questions should be answered:

1. Can the keeping quality of milk be estimated by the pyruvic-acid-determination before and after incubation?.

2. To what extent does the influence of microbial processes in raw milk affect the keeping quality of pasteurized milk?

3. At what pyruvate content can a pasteurized milk be characterized as spoiled?

4.2.1 Material and methods

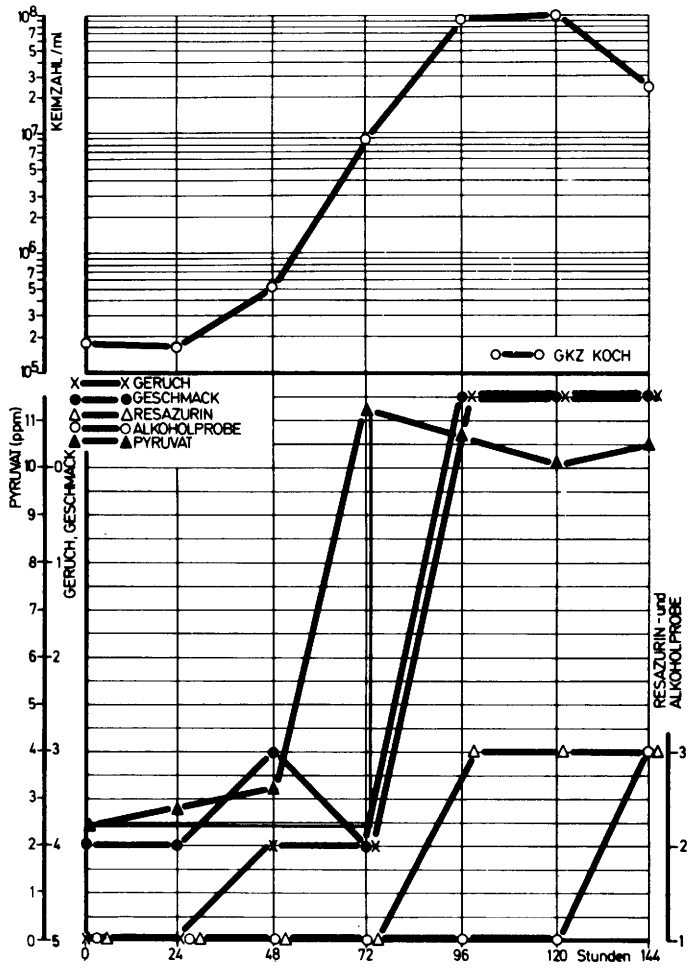
The determinations were carried out in samples of pasteurized milk from three different dairy plants in Schleswig-Holstein. The samples were stored at 10° C. The determination of pyruvate-content, lactic acid, ammonia, FFA, number of microbes and additional clot-on-boiling-test, alcohol-test and resazurin tests were carried out daily. Furthermore, organoleptic examinations were done. The latter were classified within a five-point-scheme. By definition a milk, receiving four points for the estimation of its taste, was acceptable. Three or fewer points confirmed in a following test classified the sample as below the borderline for keeping quality (see Table 4.5).

FEATURES OF REALIZED INVESTIGATIONS

S a m p l e s	T e s t	M e t h o d
Fresh pasteurized market milk samples from three different dairy plants	pyruvate lactic acid	enzymatic determination in the AutoAnalyzer System
	ammonia, FFA	AutoAnalyzer System
	total plate count	IDF-methodology
Incubation-temperature	clot-on-boiling-test	5 ml milk heated to 100° for 5 min
	alcohol-test	1 ml milk + 2 ml ethyl-alcohol (68%)
10° C	resazurine-test	1 hour at 37°C
	odour, taste	team-judgement according to the 5-point-scheme of the DLG (German Agricultural Organization) 5 points - extraordinary quality 4 points - good quality 3 points - slight defects 2 points - clear defects 1 point - distinct defects 0 point - severe defects

Table 4.5

In the following figure the principles and parameters of the determinations are shown for a pasteurized milk sample. Number of microbes and pyruvate content show the expected conformity. Before the milk was spoiled according to the given definitions, a rapid increase of pyruvic acid was observed.



74/241

PROGRESSION OF PYRUVATE-CONTENT, PLATE COUNT AND CHANGES IN THE RESAZURIN-, ALCOHOL-, AND SENSORY TEST IN A MARKET MILK (INCUBATION-TEMPERATURE 12°C)

Keimzahl/ml	=	plate count/ml
Pyruvat	=	pyruvate
Geruch	=	odour
Geschmack	=	taste
Resazurin	=	resazurin-test
Alkoholprobe	=	alcohol-test
GKZ KOCH	=	total plate count KOCH

Fig. 4.1

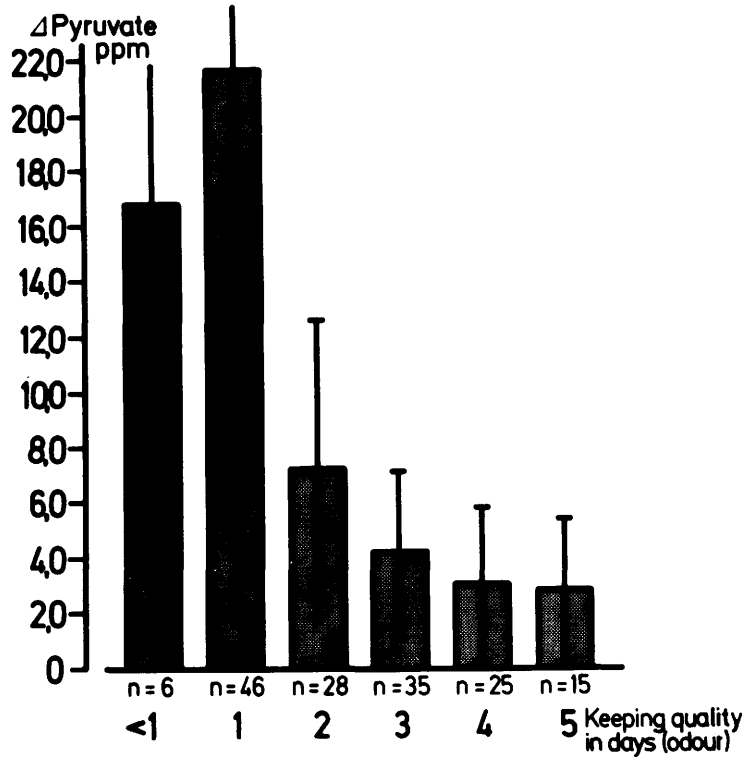
For the estimation of the spoilage-risk and bacterial activity, the pyruvate-difference-value, derived from 72-hours-incubation, was correlated with the spoilage time.

The determination of biochemical parameters was carried out by continuous flow analysis in the AutoAnalyzer System. The bacterial counts were done by the IDF-method. All other measurements were carried out with internationally approved procedures.

4.2.2 Results

4.2.2.1 Pyruvate-difference-value and keeping quality of milk

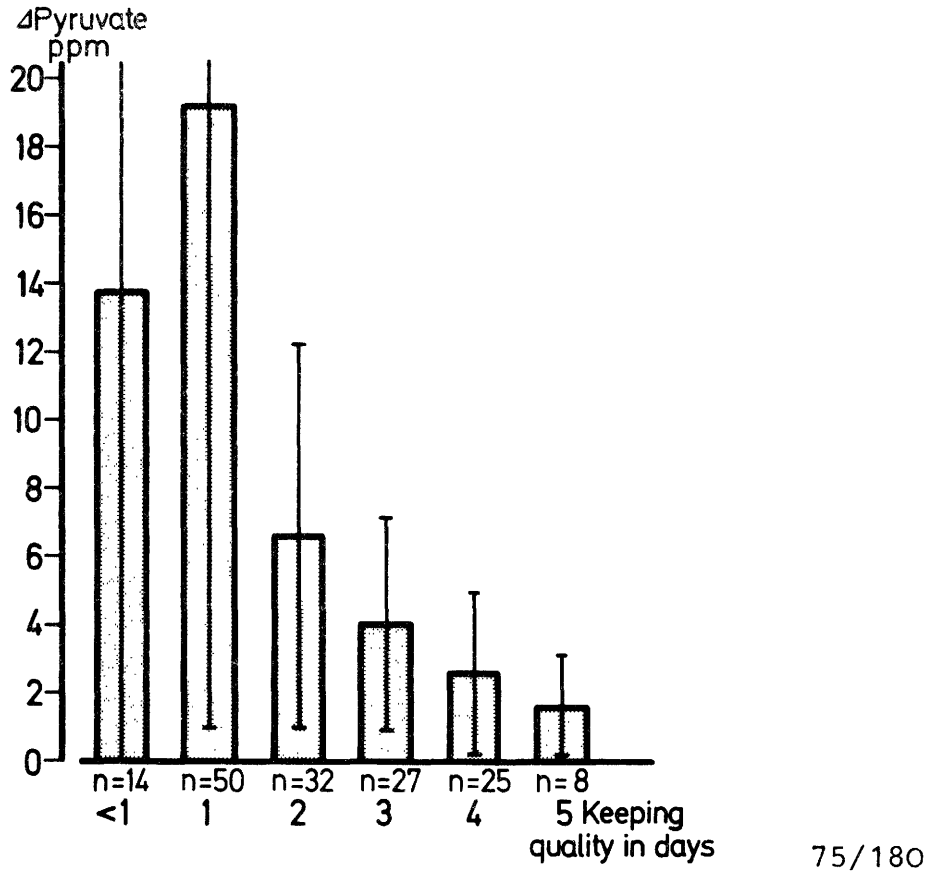
In the scope of this investigation the so-called pyruvate-difference-value (pyruvic-acid-change-value; difference between pyruvate contents before and after incubation) was correlated with the keeping time in days.
In the following figures the means of pyruvate-difference-values in samples of equal keeping time are set out.



75/223

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (ODOUR) ($\bar{X}_A$)

Fig. 4.2



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) ($\overline{X}_A$)

Fig. 4.3

With exception of the first column, the following tendencies can be seen:

The higher the pyruvate-difference-value, which means the higher the total bacterial acitivity, the poorer the keeping quality. A keeping time of only one day is observed with a pyruvate difference-value of 19 ppm. On the contrary keeping time of 5 days relates to a pyruvate-difference-value of 1.6 ppm. Milk, classified as spoiled in the preliminary organoleptic examination, showed several samples with a high bacterial activity. The

greater part of these milk samples however featured only a low pyruvate-difference-value. The spoilage of these samples is mainly due to non-microbial influences. Therefore the mean pyruvate-difference-value of these samples was lower than 14 ppm. It cannot be excluded, that also in the samples, already being spoiled on the first day, a primary and very intensive glycolysis began. The data are set out in Table 4.6.

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (incubation 72h/10°C)WITH REGARD TO THE KEEPING QUALITY (TASTE)

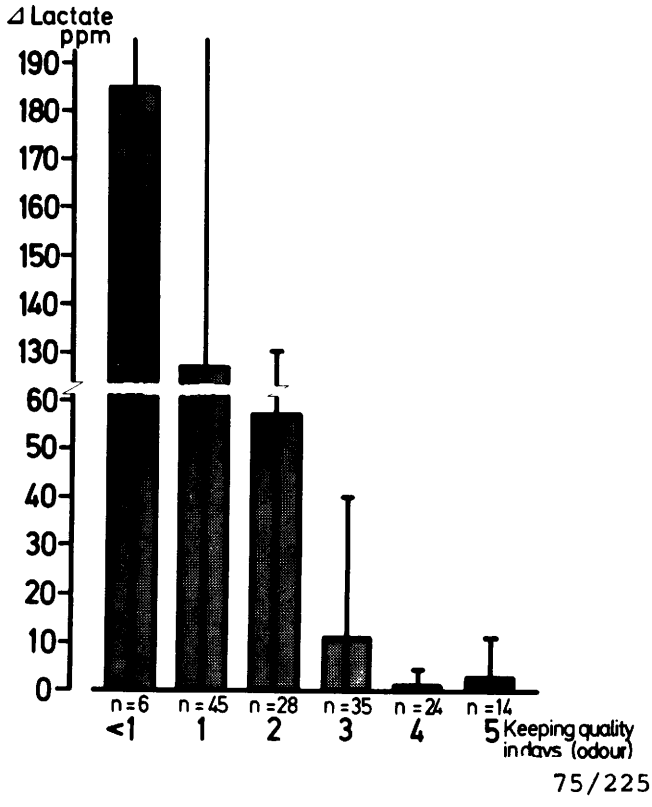
Keeping quality in days	Difference value of pyruvate (ppm)		
	n	$\bar{x}_A$	s_a
<1	14	13,8	21,01
1	50	19,2	18,19
2	32	6,6	5,61
3	27	4,0	3,12
4	25	2,6	2,35
5	8	1,6	1,54

75/167 Wi

Table 4.6

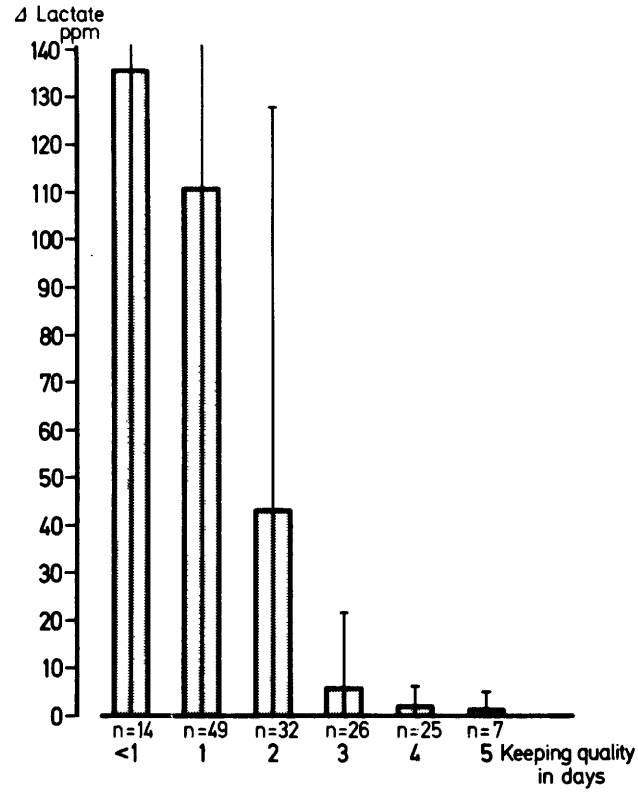
This situation is obvious from the lactic-acid-difference-value of the same samples, shown in Fig. 4.4 and 4.5. The negative correlation of lactic-acid-difference-value (in ppm) and keeping quality duration (in days) shows a good correlation. Due to fundamental considerations lactic acid seems to be a less suitable parameter than pyruvate, as with it an exclusively psychrotrophic flora (pseudomonads) cannot be included. The variation within the groups is naturally rather high due to the numerous nonmicrobial influences and the relatively long space of time between the determinations. Nevertheless the results

permit the definite statement, that pasteurized milk can be kept longer, the lower its pyruvate-difference-value. Under special microbiological conditions (processing of uncooled milk) the lactic-acid-difference-value could also be useful.



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF LACTATE (INCUBATION 72 h/10°C) WITH REGARD TO THE KEEPING QUALITY ($\bar{x}_A$)

Fig. 4.4



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF LACTATE (INCUBATION 72 h/10°C) WITH REGARD TO THE KEEPING QUALITY ($\bar{x}_A$)

Fig. 4.5

The data measured see Table 4.7.

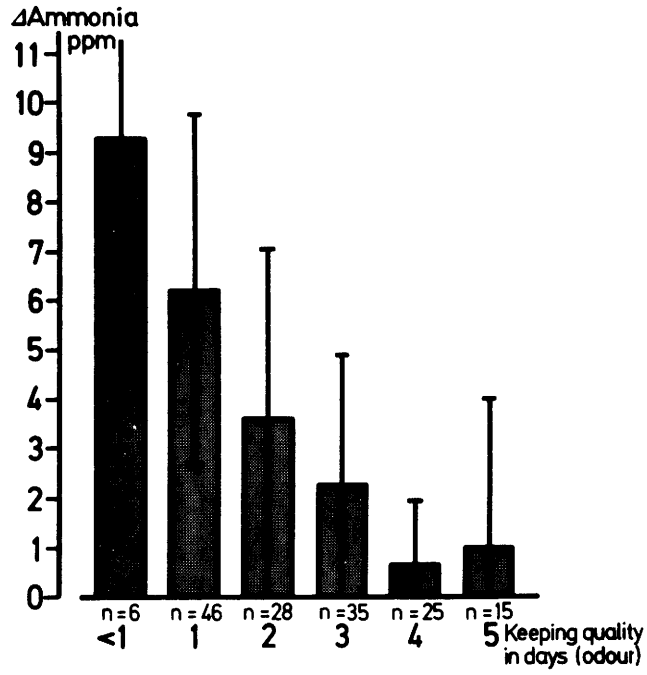
RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF LACTATE (incubation 72h/10°C) WITH REGARD TO THE KEEPING QUALITY (TASTE)

Keeping quality in days	Difference value of lactate (ppm)		
	n	$\bar{X}_A$	s_a
< 1	14	136	250, 7
1	49	112	263, 7
2	32	46	82, 3
3	26	6	15, 7
4	25	2	6, 5
5	7	2	3, 7

75/170 Wi

Table 4.7

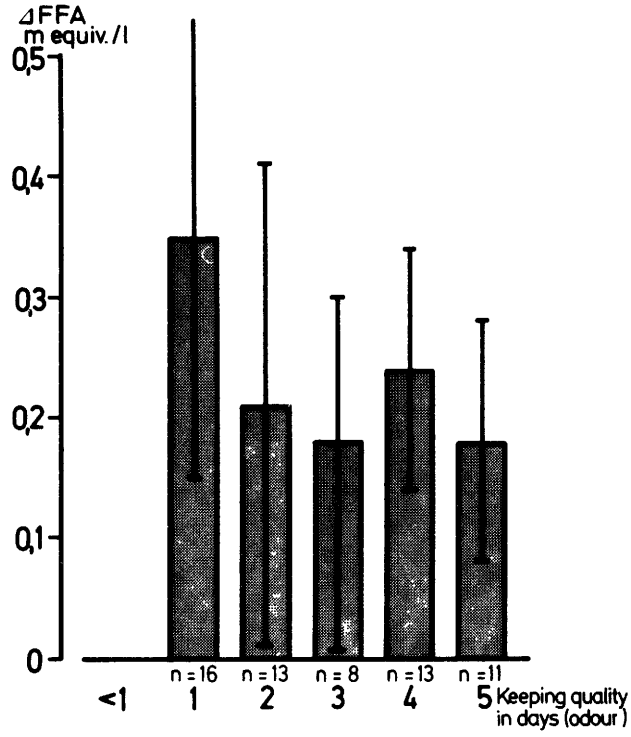
The difference-values of ammonia and free fatty acids show a comparatively uncertain figure in their relationship to the keeping quality (taste and odour). Especially the free fatty acids show the dominating power of non-microbial influences.



75/233

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF AMMONIA (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (ODOUR) ($\bar{X}_A$)

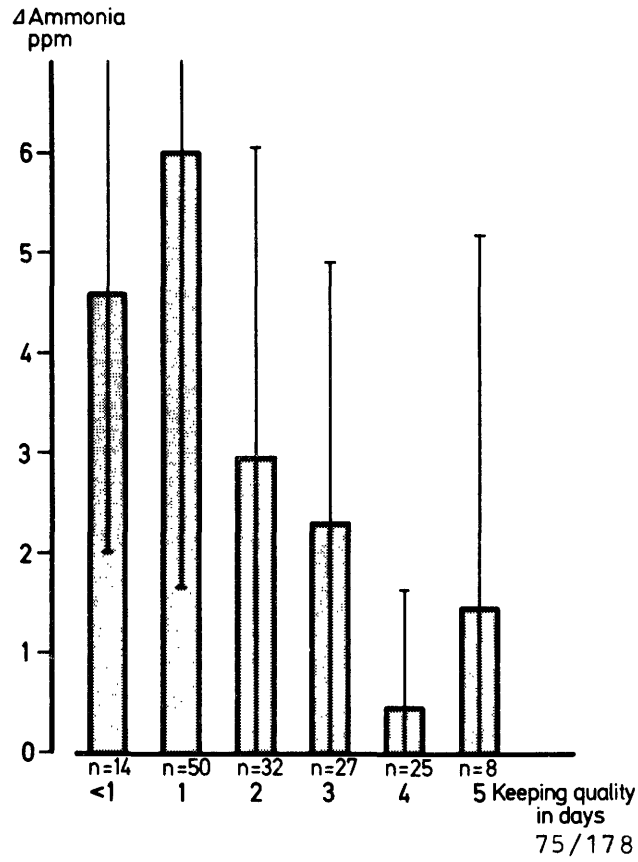
Fig. 4.6



75/224

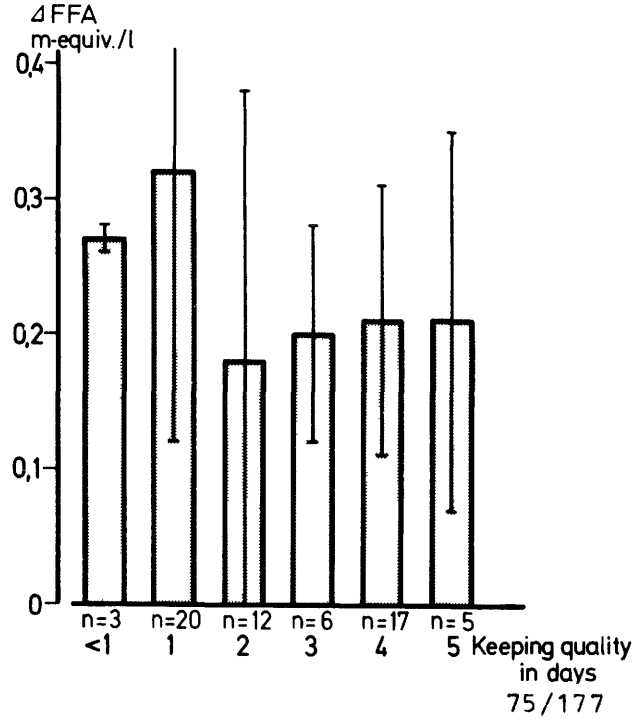
RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF FFA (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (ODOUR) ($\bar{X}_A$)

Fig. 4.7



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF AMMONIA (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) ($\overline{X}_A$)

Fig. 4.8



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF FFA (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) ($\overline{X}_A$)

Fig. 4.9

The data measured for ammonia and FFA are given in Tables 4.8 and 4.9.

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF AMMONIA (incubation 72h/10°C) WITH REGARD TO THE KEEPING QUALITY (TASTE)

Keeping quality in days	Difference value of ammonia (ppm)		
	n	$\bar{X}_A$	s_a
< 1	14	4, 8	2, 79
1	50	6, 0	4, 35
2	32	2, 9	3, 12
3	27	2, 3	2, 61
4	25	0, 4	1, 17
5	8	1, 4	3, 75

75/168 Wi

Table 4.8

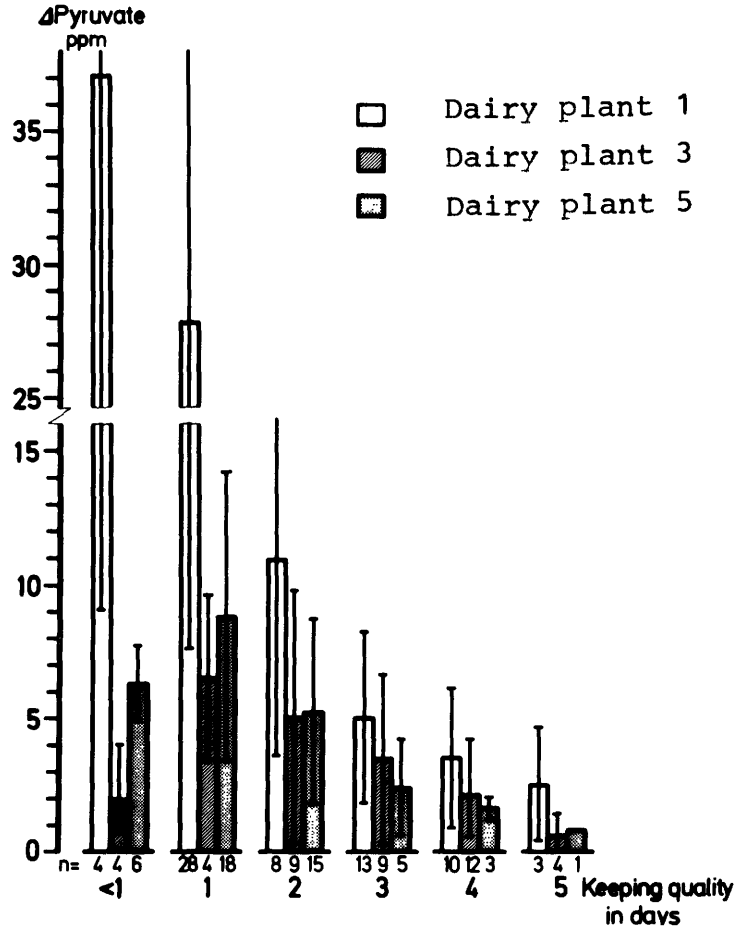
RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF FFA (incubation 72h/10°C) WITH REGARD TO THE KEEPING QUALITY (TASTE)

Keeping quality in days	Difference value of FFA (m-equiv. /l)		
	n	$\bar{X}_A$	s_a
< 1	3	0, 27	0, 01
1	20	0, 32	0, 20
2	12	0, 18	0, 20
3	6	0, 20	0, 08
4	17	0, 21	0, 10
5	5	0, 21	0, 14

75/169 Wi

Table 4.9

Splitting the total material on the three dairy plants under study shows uniform trends (Fig. 4.10 and Table 4.11).



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND DIFFERENT DAIRY PLANTS

Fig. 4.10

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND DIFFERENT DAIRY PLANTS

Keeping quality in days	Dairy 1			Dairy 3			Dairy 5		
	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
< 1	4	37,03	27,86	4	1,90	1,12	6	6,32	1,42
1	28	27,75	20,13	4	6,55	3,09	18	8,81	5,42
2	8	10,90	7,30	9	5,01	4,75	15	5,23	3,45
3	13	5,03	3,16	9	3,48	3,14	5	2,40	1,78
4	10	3,49	2,59	12	2,06	2,16	3	1,57	0,38
5	3	2,53	2,17	4	0,55	0,87	1	0,80	0

75/106 Wi

Table 4.10

The levels of the means of pyruvate/keeping duration inter-relations are different for the individual dairy plants. This may be the result of a changing combination of microbial and nonmicrobial factors. The present data show, that in dairy no. 3 the non-microbial factors dominate as the pyruvate-difference-values are on a relatively low level.

A quite good differentiation, though rather variable in the individual levels, is shown by the lactic-acid-difference-values from the three dairy plants. A high glycolytic metabolic activity of the microflora is observed only in dairy no. 5 (Table 4.11).

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF LACTATE (INCUBATION 72 h/10°C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND DIFFERENT DAIRY PLANTS

Keeping quality in days (taste)	Dairy 1			Dairy 3			Dairy 5		
	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
< 1	4	75, 8	14, 1	4	0, 03	0, 20	6	267, 2	31, 8
1	28	40, 3	18, 1	4	88, 5	14, 27	17	236, 7	34, 25
2	8	46, 3	1, 56	9	24, 3	4, 83	15	81, 7	10, 2
3	12	0, 02	0, 62	9	5, 7	0, 80	5	18, 8	2, 87
4	10	- 0, 04	0, 34	12	4, 3	0, 81	3	2, 0	0, 29
5	3	- 0, 03	0, 17	3	1, 3	0, 34	1	8, 0	-

75/105 Wi

Table 4.11

The results of the determination of ammonia - and free fatty acid-difference-values are presented in Tables 4.12 and 4.13.

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF AMMONIA (INCUBATION 72 h/10°C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND DIFFERENT DAIRY PLANTS

Keeping quality in days (taste)	Dairy 1			Dairy 3			Dairy 5		
	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
<1	4	6, 8	2, 23	4	1, 13	0, 44	6	5, 92	1, 33
1	28	7, 0	4, 53	4	3, 83	4, 19	18	4, 77	3, 54
2	8	2, 98	2, 51	9	1, 12	3, 15	15	4, 01	2, 88
3	13	3, 63	3, 01	9	1, 18	1, 49	5	0, 82	0, 38
4	10	0, 17	1, 12	12	0, 43	1, 13	3	1, 4	0, 94
5	3	3, 23	5, 57	4	0, 55	0, 82	1	-0, 4	-

751103 Wi

Table 4.12

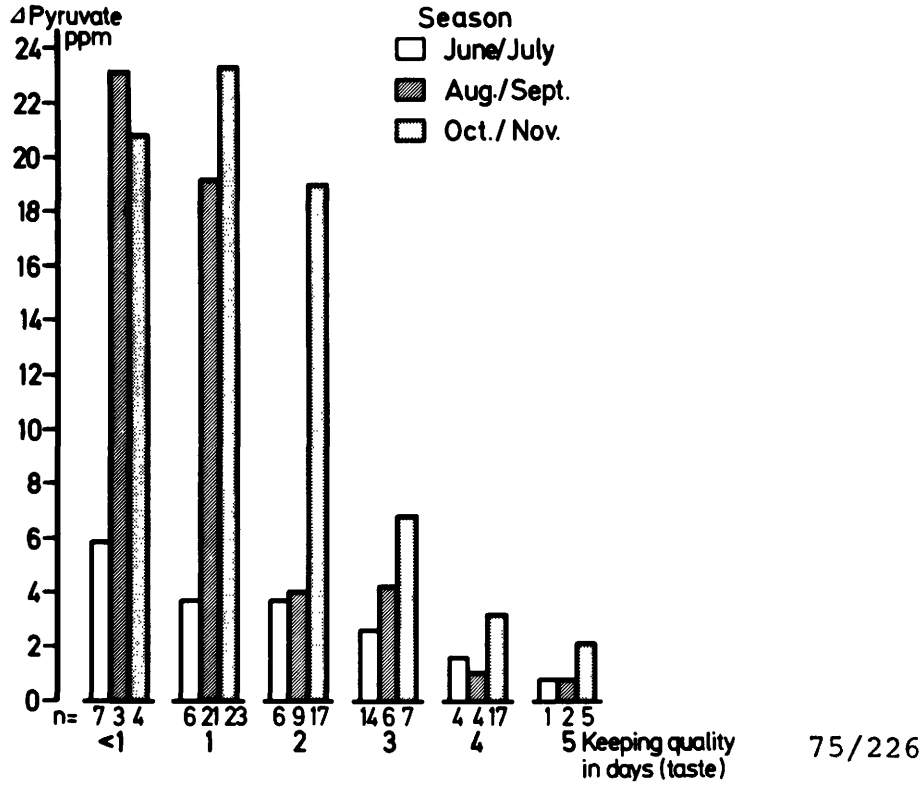
RESULTS OF THE DETERMINATION OF THE DIFFERENT VALUE OF FFA (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND DIFFERENT DAIRY PLANTS

Keeping quality in days (taste)	Dairy 1			Dairy 3			Dairy 5		
	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
<1	0	-	-	2	0,28	0,0	1	0,25	0
1	8	0,39	0,18	1	0,11	0,0	11	0,30	0,19
2	3	0,30	0,10	3	0,26	0,20	6	0,08	0,19
3	3	0,24	0,05	3	0,16	0,07	0	-	-
4	10	0,20	0,08	7	0,23	0,12	0	-	-
5	3	0,23	0,18	2	0,19	0,04	0	-	-

75/104 Wi

Table 4.13

The results of the determination of the pyruvate-difference-value with respect to keeping quality (taste) and seasonal influences are shown in Fig. 4.11.



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND THE SEASON

Fig. 4.11

The trend for the above mentioned conditions remains unchanged. The role of microbial processes in the risk of spoilage seems to be different in the individual months. Microbial acitivities are greater in August and October under study. Tables 4.14 and 4.15 give the data for pyruvate-difference-values and lactic-acid-difference-values.

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (Incubation 72h/10°C) WITH REGARD TO THE KEEPING QUALITY (Taste) AND THE SEASON

Keeping quality in days	June/July			Aug./Sept.			Oct./Nov.		
	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
< 1	7	5, 89	3, 75	3	23, 10	8, 92	4	20, 78	18, 13
1	6	3, 74	2, 05	21	19, 20	17, 31	23	23, 28	13, 94
2	6	3, 65	0, 91	9	4, 06	3, 36	17	18, 96	5, 94
3	14	2, 56	2, 35	6	4, 15	1, 03	7	6, 84	3, 46
4	4	1, 60	0, 31	4	1, 00	0, 66	17	3, 17	2, 60
5	1	0, 80	-	2	0, 75	0, 04	5	2, 10	1, 55

75/171 W

Table 4.14

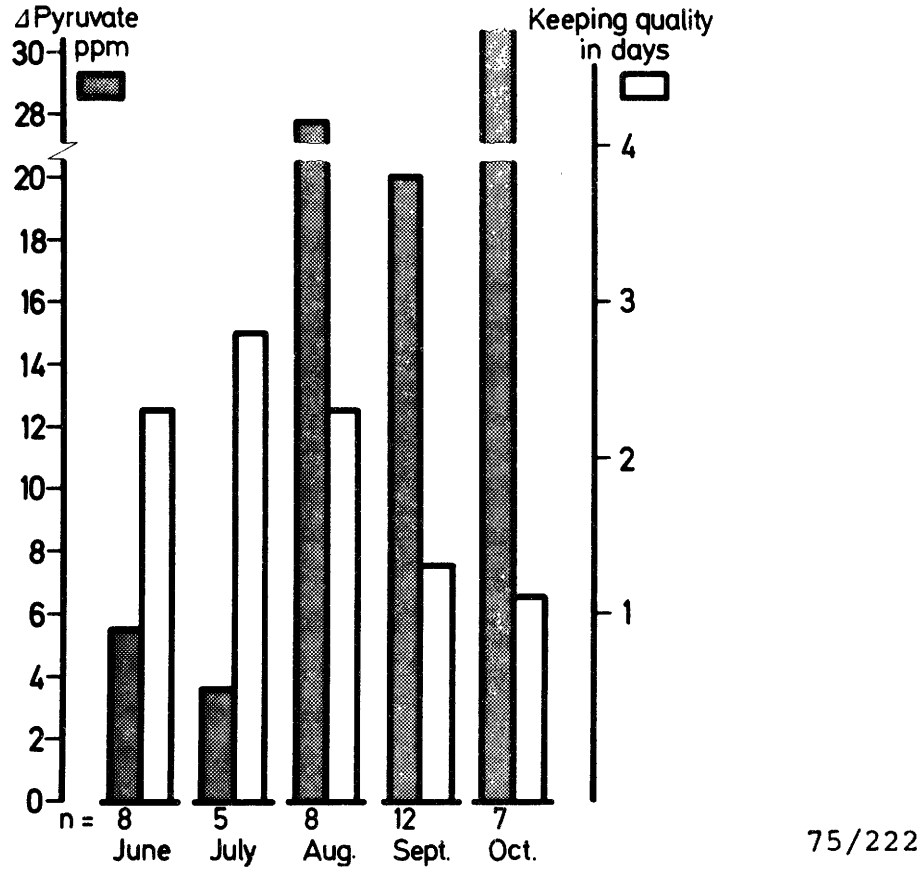
RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF LACTATE (Incubation 72h/10°C) WITH REGARD TO THE KEEPING QUALITY (Taste) AND THE SEASON

Keeping quality in days	June/July			Aug./Sept.			Oct./Nov.		
	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
< 1	7	54, 3	75, 40	3	357, 7	327, 0	4	113, 5	114, 0
1	6	239, 9	29, 41	21	115, 8	203, 8	22	74, 5	177, 9
2	6	32, 9	48, 27	9	30, 3	65, 33	17	59, 6	81, 8
3	14	2, 3	5, 49	6	16, 8	2, 37	6	2, 33	1, 8
4	4	3, 3	2, 66	4	2, 3	5, 75	17	1, 82	5, 39
5	1	8, 0	-	2	-1, 0	0, 94	5	0, 88	0, 33

75/118 W

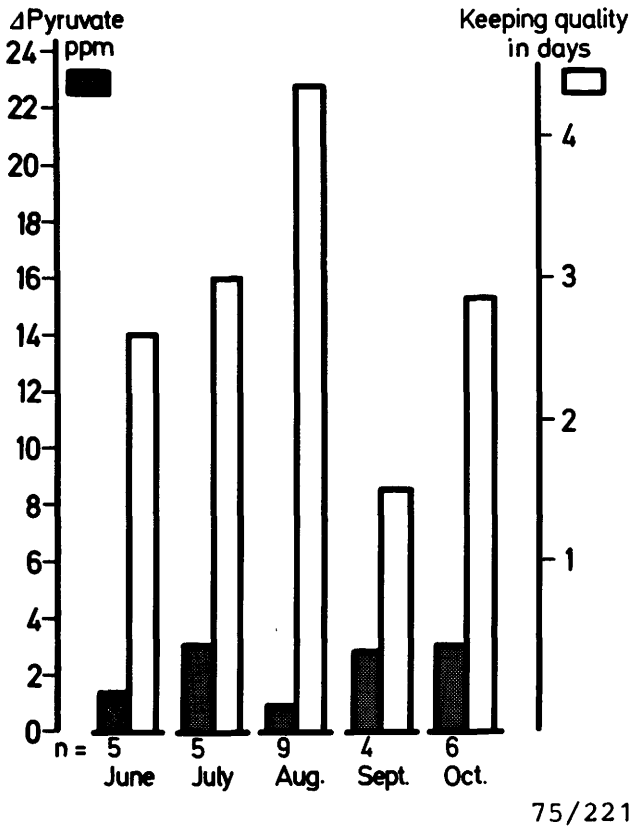
Table 4.15

The pyruvate-difference- and keeping-time-values for the individual dairies over a 5 month period are given in Fig. 4.12, 4.13 and 4.14.



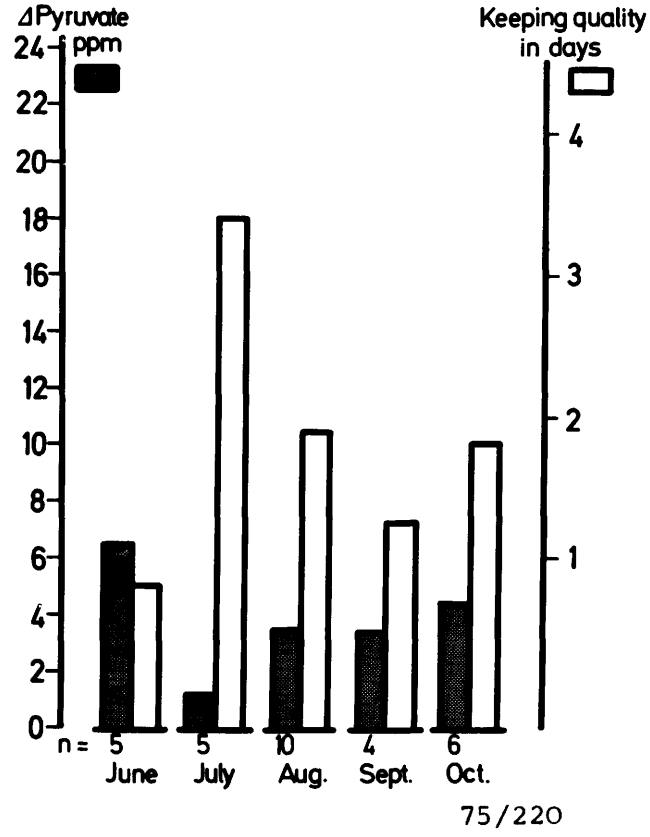
KEEPING QUALITY IN DAYS AND DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) AT DAIRY PLANT 1 WITHIN 5 MONTHS ($\bar{X}_A$)

Fig. 4.12



KEEPING QUALITY IN DAYS AND DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) AT DAIRY PLANT 3 WITHIN 5 MONTHS ($\bar{x}_A$)

Fig. 4.13

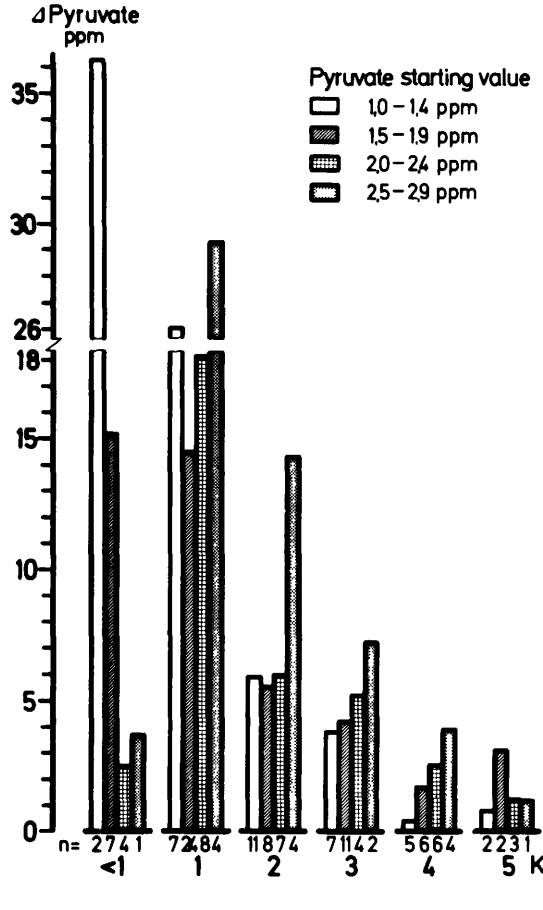


KEEPING QUALITY IN DAYS AND DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) AT DAIRY PLANT 5 WITHIN 5 MONTHS ($\bar{x}_A$)

Fig. 4.14

4.2.2.2 Microbial processes in raw milk and the keeping quality of pasteurized milk

The relationship between the influence of microbial processes in raw milk and the keeping quality of pasteurized milk is shown in Fig. 4.15.



RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (INCUBATION 72 h/10° C) WITH REGARD TO THE KEEPING QUALITY (TASTE) AND THE PYRUVATE-STARTING-VALUE ($\bar{X}_{A)}$

Fig. 4.15

The pyruvate-difference-values for distinct levels of keeping quality are divided into four groups, according to primary pyruvate levels. The milk samples under study were freshly pasteurized. As pyruvate is stable against the pasteurization temperatures, the pyruvate content corresponds to that in raw milk.

Fig. 4.15 demonstrates in general the following facts:

The lower the primary pyruvic acid level, i.e. the lower the bacterial contamination of raw milk, the higher the pyruvate-difference-value at equal span of keeping quality. That means, that a pasteurized milk, obtained from a good raw milk, needs higher microbial activity for spoilage than vice versa. So the influence of the quality of raw milk on the keeping quality of pasteurized milk is confirmed (in tendency). Naturally the variations within the groups are very high, and especially the data for a basic pyruvate content between 2.5 and 2.9 ppm do not fit the general pattern (see Table 4.16).

RESULTS OF THE DETERMINATION OF THE DIFFERENCE VALUE OF PYRUVATE (Incubation 72h/10°C) WITH REGARD TO THE KEEPING QUALITY (Taste) AND THE PYRUVATE STARTING VALUE ($\bar{X}_A$)

Pyruvate starting value	1,0 - 1,4 ppm			1,5 - 1,9 ppm			2,0 - 2,4 ppm			2,5 - 2,9 ppm			> 2,9 ppm		
Keeping quality in days (taste)	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a	n	$\bar{X}_A$	s_a
< 1	2	36,7	32,9	7	15,21	18,21	4	2,50.	2,12	1	3,70	-	0	-	-
1	7	26,03	24,45	24	14,48	11,95	8	18,17	19,02	4	29,25	24,59	4	22,5	16,11
2	11	5,93	3,96	8	5,49	2,85	7	6,04	4,52	4	14,33	8,75	1	0,30	-
3	7	3,77	3,40	11	4,21	2,14	4	5,20	3,54	2	7,25	1,15	3	0,23	1,76
4	5	0,38	1,69	6	1,70	0,74	6	2,55	2,93	4	3,93	2,92	4	4,20	2,05
5	2	0,8	0	2	3,15	2,45	3	1,23	0,37	1	1,20	0	-	-	-

75/107 W.

Table 4.16

4.2.2.3 Pyruvate-threshold-value and spoilage of pasteurized milk

Fig. 4.16 to 4.19 set out pyruvate and other metabolites of bacterial catabolism (lactic-acid, ammonia and free fatty acids (FFA)) with respect to the number of points in the organoleptic evaluation (taste) of the respective milk samples.

The numerical values are compiled in Table 4.17.

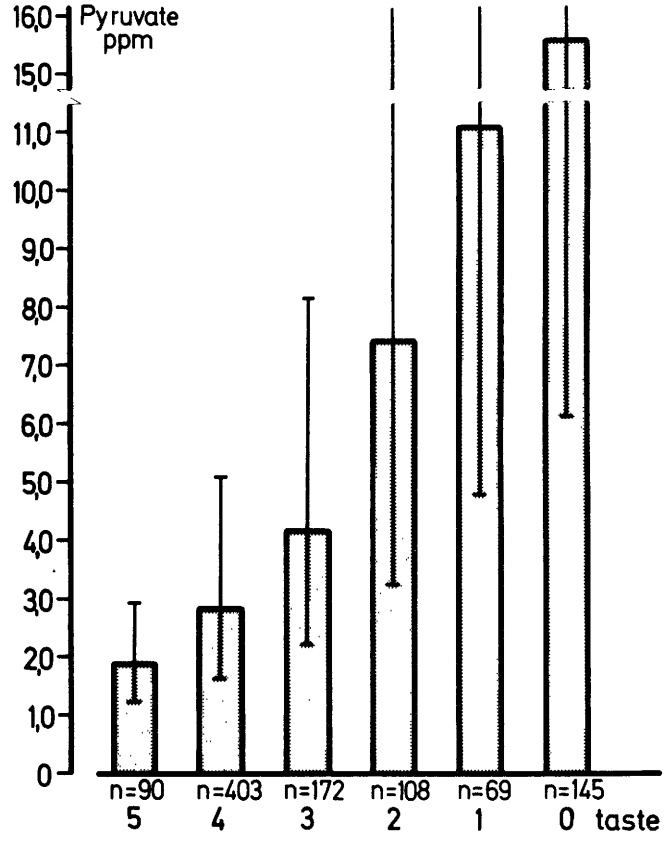
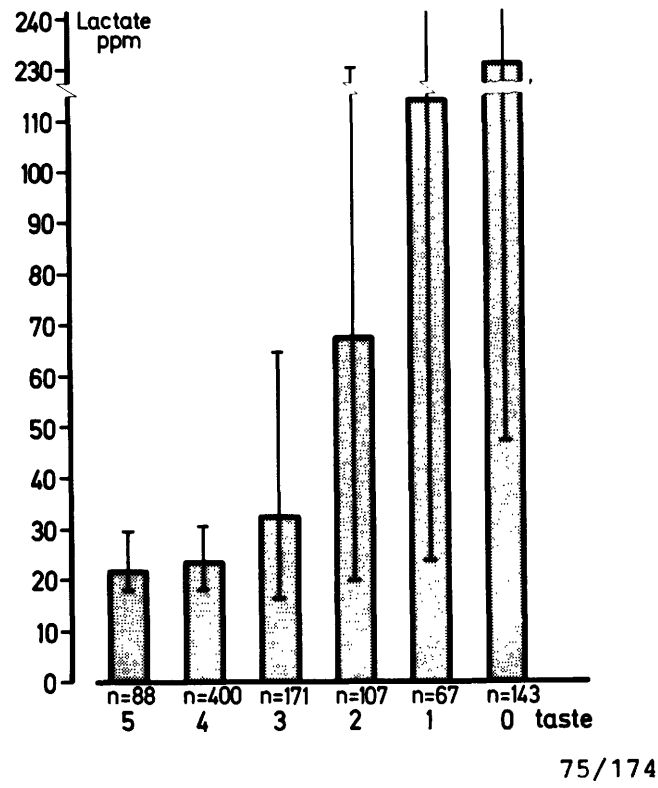
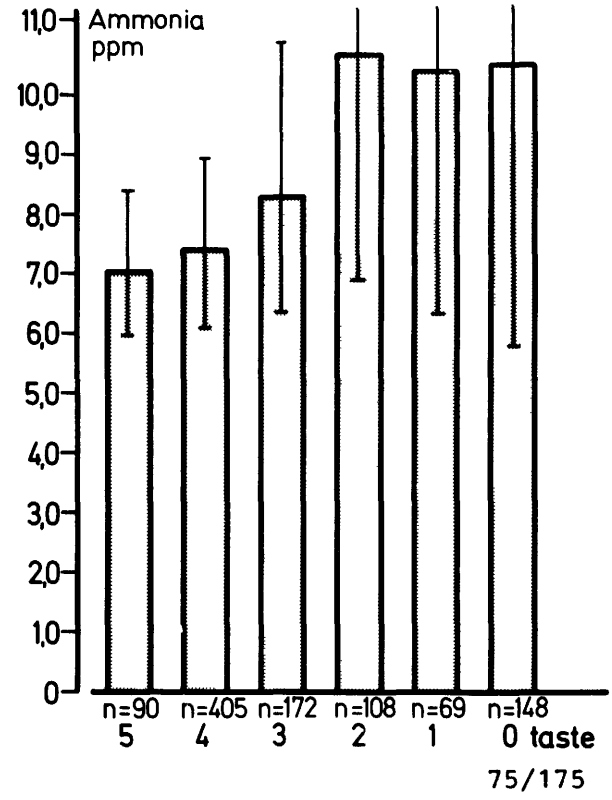


Fig. 4.16



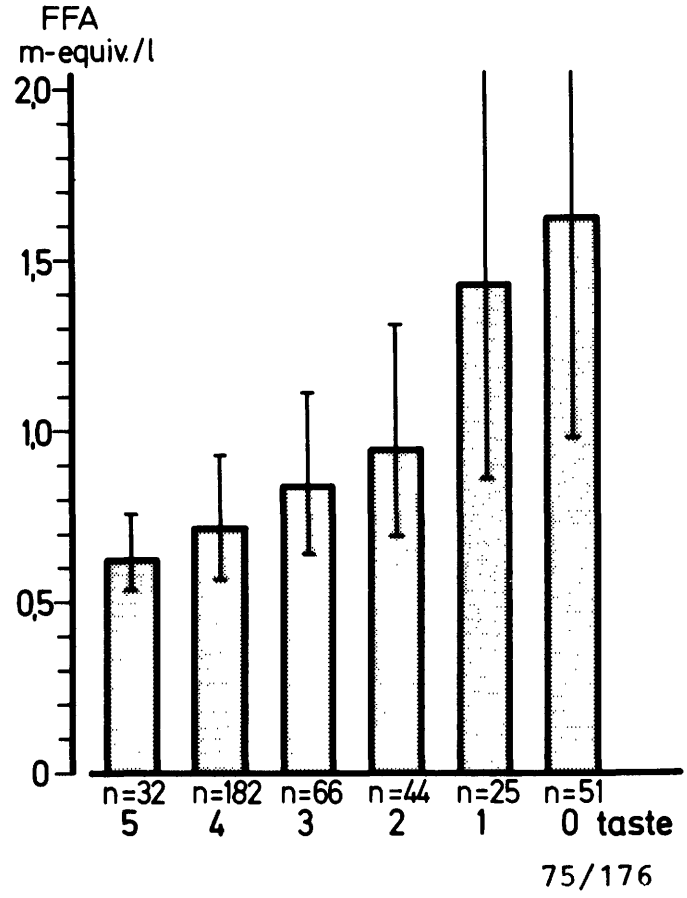
COMPARISON OF TASTE WITH THE LACTATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.17



COMPARISON OF TASTE WITH THE AMMONIA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.18



COMPARISON OF TASTE WITH THE FFA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.19

COMPARISON OF TASTE WITH THE LACTATE-, PYRUVATE-, AMMONIA- AND FFA-CONCENTRATIONS

Taste	Lactate ppm			Pyruvate ppm			Ammonia ppm			FFA (m-equiv./l)		
	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g
5	88	22,7	1,26	90	1,91	1,55	90	7,07	1,19	32	0,63	1,20
4	400	23,3	1,31	403	2,85	1,80	405	7,39	1,21	182	0,72	1,29
3	171	31,9	2,02	172	4,22	1,93	172	8,31	1,31	66	0,84	1,32
2	107	67,7	3,46	108	7,43	2,29	108	10,64	1,54	44	0,95	1,38
1	67	114,0	4,85	69	11,09	2,33	69	10,41	1,64	25	1,43	1,66
0	143	231,0	4,39	145	15,57	2,56	148	10,50	1,80	51	1,62	1,65

75/161 Wi

Table 4.17

A milk with four "taste-points" features in average the following contents: lactic acid 23.3 ppm, pyruvate 2.85 ppm, ammonia 7.39 ppm, free fatty acids 0.72 mequ./l. The total numbers of micro-organisms, pseudomonads and coliforms as the corresponding geometric means are given in Table 4.18.

COMPARISON OF TASTE WITH TOTAL COLONY COUNT, PSEUDOMONADS AND COLIFORMS

	Total colony count/ml			Pseudomonads/ml			Coliforms/ml		
Taste	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g
5	85	206×10^3	3,95	68	131×10^1	57,81	59	98×10^1	52,31
4	182	38×10^4	6,22	383	94×10^2	70,03	288	91×10^1	46,28
3	161	201×10^4	10,57	136	157×10^3	38,49	147	106×10^2	58,58
2	103	124×10^5	6,67	90	284×10^4	6,44	90	56×10^4	13,99
1	67	31×10^6	6,98	58	44×10^5	7,88	54	93×10^4	33,93
0	139	54×10^6	5,99	115	290×10^4	9,17	130	257×10^4	18,60

75/160 Wi

Table 4.18

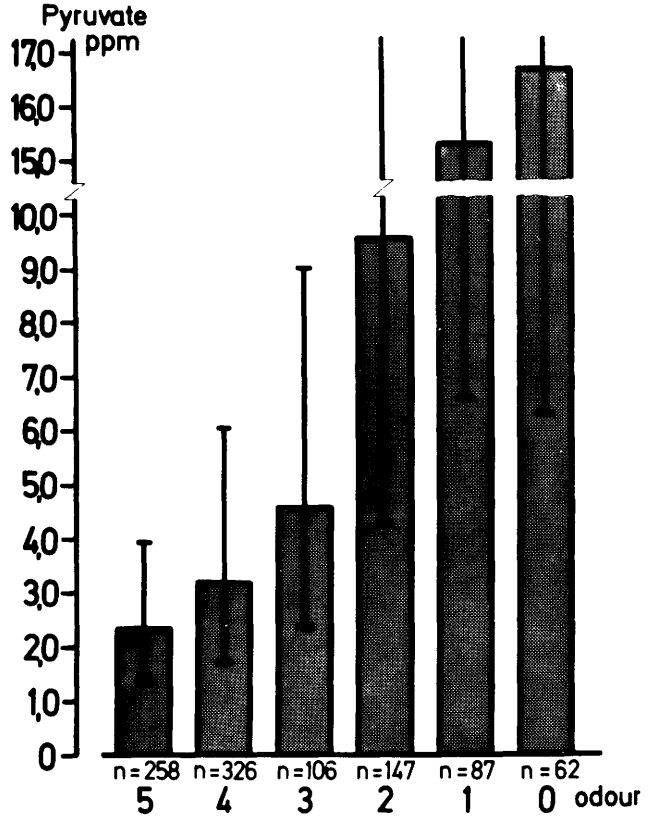
Similar data, based on the parameter "odour" are shown in the Tables 4.19 and Figures 4.20 to 4.23.

COMPARISON OF ODOUR WITH LACTATE-, PYRUVATE-, AMMONIA- AND FFA-CONCENTRATIONS

	Lactate ppm			Pyruvate ppm			Ammonia ppm			FFA (m-equiv./l)		
Odour	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g
5	253	22,66	1,28	258	2,33	1,70	258	7,16	1,19	110	0,67	1,25
4	326	24,73	1,48	326	3,22	1,88	328	7,60	1,24	140	0,77	1,31
3	105	51,96	2,93	106	4,63	1,95	107	9,19	1,41	38	0,87	1,35
2	145	102,1	4,63	147	9,55	2,26	148	10,19	1,62	67	1,12	1,52
1	85	158,5	5,26	87	15,3	2,34	88	11,07	1,66	28	1,54	1,72
0	62	177,82	4,87	62	16,7	2,66	63	10,78	1.86	17	1,97	1,69

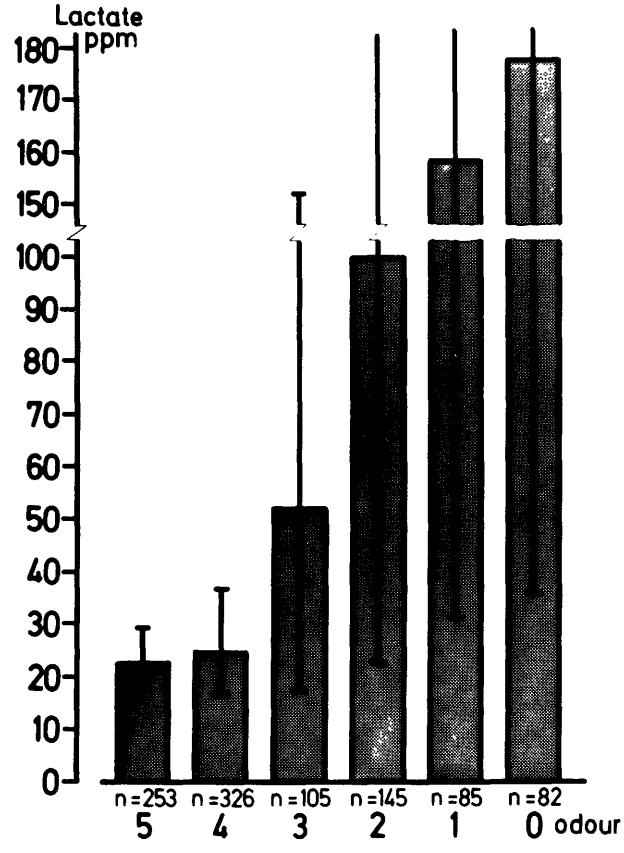
75/159 Wi

Table 4.19



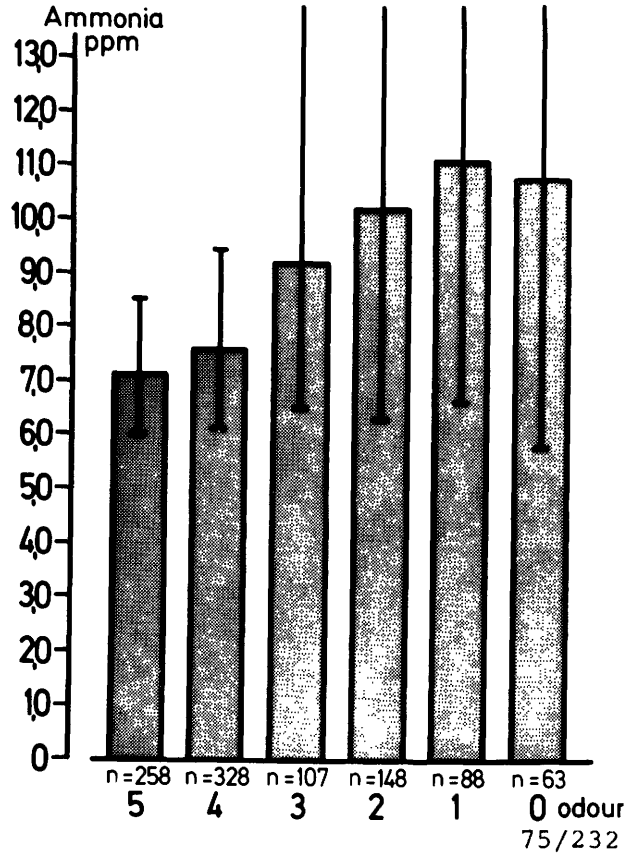
COMPARISON OF ODOUR WITH REGARD TO THE RESULTS OF THE PYRUVATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.20



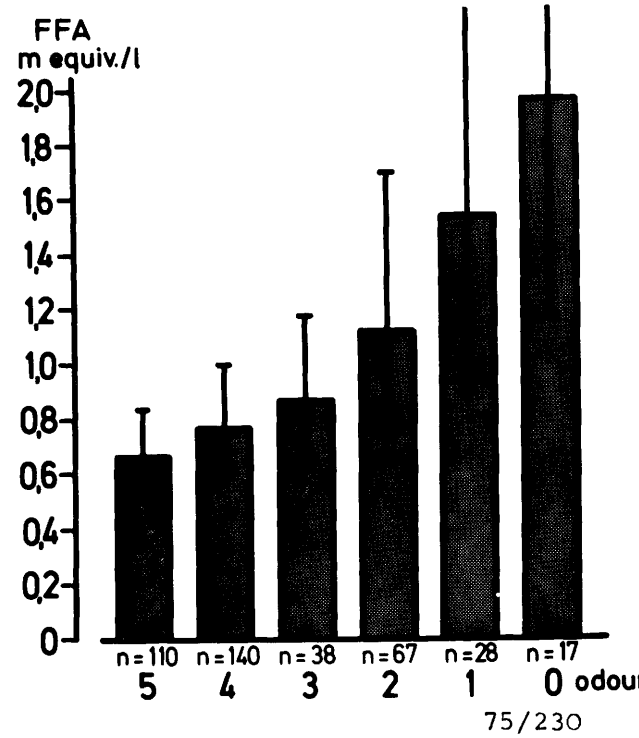
COMPARISON OF ODOUR WITH REGARD TO THE RESULTS OF THE LACTATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.21



COMPARISON OF ODOUR WITH REGARD TO THE RESULTS OF THE AMMONIA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.22



COMPARISON OF ODOUR WITH REGARD TO THE RESULTS OF THE FFA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.23

COMPARISON OF ODOUR WITH TOTAL COLONY COUNT, PSEUDOMONADS AND COLIFORMS

Odour	Total colony count/ml			Pseudomonads/ml			Coliforms/ml		
	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g
5	241	267×10^3	4,92	198	35×10^2	71,14	173	10×10^1	49,49
4	311	53×10^4	8,27	274	175×10^2	67,79	254	118×10^1	47,92
3	102	60×10^5	7,32	88	73×10^4	11,28	91	91×10^3	32,01
2	142	230×10^5	6,76	124	40×10^5	6,62	122	83×10^4	20,18
1	84	37×10^6	6,56	72	232×10^4	10,38	73	278×10^4	15,33
0	58	54×10^6	6,68	45	40×10^5	7,06	55	225×10^4	24,03

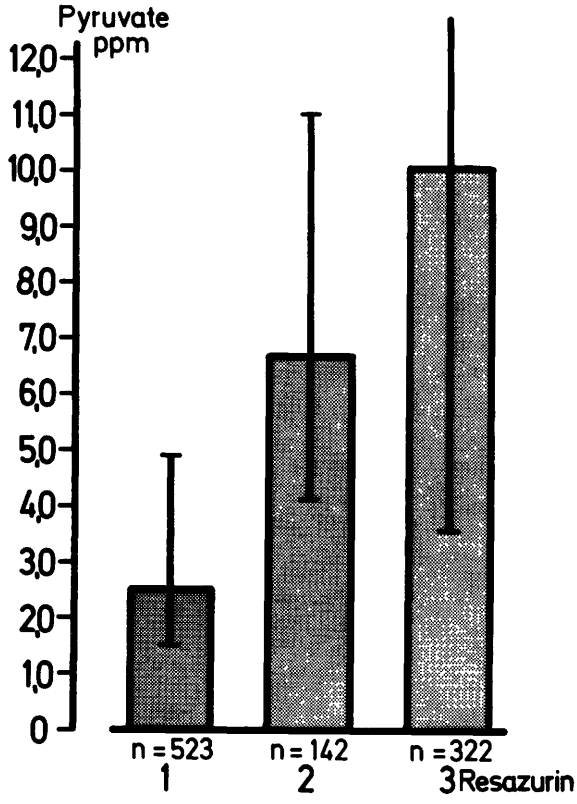
75/162 W

Table 4.20

It seems, that the parameter "taste" is slighly more sensitive to the limit of bacterial metabolites then a classification with respect to "odour".

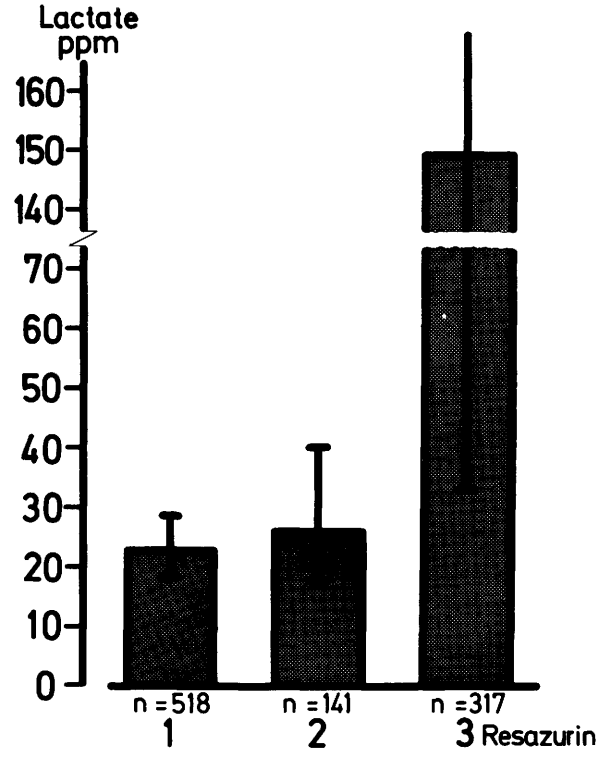
4.2.2.4 Reduction-test, pyruvate and metabolites of microbial catabolism

The interrelations between the resazurin-reduction-test and the parameters of bacterial catabolism (pyruvate, lactic-acid, ammonia and free fatty acids) are shown in Fig. 4.24 to 4.27.



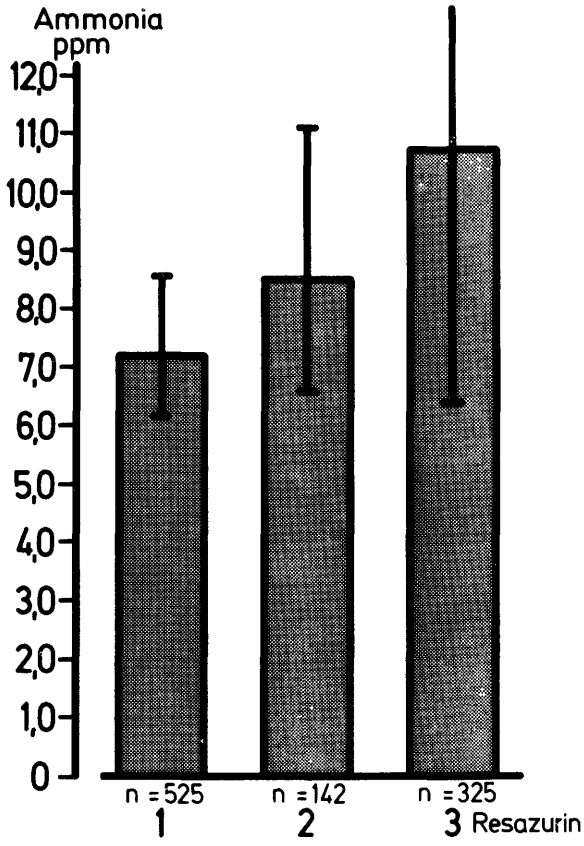
COMPARISON OF RESAZURIN REDUCTION TEST WITH THE PYRUVATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.24



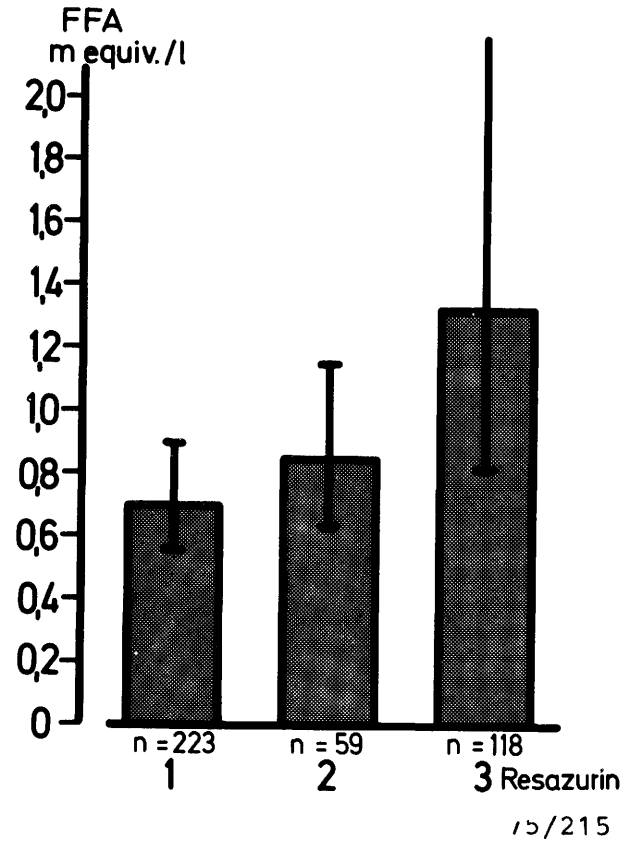
COMPARISON OF RESAZURIN REDUCTION TEST WITH THE LACTATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.25



COMPARISON OF RESAZURIN REDUCTION TEST TEST WITH AMMONIA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.26



COMPARISON OF RESAZURIN REDUCTION TEST WITH FFA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.27

The numerical values in Tables 4.21 and 4.22 show quite a good differentiation between stages 2 and 3 of the resazurin-reduction-test and lactic acid (26 and 149 ppm respectively). At a pyruvate-mean of 2.56 ppm the resazurin-test is still negative. A positive result corresponds with a total number of bacteria of 61 x 10^5/ml in the material under study. The number of pseudomonads was about 138 x 10^4/ml. This show the poor sensitivity of the reduction test, in the material under study.

COMPARISON OF RESAZURIN TEST WITH LACTATE-, PYRUVATE-, AMMONIA- AND FFA-CONCENTRATIONS

Resazurin reduction test	Lactate (ppm)			Pyruvate (ppm)			Ammonia (ppm)			FFA (m-equiv./l)		
	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g
1	518	23,04	1,27	523	2,56	1,72	525	7,24	1,18	223	0,70	1,27
2	141	26,08	1,53	142	6,71	1,65	142	8,54	1,30	59	0,85	1,35
3	317	149,7	4,50	322	10,12	2,84	325	10,75	1,68	118	1,33	1,64

75/166 W.

Table 4.21

COMPARISON OF RESAZURIN REDUCTION TEST WITH TOTAL COLONY COUNT, PSEUDOMONADS AND COLIFORMS

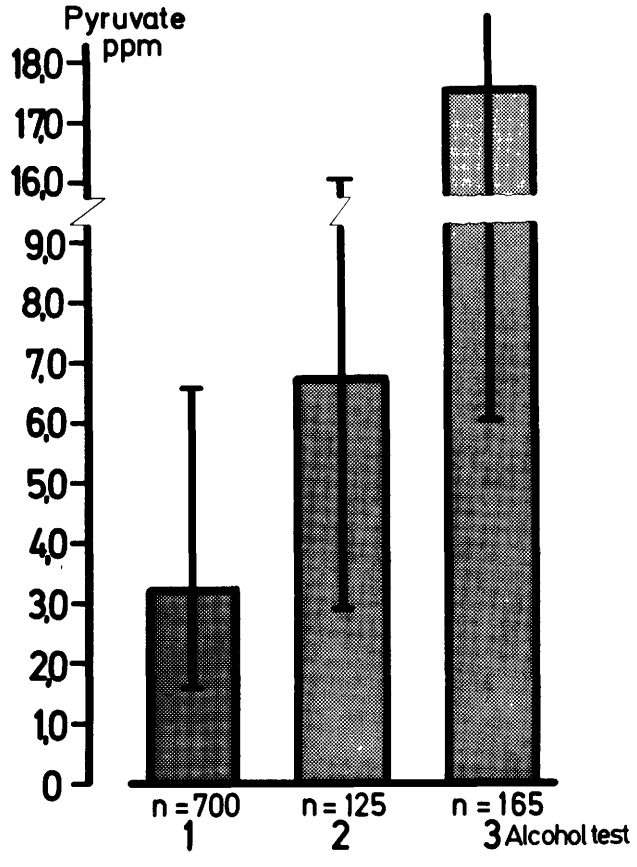
Resazurin reduction test	Total colony count/ml			Pseudomonads/ml			Coliforms/ml		
	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g
1	498	283×10^3	5,20	431	59×10^2	66,60	374	84×10^1	49,33
2	132	61×10^5	5,60	106	138×10^4	9,00	124	46×10^3	39,33
3	308	290×10^5	7,55	264	230×10^4	10,85	270	100×10^4	26,39

75/165 W.

Table 4.22

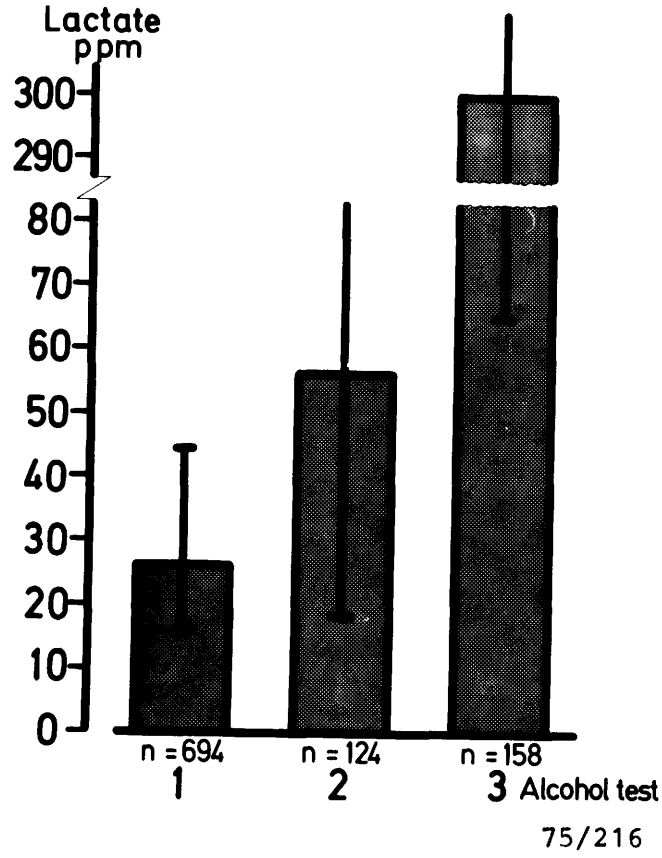
4.2.2.5 Alcohol-test, pyruvate and metabolites of bacterial catabolism

In Fig. 4.28 to 4.31 the correlations between the so called double alcohol-test and the metabolites of bacterial origin are illustrated. Lactic acid and pyruvate show a good differentiation in the single classes of the alcohol-test (1-3). Nevertheless the alcohol test gives a positive result at a pyruvate content of nearly 7 ppm. Even with respect to a certain pyruvate content in the raw milk, the poor sensitivity of this test is demonstrated. The numerical data are listed in Tables 4.23 and 4.24.



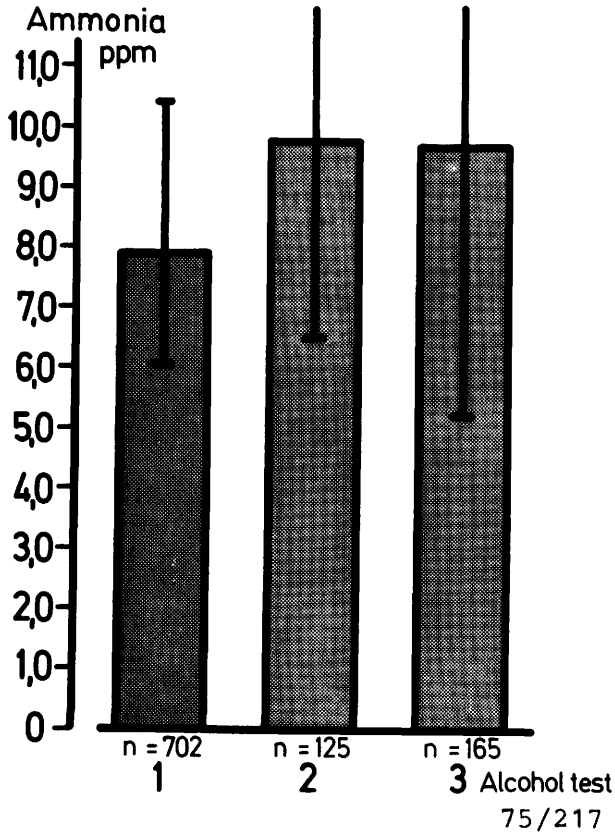
COMPARISON OF ALCOHOL TEST WITH PYRUVATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.28



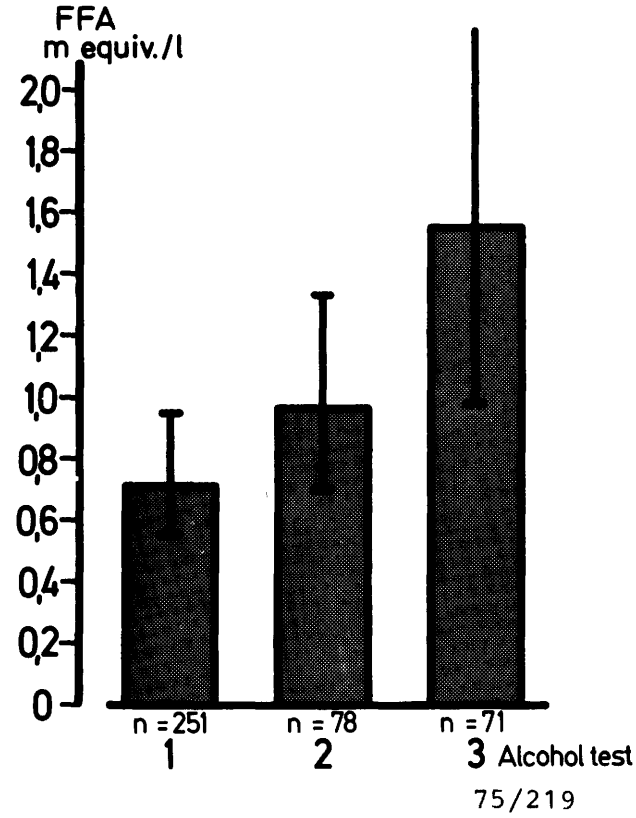
COMPARISON OF ALCOHOL TEST WITH LACTATE-CONCENTRATION ($\bar{X}_G$)

Fig. 4.29



COMPARISON OF ALCOHOL TEST WITH AMMONIA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.30



COMPARISON OF ALCOHOL TEST WITH FFA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.31

COMPARISON OF ALCOHOL TEST WITH LACTATE-, PYRUVATE- AMMONIA- AND FFA-CONCENTRATIONS

Alcohol test	Lactate (ppm)			Pyruvate (ppm)			Ammonia (ppm)			FFA (m-equiv./l)		
	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g
1	694	26, 32	1, 70	700	3, 23	2, 03	702	7, 94	1, 31	251	0, 72	1, 32
2	124	56, 56	3, 08	125	6, 82	2, 35	125	9, 80	1, 51	78	0, 97	1, 38
3	158	302, 5	4, 63	165	15, 74	2, 61	165	9, 75	1, 86	71	1, 56	1, 59

75/164 Wi

Table 4.23

COMPARISON OF ALCOHOL TEST WITH TOTAL COLONY COUNT, PSEUDOMONADS AND COLIFORMS

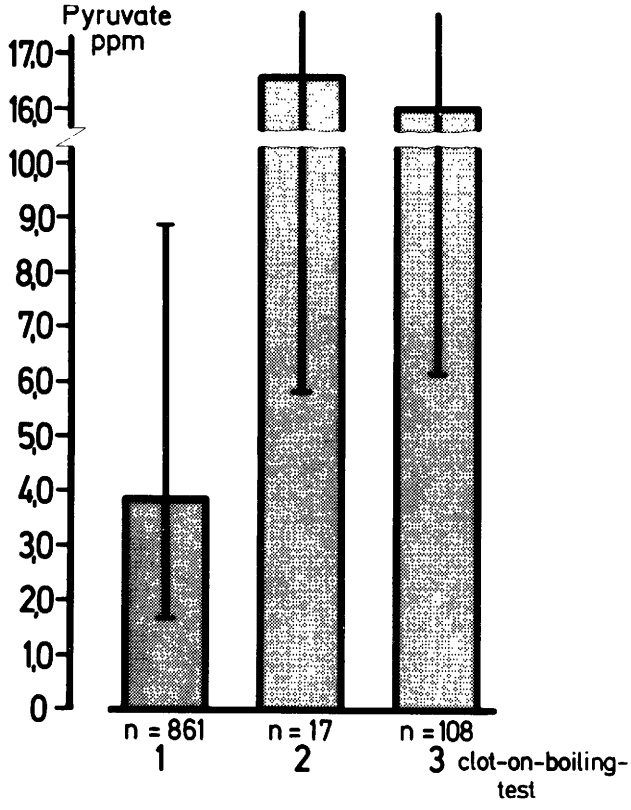
Alcohol test	Total colony count/ml			Pseudomonads/ml			Coliforms/ml		
	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g	n	$\bar{X}_G$	s_g
1	666	74×10^4	8,95	557	231×10^2	89,79	530	47×10^2	79,92
2	118	55×10^5	17,43	107	67×10^4	26,29	98	40×10^3	137,02
3	154	64×10^6	5,89	137	38×10^5	7,92	140	242×10^4	21,23

75/163W

Table 4.24

4.2.2.6 Clot-on-boiling-test, pyruvate and metabolites of bacterial catabolism

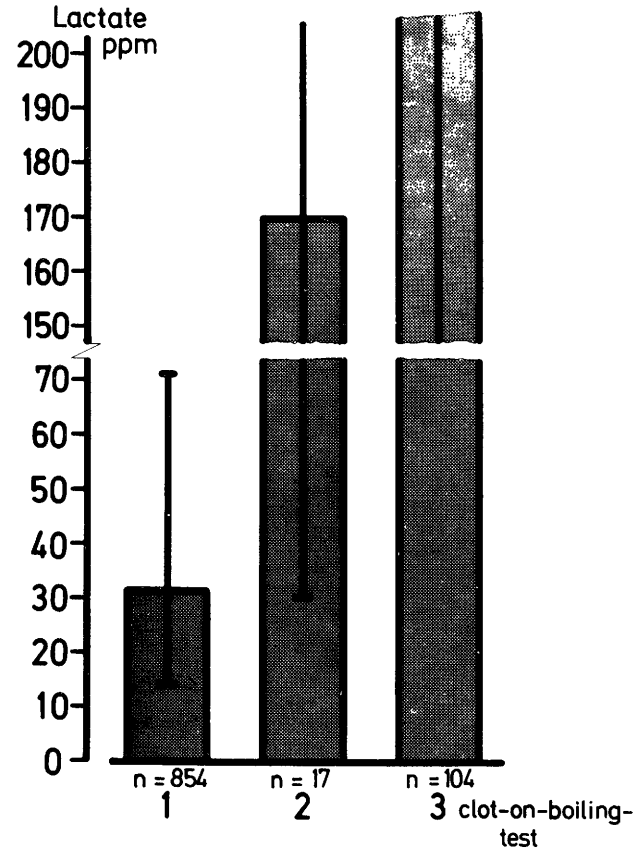
The results of the boiling-test and related determinations of bacterial metabolites are shown in Fig. 4.32 to 4.35.



75/209

COMPARISON OF CLOT-ON-BOILING-TEST WITH PYRUVATE-CONCENTRATION ($\bar{X}_G$)

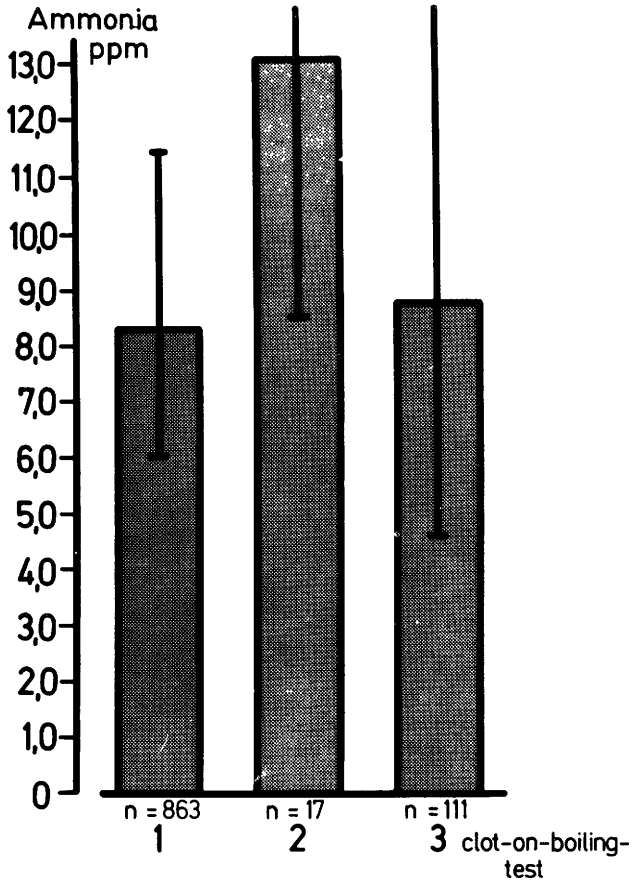
Fig. 4.32



75/208

COMPARISON OF CLOT-ON-BOILING-TEST WITH LACTATE-CONCENTRATION ($\bar{X}_G$)

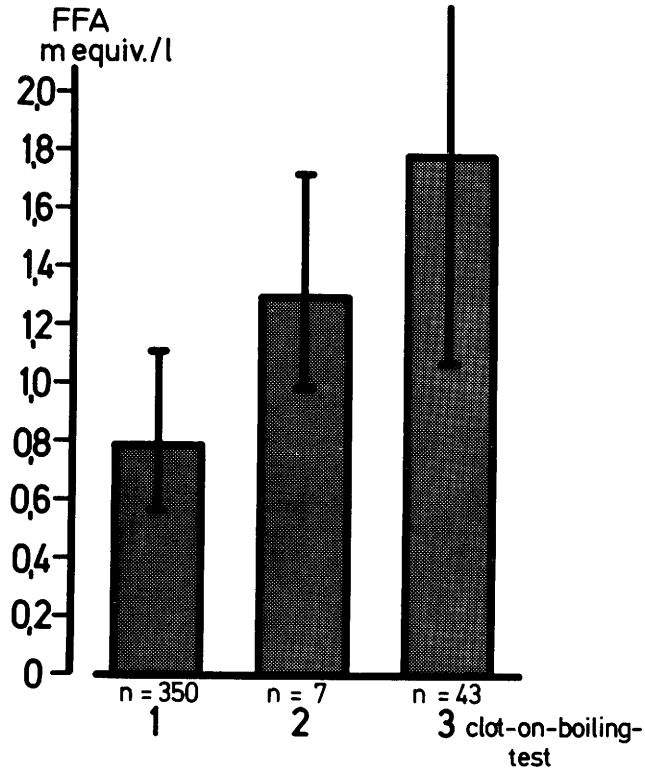
Fig. 4.33



75/211

COMPARISON OF CLOT-ON-BOILING-TEST WITH AMMONIA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.34



75/210

COMPARISON OF CLOT-ON-BOILING-TEST WITH FFA-CONCENTRATION ($\bar{X}_G$)

Fig. 4.35

A positive result of the boiling-test can only be obtained (80° C/5 min) when the contents of the metabolites indicate a practically spoiled substrate.

This is obvious from the data in Tables 4.25 and 4.26.

COMPARISON OF THE CLOT-ON-BOILING-TEST WITH LACTATE-, PYRUVATE-, AMMONIA- AND FFA-CONCENTRATIONS

clot-on-boiling test	Lactate ppm			Pyruvate ppm			Ammonia ppm			FFA (m-equiv./l)		
	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g
1	854	31,6	2,25	861	3,85	2,30	863	8,32	1,38	350	0,79	1,40
2	17	169,0	5,62	17	16,61	2,85	17	13,06	1,54	7	1,30	1,32
3	104	442,9	4,18	108	15,96	2,60	111	8,79	1,93	43	1,78	1,68

75/157 Wi

Table 4.25

COMPARISON OF THE CLOT-ON-BOILING-TEST WITH TOTAL COLONY COUNT, PSEUDOMONADS AND COLIFORMS

clot-on-boiling test	Total colony count/ml			Pseudomonads/ml			Coliforms/ml		
	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g	n	$\bar{x}_G$	s_g
1	815	117×10^4	12,15	698	50×10^3	91,63	658	82×10^2	98,95
2	17	214×10^5	7,87	13	115×10^4	8,45	14	69×10^4	34,64
3	105	84×10^6	5,76	90	44×10^5	7,94	95	43×10^5	15,38

75/158 Wi

Table 4.26

5. Discussion and summary of chapters 3. and 4. (TOLLE and HEESCHEN)

Because of its complex composition and high water activity milk is a sheer ideal growth medium for bacteria the catabolic processes of which in the substrate may be summarized as glycolysis, proteolysis, and lipolysis. In the foreground of discussions held up to the twenties of this century about the assessment of saprophytic-bacterial contamination of milk were the morphologic criteria such as the number of colony-forming units. Due to the sources of errors involved and the required material in plate cultures, reduction tests have then succeeded later in the thirties in order to measure the glycolytic changes occurring in the substrate. The gram-positive lactic acid formers developing in insuffiently cooled or uncooled milk, indicated by such reduction tests, even have a certain protective function due to the suppression of the gram-negative flora with its undesirable processes of lipolytic and proteolytic degradation. Thus, to a certain extent, reduction tests point to microorganisms which are not absolutely undesirable from the hygienic point of view.

Today in the EEC member countries, milk is mechanically refrigerated, though to a different extent, seen on the whole however, to a considerable degree. Refrigeration involves the development of psychrotrophic organisms which cannot be determined by means of reduction tests; these organisms, however, initiate undesired catabolic processes such as gram-positive lactic acid formers.

Hence, this evolution involves the principal question whether

1. colony counts technically improved (e.g. in form of the plate/loop procedure, the automated preparation of dilution plates, mechanization of the counting process by electronic microcolony counting or the computer image analysis of plate cultures) should be resumed or

wether

2. parameters others than morphological ones, such as biochemical parameters, would supply better evidence and be more practicable.

The present expertise deals with the question whether pyruvate - the salt of pyruvic acid as a central parameter of microbial metabolism would be suitable to describe the saprophytic-bacteriological contamination of milk and to give indications as to the keeping quality of milk after heat-treatment.

After representation of the biochemical principles of microbial catabolism, a model scheme is described to explain and to interpret the extracellular accumulation of pyruvate. According to this model scheme, a systematic accumulation of pyruvate in milk takes place in dependence on number and activity of the microorganisms present, and the amount of pyruvate released by the cell may be looked upon as a direct "parameter of activity". Automated or manual procedures may be used to measure the pyruvate pool in milk. For the automated procedure in the continuous flow analysis it is recommended to use the colorimetric process with blanks since this is in accordance also with the manual procedure.

The enzymatic determination of pyruvate is of high specifity. Since pyruvate is a substance dissolved in milk, sampling for it is facilitated as compared with that for bacterial counting, and this with reference to the representativeness of sampling as well as to errors of displacement of values. Samples for fixation of the pyruvate pool may be preserved by formaldehyde for a period of up to 24 hours, prolonged fixation of the pyruvate pool may be made by the addition of perchloric acid or trichloracetic acid. The physiologic pyruvate values in milk do not exceed 0.5 ppm in case of the black-pied lowland cattle. Any excess of this value is exclusively due to bacterial activity. As a thermostable substance pyruvate endures pasteurization without being destroyed, and thus it is possible to record a continuously additive picture of the bacteriological quality of milk from production till consumption.

The correlations between pyruvate pool and bacterial activity of saprophytic bacteria were studied in extensive time series by using pure cultures of the most different species. All microorganisms present in milk are practically able to produce pyruvate.

The results obtained under practical conditions have shown that systematic accumulation of pyruvate takes place between production of milk and its heat-treatment, the accumulation being dependent on the care taken in refrigeration and the length of time of storage of milk in the dairy processing plant. From the data available at this time it appears justifiable to suggest that three quality grades be established on milk producer level, i.e. up to 1 ppm, up to 2 ppm, and up to 3 ppm. Milk from producer level with a pyruvate content of more than 3.0 ppm does not appear to meet the requirements for this substrate. Just before heat-treatment of milk, the mean values of several analysis for pyruvate should not exceed 4 - 5 ppm.

Detailed studies have dealt with the question of the suitability of the pyruvate pool for the determination of the keeping quality of milk. After discussion of principles and of the numerous factors of influence on the keeping quality of heat-treated milk, the following complexes were dealt with:

1. Assessment of the keeping quality of pasteurized milk by determinations of pyruvate before and after incubation of milk samples (pyruvate difference value);

2. Influence of the microbial processes having taken place in raw milk on the keeping quality of pasteurized milk for consumption;

3. Impairment of value of pasteurized milk by microbial processes and pyruvate limit-value.

to 1)
In order to estimate the risk of spoilage by assessment of the bacterial activity in pasteurized milk, the pyruvate difference value - resulting from the initial value and from the value obtained after 72 hours incubation - was correlated to the keeping quality in days. The keeping quality was determined sensorically in accordance with a 5-scores-scheme. It may be concluded from the studies that the keeping quality is reduced

with increasing pyruvate difference value, i.e. with increasing bacterial activity. Thus, a keeping quality of only one day and a mean pyruvate difference value of 19.2 ppm contrasts with a keeping quality of 5 days and a pyruvate difference value of 1.6 ppm.

to 2)
As regards the question of the influence of microbial processes in raw milk on the keeping quality of pasteurized milk, samples were taken from a dairy; the pyruvate difference values were determined and the samples divided into 3 groups according to their initial pyruvate values. Due to the heat stability of pyruvate towards pasteurization, the initial pyruvate value of freshly pasteurized milk samples is in accordance with that of the raw milk taken for pasteurization. It is clear from the analysis of the results that 16 % of the milk samples with an initial pyruvate value of below 1.5 ppm have a keeping quality of more than 4 days, whereas at higher initial values only 10 % and 3 % respectively of the samples will reach this keeping quality of more than 4 days. Thus, the number of samples with a keeping quality of more than 4 days will be the lower, the higher was the microbial contamination of the raw milk.

to 3)
Putting into correlation the scores of sensoric grading of pasteurized milk with the respective pyruvate values will result in a good graduation within the scheme of scores from 1 - 5. The full number of scores (5) in flavour grading is in accordance with an average pyruvate content of 1.9 ppm. If not more than 3 scores are attributed to a pasteurized milk, which is thus clearly reduced in value, this corresponds with an average pyruvate value of 4.2 ppm. In the case of pyruvate values of 8 ppm and more, the sensoric properties are adversely affected to an extent that the milk in question is no longer suitable for consumption. Comparative studies of pyruvate values and results of the clot-on-boiling and alcohol tests have shown that the latter tests supply positive results not before pyruvate

values have already characterized a substrate as clearly reduced in value. Therefore, neither clot-on-boiling nor alcohol test are suitable to describe the bacteriological quality of raw milk in any phase between production and heat-treatment.

Summarizing

It may be said that the measurement of the pyruvate pool is well superior to colony counting procedures, and this from the point of view of theorical conception as well as from that of the results of tests and studies. Pyruvate in milk is a parameter of activity suitable for the description of the actual microbial contamination as well as for predicting the keeping quality of heat-treated milk. It goes without saying that such complex and difficult problematics may not fully be dealt with within a period of time of only one year. The results available so far should, therefore, be interpreted to the effect that they may be used as the basis for further common studies within the European Community.

6. Collaborative experiments between Institut für Hygiene der Bundesanstalt für Milchforschung, Kiel, and the National Institute for Research in Dairying, Shinfield (MABBIT)

6.1 Introduction

To study in more detail pyruvate levels and the factors which might affect them in refrigerated raw milk from individual farms and commercially pasteurized bottled milk, collaborative work between NIRD and Kiel has been undertaken. This report describes the experiments and results with raw and pasteurized milks sampled and analysed at NIRD, and preserved samples sent to Kiel for pyruvate and lactate determinations.

6.1.1 Raw milks

An earlier trial had provided valuable information on the feasibility of such collaborative studies, problems with transportion and preservation and, more important, data on the changes to be expected in bacterial counts during laboratory storage at different temperatures; for example, with many of the milks there was no significant increase in total counts until they had been stored for 4 days at 5^{o} although the psychrotroph counts showed an increase after 3 days. The bacteriological analyses of the raw milks showed the diversitiy in the initial counts, the microfloras of the milks and the changes which occurred during storage at 5^{o} and at 20^{o}.

In the series to which this report refers it was therefore decided that after the initial examination colony counts and other tests would not be made until the fourth day of storage at 5^{o} and, because of the relatively good bacteriological quality of many of the samples the storage period would be extended to 5 days. Furthermore determination of the predominant groups of organisms in the microfloras before and after storage was considered worthwhile.

The value of dye reduction tests and colony counts on raw milks after pre-incubation for detecting poor hygiene in production is currently under investigation in the U.K.; samples were therefore

also pre-incubated at 20° for 17h before applying bacteriological and biochemical tests.

Determination of pyruvate using a manual spectrophotometric method were made at NIRD for comparison with the automated fluorimetric and colorimetric methods employed at Kiel.

6.1.2 Pasteurized milks

The object of this trial, concerning bottled pasteurized milk from three local dairies, was to determine whether increases in pyruvate (Δ pyruvate) values during storage of the milks at 10° for 2 days (48±1h), for example, could measure or indeed predict any deterioration in the bacteriological quality of the milks at this temperature, the quality being judged by increases in total and psychrotrophic counts.

Based upon previous reports (Thomas, 1969; Page 90 of this report) it has been assumed that pasteurized milk in which the bacterial count has reached $\geq 10^6$/ml is unacceptable because of the risk of taints or other defects of bacterial origin.

Preserved samples of the bottles milks were analysed for pyruvate in Kiel by both the automated fluorimetric and colorimetric methods. Samples were also deep-frozen for up to 6 weeks and analysed by the automated fluorimetric method at NIRD.

6.2 Experimental Methods

6.2.1 Milk samples and pre-test storage

6.2.1.1 Raw milks

Twelve refrigerated bulk tank milks (four/week) were examined. Three of these were from NIRD farms, the remainder from local commercial farms, chosen for convenience in collecting samples. All the farms were on everyday collection, and the samples taken were of mixed evening and morning milk at $\leq 7°$. After at least two

minutes agitation of the bulk tank milk, a 500ml sample was taken into a sterile bottle using a dipper, then transported to the NIRD in an insulated box at refrigeration temperature.

On arrival in the laboratory, 50ml milk was placed in a sterile bottle and stored in iced water for subsequent incubation at 20° for 17h. Another 60ml was removed and stored in iced water, to be used for the initial plating, pyruvate determination, and for the somatic cell count. A sediment test was carried out. The remaining milk was placed in a sterile flask and transferred to an incubator at $5^{\circ} \pm 1^{\circ}$, to be reexamined bacteriologically and for pyruvate after storage for 4 and 5 days. For transport to Kiel for pyruvate and lactate determinations, 4ml milk was placed in a labelled plastic tube and an equal volume of 1M perchloric acid was added.

6.2.1.2 Pasteurized milks

Thirty bottles of milk at the point of "door-step" delivery, 10 from each dairy, were tested. The samples from dairies A and C had been cold-stored for 24h and those from dairy B for 48h after pasteurization.

After refrigerated transport to NIRD and the initial plating, portions were deep-frozen for subsequent pyruvate determination. The milks were stored at 10° and were re-examined after 2,3 and 4 days. Milks, initially and after 2d at 10° were despatched to Kiel for pyruvate determinations; 4ml-samples in labelled plastic tubes were preserved by the addition of 2ml of 10% trichloracetic acid.

6.2.2 Bacteriological analyses (Ministry of Agriculture, Fisheries & Food, 1968)

The following bacteriological tests as indicated were carried out on the samples initially, after storage at $5^{\circ} \pm 1^{\circ}$ or $10^{\circ} \pm 1^{\circ}$, and after incubation at 20°. The pour plate method and serial dilutions made in quarter strength Ringer's solution were used for all colony counts.

6.2.2.1 Total Count (TC)

Plate Count Agar (PCA) was used, and the plates (in duplicate) were incubated at $30^{\circ} \pm 1^{\circ}$ for 72h.

6.2.2.2 Coli-aerogenes (Coliform) Count (CAO)

Violet-red-bile-agar (VRBA) was used, and the plates were incubated at $30^{\circ} \pm 1^{\circ}$ for 24h.

6.2.2.3 Psychrotrophic Count (PC)

PCA was used and the plates (in duplicate) were incubated at $5^{\circ} \pm 1^{\circ}$ for 10d.

6.2.2.4 Thermoduric Count (LPC)

Sterile test tubes, containing 10ml milk, were immersed in a water bath at $63.5^{\circ} \pm 0.5^{\circ}$ for 33 min. After rapid cooling to 17° - 18° the milk was plated on PCA. The plates were dried at 37° for 30 min to minimise the incidence of spreading colonies and were then incubated at 30° for 3d.

6.2.2.5 Total Aerobic Spore Count (TS)

This test was not carried out on the milks incubated at 20° for 17h. 4ml milk in a small sterile screw cap bottle were immersed in water at $80^{\circ} \pm 0.5^{\circ}$ for 10 min, then cooled, plated on PCA, dried as above and incubated at 30° for 3d.

6.2.2.6 Streptococcal Count (SSM)
(NIRD Report 1971-72)

Streptococcal Selective Medium (Difco Brain Heart Infusion Agar + 0.1% thallous acetate + 1 ppm crystal violet) was used and duplicate plates were prepared. One set of plates was incubated at 30° for 3d to detect mastits and faecal streptococci. The other set was incubated at 45° for 3d (in plastic bags) to detect faecal streptococci only.

6.2.2.7 Resazurin Test
(Hygiene Test for England and Wales)

The initial but not the stored samples were refrigerated for 24h prior to testing. Disc readings[+)] were taken after 2 and 2 1/2 h incubation at 37°. The milks were then examined half hourly until reduction was complete or read again after 6h.

+)
6 = lilac blue; O = colourless; 1/2 = partial decolorization

6.2.2.8 Methylene Blue (MB) Test
(Statutory test for consumer milk in England and Wales)

After pre-incubation at 18° for 18h, 10ml milk with MB added is incubated at 37°; MB reduction within half-an-hour constitutes a failure.

6.2.2.9 Alcohol Precipitation Test (APT)

One ml milk was added to 1ml reagent (68% alcohol + 0.01% bromo-cresol-purple); the mixture was examined for precipitation and acid. Absence of precipitation was recorded as a negative result.

6.2.3 Somatic cell counts

All counts were determined using a Coulter Counter model F, and the IDF (1971) method.

6.2.4 Examination of microfloras

Colonies were picked for identification from the TC plates of the initial samples, the samples held at 5° for 5d and the samples incubated at 20° for 17h.

Ten colonies, chosen at random, were picked off into Yeast Dextrose Broth. After incubation at 30° for 24h, each broth was streaked onto the surface of a dried Yeastrel Milk Agar plate; after incubation at 30° for 24h, the streaks were examined for purity and

any showing mixed growth were discarded. Each culture was Gram stained and the Gram reaction and morphology of the culture noted. Gram-negative organisms were inoculated into MacConkey Broth (MCB) and incubated at 30° for 48h. The catalase reaction was determined by mixing some growth with a drop of H_2O_2 on a slide. Formation of gas bubbles was recorded as a positive reaction. These results were used to classify the organisms into the groups shown in the following table.

Classification of cultures isolated from TC plates

Morphology	Gram reaction	Catalase reaction	McB	Presumptive Groups
Cocci	+	+	NA	Micrococci
Cocci	+	-	NA	Streptococci
Non-sporing rods	+	+	NA	Coryneforms
Sporing rods	+	+	NA	Spore-formers
Rods- or cocco-bacilli	-	+	-	Gram -ve rods
Rods- or cocca-bacilli	-	+	+	Coliforms

NA = test not applicable

6.2.5 Biochemical analyses

6.2.5.1 Pyruvate

6.2.5.1.1 NIRD colorimetric manual method (NIRD)

6.2.5.1.2 Kiel colorimetric automated method (Kc)

6.2.5.1.3 Kiel and NIRD fluorimetric automated method (Kf and NIRDf)

6.2.5.2 Lactate

6.2.5.2.1 Kiel fluorimetric automated method

Details of these methods are given in Appendix

6.3 Results and discussion

6.3.1 Raw milks

6.3.1.1 Bacteriological results

6.3.1.1.1 Initial values

6.3.1.1.1.1 Total counts (TC)

The milks were of good bacteriological quality; all were $< 10^5$/ml and only three exceeded 7×10^3/ml (Table 1).

TABLE 1. Results for initial milk samples examined at 0h

Sample	Initial log colony count/ml						Somatic cell count/ml x 1000	Pyruvate (ppm)			Lactate (ppm)
	TC	PC	SSM 30°	CAO	LPC	TS		Kiel (F)	Kiel (C)	NIRD	
A	3.0	1.7	c.2.5	0.8	1.9	1.7	228	0	NR	1.8	8
B	3.7	2.9	c.2.8	0.3	2.4	1.6	262	0	NR	2.2	9
C	4.0	2.6	3.9	1.6	2.5	1.7	395	0	NR	2.1	10
D	3.4	2.3	3.1	1.3	2.5	2.1	308	0	NR	1.8	9
E	3.5	2.3	c.2.0	1.5	1.3	0.9	378	0	0.8	2.2	10
F	3.8	3.0	3.3	1.5	3.1	0.9	388	0	1.6	2.3	12
G	4.9	3.3	c.4.9	1.7	2.3	0.0	756	0	1.5	1.2	16
H	3.4	1.7	c.2.9	1.4	2.3	1.8	490	0	1.1	2.1	13
J	2.9	2.3	c.2.5	0.6	1.7	0.6	218	0	1.0	2.4	4
K	4.0	2.1	.3.9	1.6	1.2	1.0	609	0.6	1.5	2.5	14
L	3.7	3.5	c.2.0	1.6	3.0	2.1	261	0	0.9	2.5	8
M	3.2	2.7	c.2.0	1.5	1.2	0.7	321	0.4	1.4	2.5	7

NR = no result

TC = total count

PC = psychrotroph count

SSM 30° = selective streptococcal count at 30°

CAO = coliform (coli-aerogenes) organisms

LPC = Laboratory pasteurised count

TS = total spores

Figure 16 (a,b) shows the predominant groups comprising the microflora obtained by isolating colonies from the TC plates. In eight of the samples, micrococci comprised 50% or more of the microflora, in three samples, (C,G. & K), streptococci were predominant ($>$ 70%), three samples, J, L & M, contained $\geq$ 33% of Gram -ve rods, and the remaining sample (F) contained micrococci, streptococci, coryneforms and Gram -ve rods.

6.3.1.1.1.2 Streptococcal counts (SSM counts)

The counts of faecal streptococci (SSM at 45°) were all $<$ 400/ml and most were $<$ 100/ml and need not be considered (Table 2). In three samples (C,G & K), mastitis streptococci accounted for 85 % of the total count; these three had the highest total counts and it is of interest that streptococci were responsible (Table 1; Fig. 16 (a,b)).

6.3.1.1.1.3 Psychrotroph counts (PC)

Only three milks (F,G. & L) had counts of $>10^3$/ml (Table 1).

6.3.1.1.1.4 Coliform counts (CAO)

Counts were all $<$ 100/ml (Table 1).

6.3.1.1.1.5 Thermoduric counts (LPC)

Ten samples had counts $<$ 500/ml, samples F & L had counts of $\underline{c}$ 1000/ml. Sporeformers were isolated only in sample A (Table 1).

6.3.1.1.1.6 Resazurin test (RT)

Resazurin readings after 2 h and 2 1/2 h were all disk 5 or 5 1/2, except for G (4 1/2 after 2 1/2 h) (Table 3).

6.3.1.1.2 After storage at 5° for 4 and 5 days

6.3.1.1.2.1 Total and psychrotroph counts (TC and PC)

TC and PC were of a similar order after both 4 and 5 days at 5°

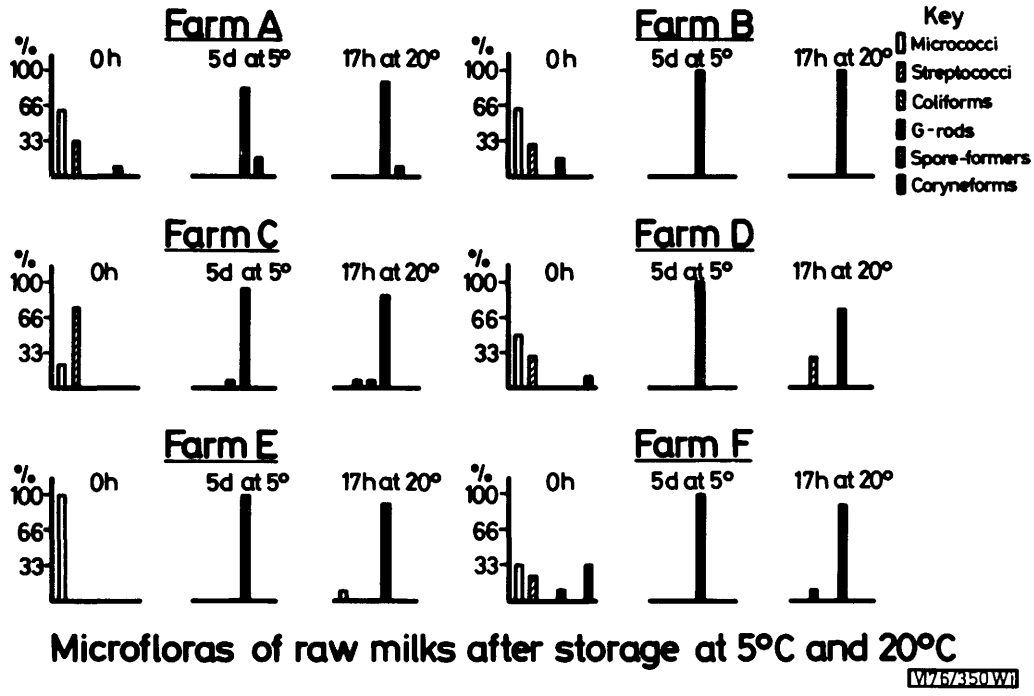


Fig. 16 a

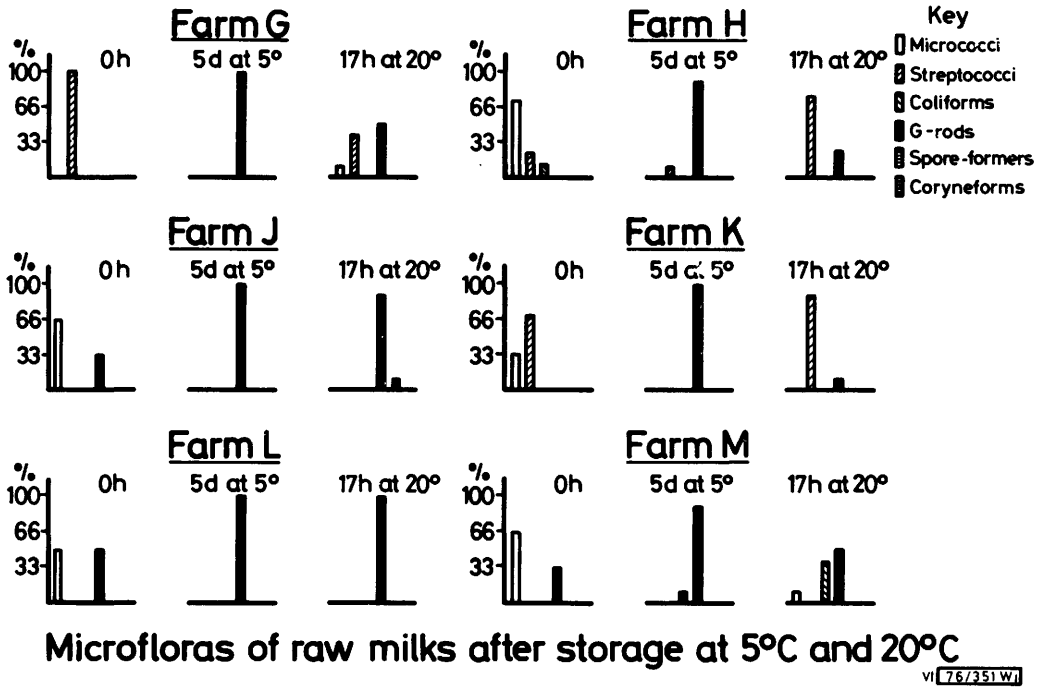


Fig. 16 b

TABLE 2. Table showing change in streptococcal count after storage at 20° for 17h

Sample	Log colony count/ml at 0h		Log colony count/ml after 17h at 20°	
	SSM 30°	SSM 45°	SSM 30°	SSM 45°
A	c 2.5	<2.0	3.2	2.8
B	2.8	<2.0	4.9	4.0
C	3.9	2.5	3.8	3.0
D	3.1	2.0	4.1	3.9
E	c 2.0	1.0	3.4	3.3
F	3.3	1.8	4.4	3.9
G	4.9	<1.0	6.3	2.7
H	2.9	1.5	4.5	4.5
J	c 2.5	1.5	2.8	2.6
K	3.9	1.0	5.7	5.6
L	c 2.0	<1.0	4.2	3.4
M	c 2.0	<1.0	2.8	2.5

SSM 30° = selective streptococcal count at 30°

SSM 45° = " " " " 45°

TABLE 3. Resazurin readings after 2 h at 37° on milk samples

Sample	at 0 h	after 4d/5°	after 5d/5°	after 17h/20°
A	5½	5½	5½	5½
B	5½	5½	5½	3½
C	5½	5½	4	3½
D	5½	5½	5½	3½
E	5½	5½	5½	5½
F	5½	5½	½	0
G	5½	5½	3	½
H	5½	5½	5½	½
J	5½	5½	5½	5½
K	5	5½	5½	4½
L	5½	3½	0	3½
M	5½	5½	3½	5½

(Fig. 1), but the effects of storage on counts were variable (Fig. 2 (a,b,c); Table 4 & 5)). After 4d, both TC and PC had increased by <10-fold in 5 samples (A,E,H,J & K), those of B & G by between 10 and 100-fold, whereas the remainder increased by >100-fold. After a further 24h storage, TC and PC had further increased on average about 10-fold in all samples. The counts of 4 milks, F, G, L & M, exceeded 10^7/ml (Fig. 2a,b,c).

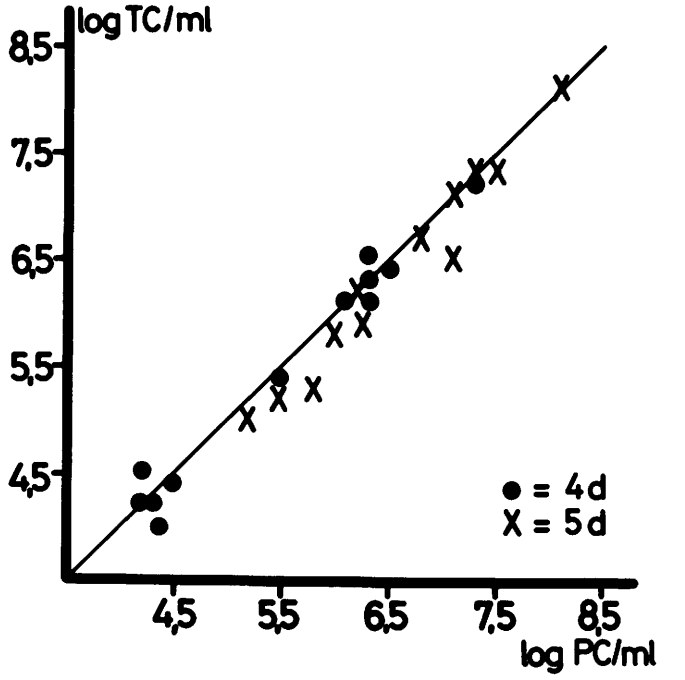
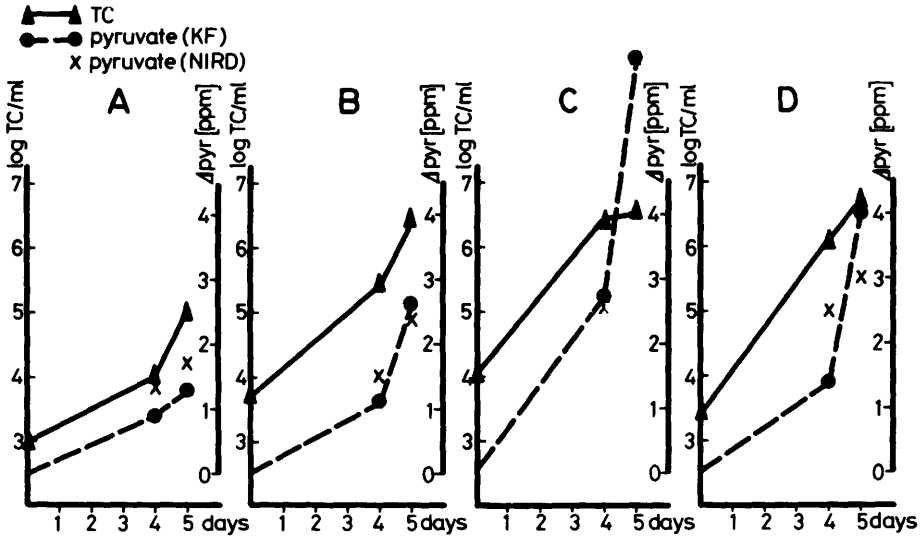


Fig. 1

6.3.1.1.2.2 Streptococcal counts (SSM)

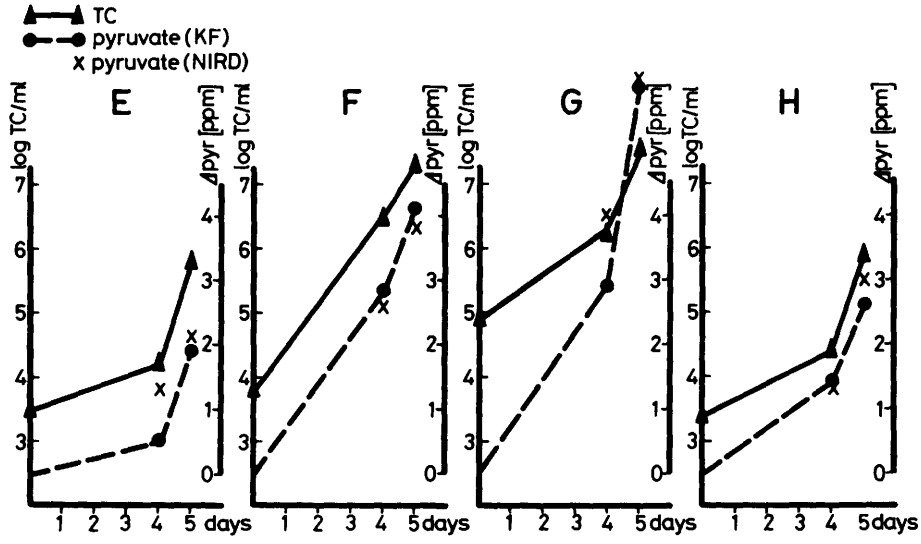
In most milks, the counts at 30° had decreased after 4 days at 5°, most markedly in samples C, G & K, which had the highest initial counts. Increases occurred in samples F & Lhowever and these two milks also showed bis increases in TC. There was little change after 5d at 5°, except that sample F showed a further marked increase and sample D also increased (Tables 4 & 5). These



Graphs showing increases in TC and pyruvate in individual raw milk samples during storage at 5°C

VI 76/354 W.i

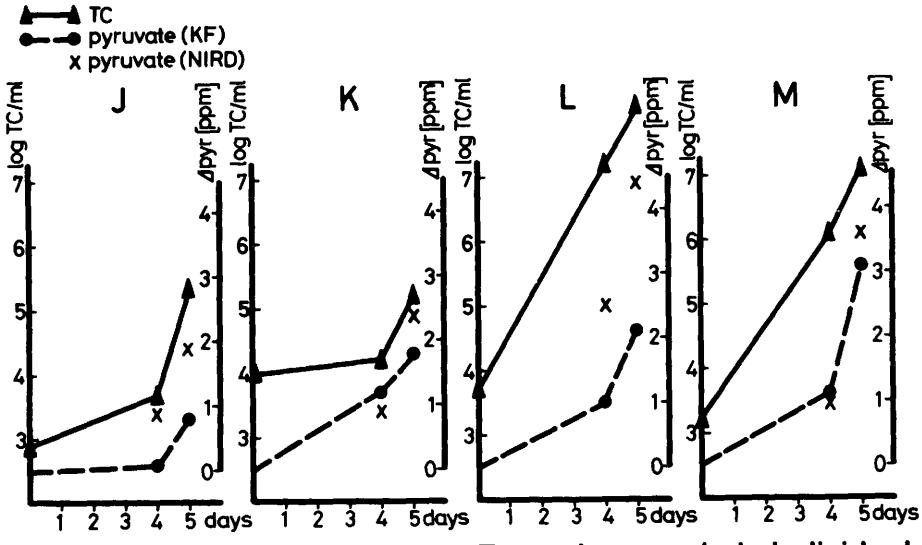
Fig. 2 a



Graphs showing increases in TC and pyruvate in individual raw milk samples during storage at 5°C

VI 76/352 W.i

Fig. 2 b



Graphs showing increases in TC and pyruvate in individual raw milk samples during storage at 5°C

VI 76/353 W.i

Fig. 2 c

TABLE 4. Results for milk stored at 5° for 4 days

Sample	Log colony count/ml				ΔPyruvate*(ppm)			ΔLactate**
					Kiel		NIRD	
	TC	PC	SSM 30°	CAO	(F)	(C)	(C)	(F) (ppm)
A	4.0	4.4	2.3	0.8	0.9	NR	1.4	-
B	5.4	5.5	2.6	2.6	1.1	NR	1.5	1
C	6.4	6.5	3.4	3.9	2.7	NR	2.6	4
D	6.1	6.1	2.9	3.1	1.4	NR	2.8	9
E	4.2	4.2	2.6	2.8	0.5	1.4	1.3	2
F	6.5	6.3	4.0	2.8	2.8	3.2	2.6	2
G	6.3	6.3	4.5	4.4	2.9	2.9	4.0	5
H	4.4	4.5	2.6	1.5	1.4	1.9	1.4	3
J	4.5	4.2	2.0	0.8	0.1	0.7	0.9	6
K	4.2	4.3	3.4	<1.0	0.6	1.0	0.9	8
L	7.2	7.3	3.5	4.1	1.0	1.7	2.6	3
M	6.1	6.3	1.4	4.2	0.7	1.3	1.1	5

*Δ Pyruvate = Increase in pyruvate value

**Δ Lactate = " " lactate "

NR = no result

TC = total count

PC = psychrotroph count

SSM 30° = selective streptococcal count at 30°

CAO = coliform (coli-aerogenes) organisms

TABLE 5. Results for milks stored at 5° for 5 days

Sample	Log colony count/ml				ΔPyruvate* (ppm)			ΔLactate**
					Kiel		NIRD	
	TC	PC	SSM 30°	CAO	(F)	(C)	(C)	(F) (ppm)
A	5.0	5.2	2.0	1.4	1.3	NR	1.7	6
B	6.2	6.2	3.0	3.6	2.6	NR	2.4	0
C	6.5	7.1	3.2	4.9	6.4	NR	6.4	3
D	6.7	6.8	4.1	4.5	4.0	NR	3.0	2
E	5.8	6.0	c<2.0	3.8	1.9	2.6	2.1	0
F	7.3	7.3	5.5	4.1	4.1	3.6	3.8	-7
G	7.3	7.5	3.5	5.7	6.0	5.4	6.1	3
H	5.9	6.3	c 2.0	2.4	2.6	2.4	2.5	3
J	5.3	5.8	2.3	1.7	0.8	1.1	1.9	6
K	5.2	5.5	3.7	2.2	1.2	1.5	2.4	8
L	8.1	8.1	3.1	5.1	2.1	2.7	4.4	2
M	7.1	7.1	1.4	5.1	2.7	3.5	3.6	5

* ΔPyruvate = Increase in pyruvate value

** ΔLactate = " " lactate "

NR = no result

TC = total count

PC = psychrotroph count

SSM 30° = streptococcal selective count at 30°

CAO = coliform (coli-aerogenes) organisms

results indicate that for milks stored for 4d or more at 5^{o} counts on SSM at 30^{o} are unreliable for indicating the number of mastitis streptococci initially present in the refrigerated milks. Counts of faecal streptococci did not increase at 5^{o} except after 5 days in one sample, K.

6.3.1.1.2.3 Coliform counts (CAO)

After 4d at 5^{o}, changes in counts were highly variable but most had increased, notably those of G, L & M which rose to $>10^{4}$/ml. There was a further increase after 5d at 5^{o} in all samples and some, those of G, L & M were quite marked, indicating the presence of psychrotrophic coliforms (Table 5).

6.3.1.1.2.4 Thermoduric counts (LPC)

These showed little change.

6.3.1.1.2.5 Resazurin Test (RT)

With the exception of sample L, which had the highest TC, after 4d at 5^{o} the resazurin disk numbers were similar to the readings on the milks initially while after 5d samples F & L reduced resazurin to colourless in 2h, and C, G & M showed a deterioration as compared with the result after 4d. Table 3 indicates that milks having a TC $>10^{7}$/ml after refrigerated storage are likely to reduce resazurin to the pink stage in 2 - 2 1/2 h, although sample D was an exception.

6.3.1.1.3 After incubation at 20° for 17 h

6.3.1.1.3.1 Total count and psychrotroph count (TC & PC)

Except for samples A, E & J which had TC and PC of $<10^6$/ml, there were quite marked differences between these two counts, the PC tending to be the lower (See Fig. 3). Milks showing the least increase in both TC and PC after incubation

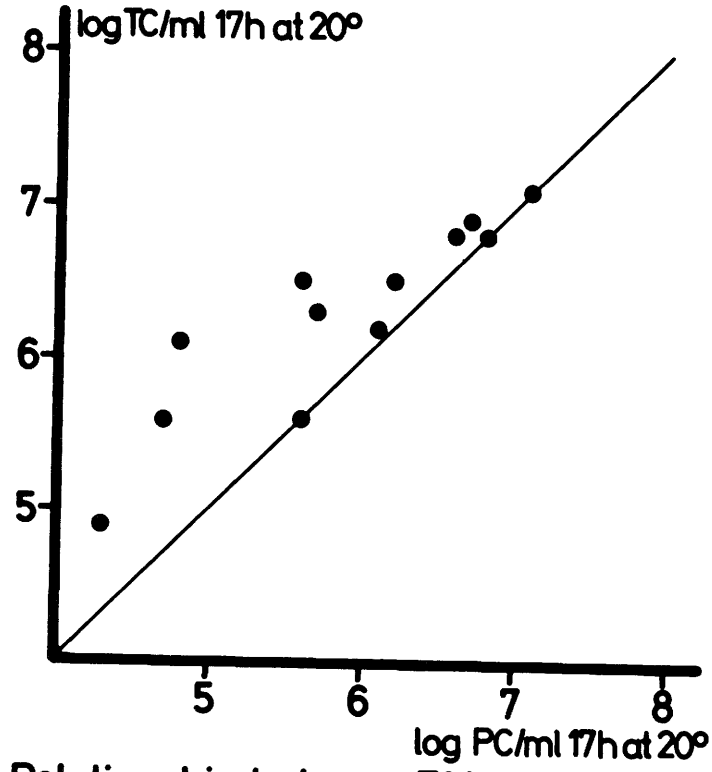
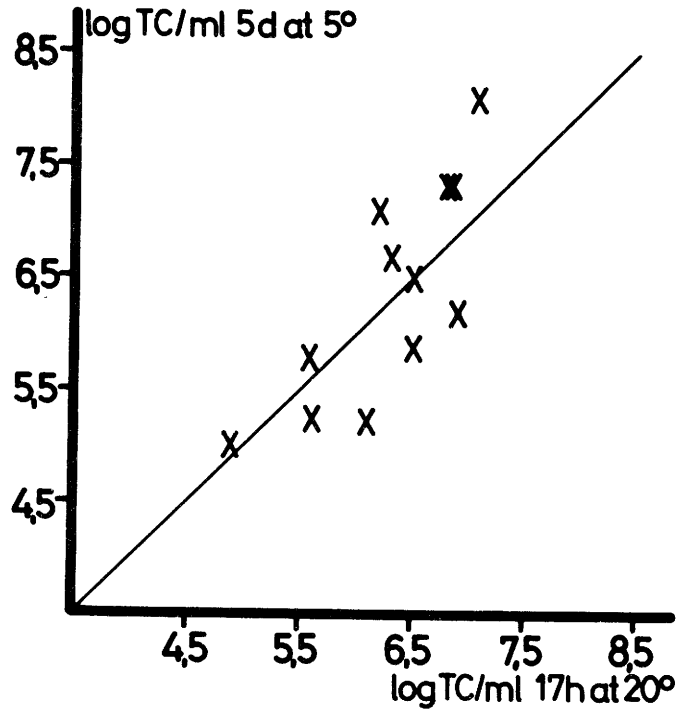


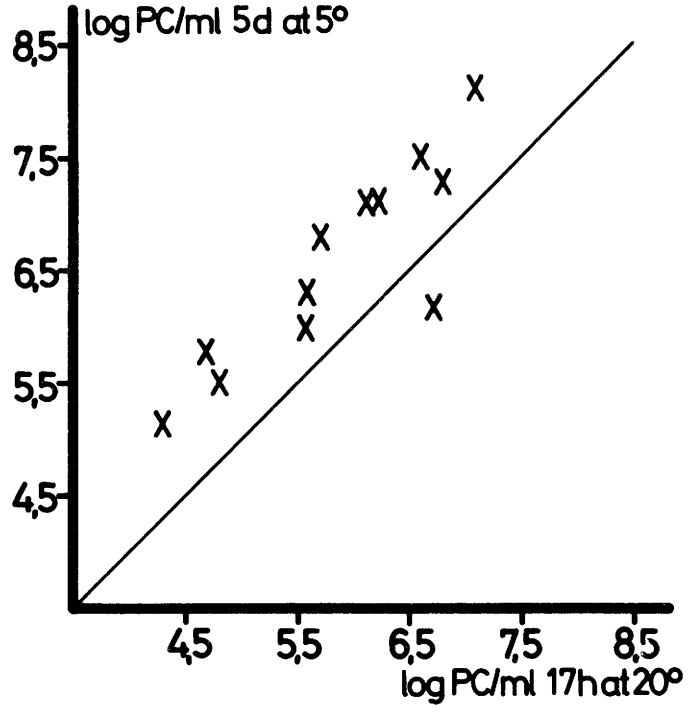
Fig. 3

at 20° for 17 h also showed the least increase in TC and PC on storage at 5° for 5 d (See Figs. 4 & 5; Tables 5 & 6).



Relationship between TC/ml after 5 days at 5°C and 17 hours at 20°C VI 76/359 Wi

Fig. 4



Relationship between PC/ml after 5 days at 5°C and 17 hours at 20°C VI 76/360 Wi

Fig. 5

TABLE 6. Results for milks stored at 20° for 17 h

Sample	Log colony count/ml				ΔPyruvate* (ppm)			ΔLactate**
					Kiel		NIRD	(F)
	TC	PC	SSM 30°	CAO	(F)	(C)	(C)	(ppm)
A	4.9	4.3	3.2	4.4	0.5	NR	1.2	1
B	6.9	6.7	4.9	3.6	1.4	NR	2.1	4
C	6.5	6.2	3.8	5.3	1.6	NR	1.7	4
D	6.3	5.7	4.1	5.3	1.4	NR	1.7	5
E	5.6	5.6	3.4	<3.0	0.1	1.3	1.9	3
F	6.8	6.8	4.4	4.6	1.8	2.3	2.8	8
G	6.8	6.6	6.3	4.9	1.7	2.1	2.0	27
H	6.5	5.6	4.5	<3.0	1.0	1.4	1.4	7
J	5.6	4.7	2.8	3.7	0.8	0.6	0.5	2
K	6.1	4.8	5.7	<3.0	1.1	1.2	1.2	14
L	7.1	7.1	4.2	5.9	1.3	1.7	2.6	2
M	6.2	6.1	2.8	5.5	0.9	1.4	2.3	5

* ΔPyruvate = increase in pyruvate value

** ΔLactate = " " lactate "

NR = no result

TC = total count

PC = psychrotroph count

SSM 30° = selective streptococcal count at 30°

CAO = coliform (coli-aerogenes) organisms

6.3.1.1.3.2 Streptococcal counts (SSM)

At 20°, faecal streptococci showed a marked increase, notably in samples D, F, H & K (Table 2) and in H & K the total SSM count consisted almost entirely of faecal streptococci. In samples B, F, G and L, the mastitis streptococci multiplied rapidly, while in the remaining 4 samples there was no significant increase. It is evident that where milk is stored at temperatures permitting the growth of faecal streptococci a count at 30° on SSM is not specific for mastitis streptococci.

6.3.1.1.3.3 Coliform counts (CAO)

In 3 samples (E,H & K), coliforms failed to multiply appreciably and counts of <1000/ml were recorded. In the remainder the increases were variable and bore no relationship to the initial counts (Tables 1 & 6).

6.3.1.1.3.4 Thermoduric counts (LPC)

These counts showed little change from the initial counts and are not shown.

6.3.1.1.3.5 Resazurin Test (RT)

Three milks, F, G & H, reduced resazurin completely in 2h. Only samples A, E, J & M did not reduce the dye to the pink stage ($\leq$ 3 1/2) and these milks, with the exception of M, had counts of $<10^6$/ml (Tables 3 & 6) at the time of the test.

6.3.1.1.4 Microfloras of the milks after storage at 5^{o} and at 20^{o}

The histograms in Fig. 16 (a,b) of results obtained by isolating colonies from TC plates, show that after 5d at 5^{o} Gram -ve rods were the predominating group in all samples, comprising 100% of the microflora in eight of them. After 17h at 20^{o} this group formed $\leq$ 50 % of the microflora in ten samples but streptococci were present in appreciable numbers in two of these, D & G, as were coliforms in M. Streptococci predominated in H & K and in both these samples the total counts exceeded the psychrotroph counts by about 10-fold (Table 6).

6.3.1.1.5 The error of the plate count

From 4 milk samples, 10 replicate platings were made, duplicate plates being poured from appropriate dilutions. The following standard deviations (s) and coefficents of variation (CV) were calculated for the 4 samples.

Sample	Mean Count/ml	S	CV %
A	6.85×10^{6}	13.58×10^{5}	19.82
B	1.14×10^{6}	8.66×10^{4}	7.58
C	8.8×10^{5}	9.18×10^{4}	10.40
D	5.3×10^{4}	6.02×10^{3}	11,24

These results indicate that the error of the plate counts as determined for this work are well within normally accepted limits (Wilson, 1935).

6.3.1.2 Somatic Cell Counts (cells)

Cell counts were all $\leq$ 500,000/ml, except for milks G & K, both of which contained a high proportion of mastitis streptococci; however sample C, similar to G & K in this respect, did not have an unduly high cell count (392,000/ml) (Table 1). There was a relationship between cell count and initial streptococcal count (Fig. 6) but they were not highly correlated.

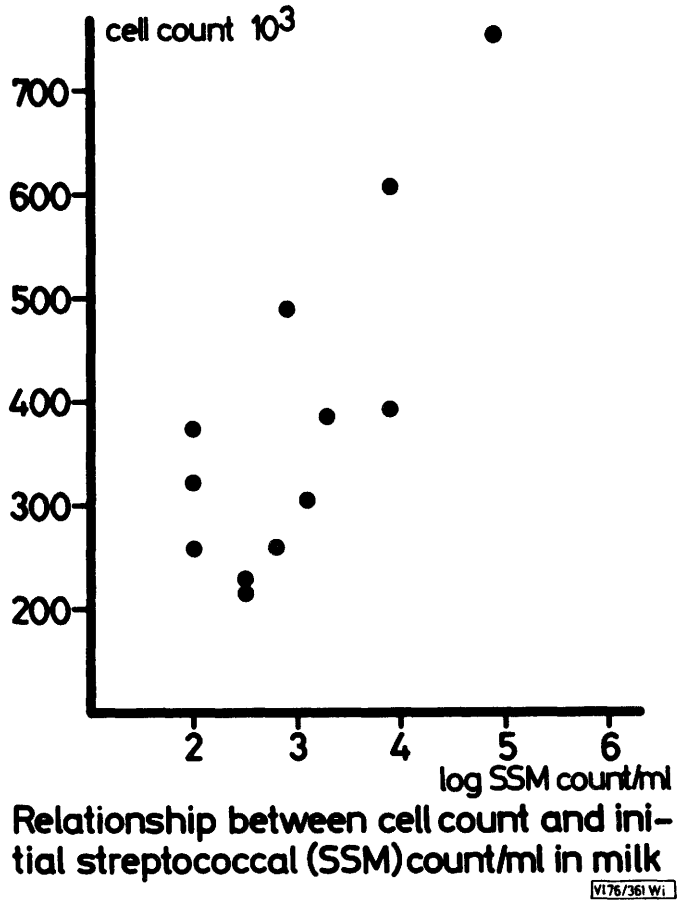


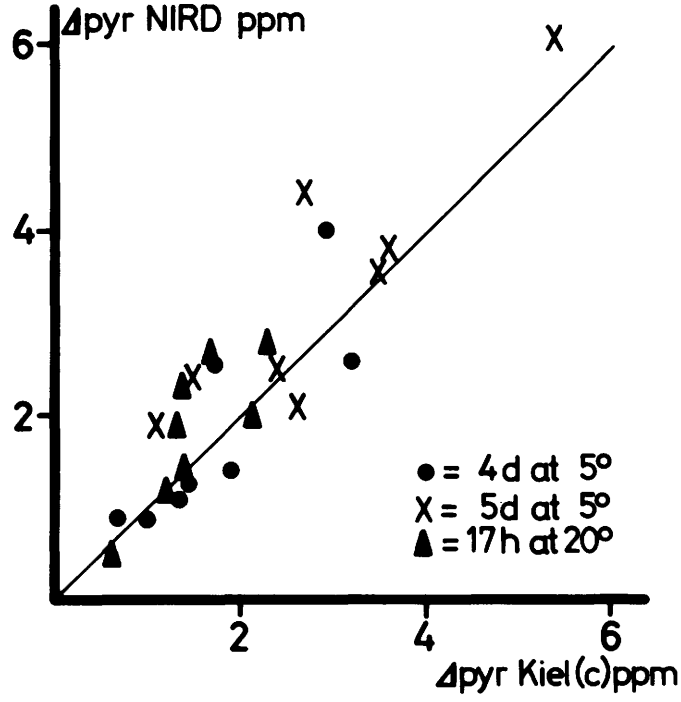
Fig. 6

This is not surprising because streptococcal infections are not the only cause of high somatic cell counts. No attempt was made to determine the incedence of coagulase +ve staphylococci in the milks and it is possible that staphylococcal infections may also have influenced cell counts.

6.3.1.3 Biochemical Results

6.3.1.3.1 Pyruvate

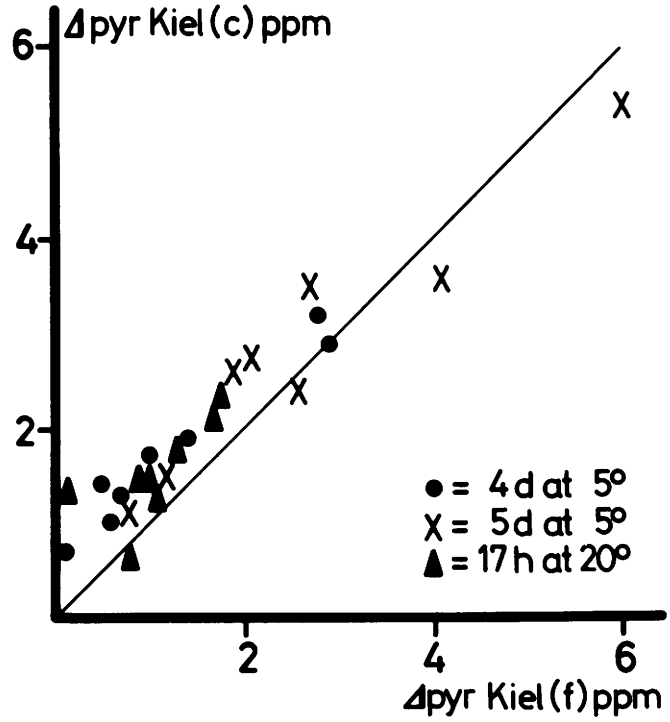
The values obtained by the different methods (see 6.2.5.1 and Appendix) applied to the initial and stored milk samples were compared by plotting graphs of the results. There was no agreement between the absolute values obtained on the initial milks by the two colorimetric methods or between the fluorimetric and colorimetric initial (absolute) values and there is no explanation for the discrepancies. However the two colorimetric methods were in good agreement for the Δ pyruvate values, i.e. measurement of increase in pyruvate, after storage of the milks at 5° and 20° (Fig. 7); the Kiel fluorimetric and colorimetric Δ pyruvate values showed very close agreement (Fig. 8). For the most part Kf values only will be considered in relation to the bacterial counts.



Relationship between Δpyruvate values given by the NIRD manual method and the Kiel automated colorimetric method

VI 76/376 WI

Fig. 7



Relationship between Δ pyruvate values given by the Kiel automated fluorimetric method and the Kiel automated colori - metric method VI 76/375 W1

Fig. 8

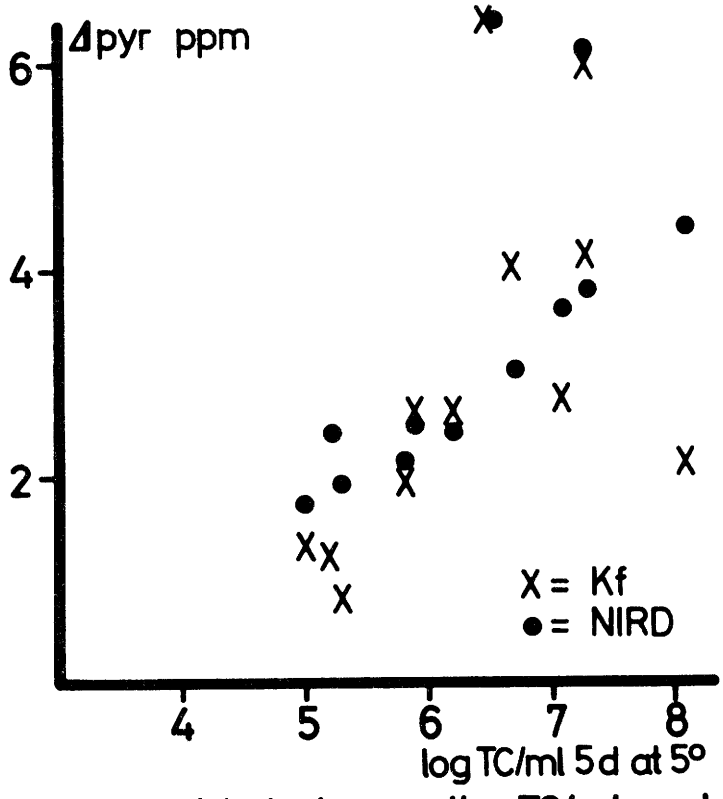
6.3.1.3.1.1 Relationship of initial pyruvate to initial status of the milks

The pyruvate values, Kf, were all 0 except for two samples K (0.6 ppm) and M (0.4 ppm); as expected these higher values were not related to initial bacterial or somatic cell counts. The colorimetric values, Kc and NIRD, were more variable and were also unrelated to the initial status of the milks (Table 1).

6.3.1.3.1.2 Relationship of Pyruvate (Δ values) to total and psychrotroph colony counts after storage at 5^{o} for 4 and 5 days

Fig. 2 (a,b,c) shows that, with the exception of J, after 4d there was an increase of $\leq$0.5 ppm in all samples and this occurred, even where in 4 milks, A, E, H & K, the total counts were $<10^{5}$/ml. Presumably during this time, the bacterial cells were metabolising and producing pyruvate although not multiplying

rapidly. However in milks B, D, L & M, the counts increased 50- to 1000-fold, but the increases in pyruvate were of the same order as for A, E, H & K, i.e. < 2ppm. Samples C, F & G with 100- to 1000-fold increases in TC and PC showed bigger increases in pyruvate (2-3 ppm). After 5 days storage, pyruvate levels increased further in all samples and, in milks C and G, reached 6.4 and 6.0 ppm respectively, although samples D and F, which had similar counts had Δ values of only 4.0 and 4.1 ppm respectively (Fig.2a, b,c). Sample L, which had a count $>10^8$/ml had a surprisingly low Δ pyruvate value, possibly because in milks in which very high bacterial counts have developed, other biochemical changes occurring may affect the analysis. The agreement between Δ pyruvate values (Kf) an total counts is fairly good, although a slightly better relationship is apparent if the NIRD pyruvate results are used (Fig. 9).



Relationship between the TC/ml and the Δpyruvate values (NIRD and Kf) after 5 days at 5°C

VI 76/374 W1

Fig. 9

6.3.1.3.1.3 Relationship of Δ pyruvate to total and psychrotroph colony counts after storage at 20° for 17h

There is some relationship between total counts and Δ pyruvate values (Fig. 10) but the scatter is large. Sample E had a very low value (0.1 ppm) and four samples B, H, L & M had relatively low pyruvate levels in relation to their total counts (Table 6). The relationship between the psychrotroph counts and pyruvate levels is even less good (Fig. 11). Thus in some cases the amount of pyruvate produced may be similar even though there is a discrepancy in count of more than 100-fold.

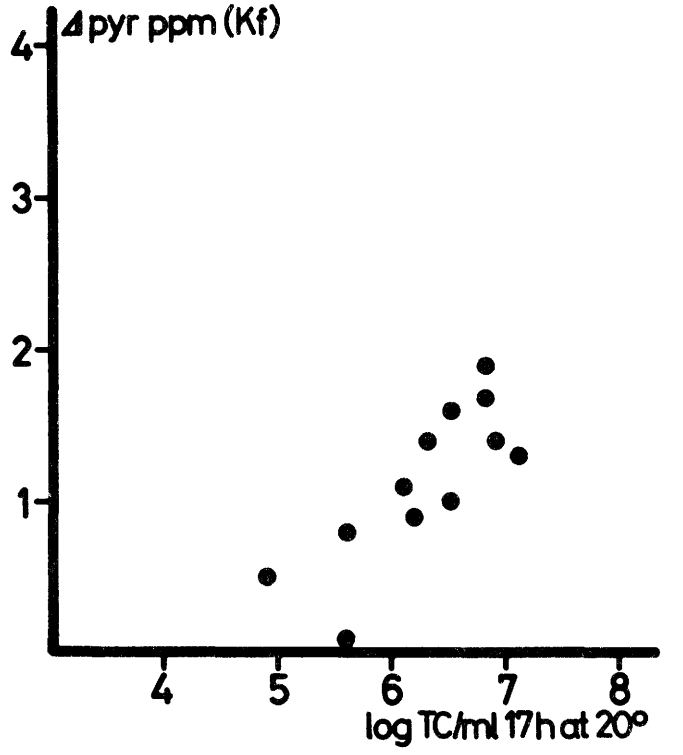


Fig. 10

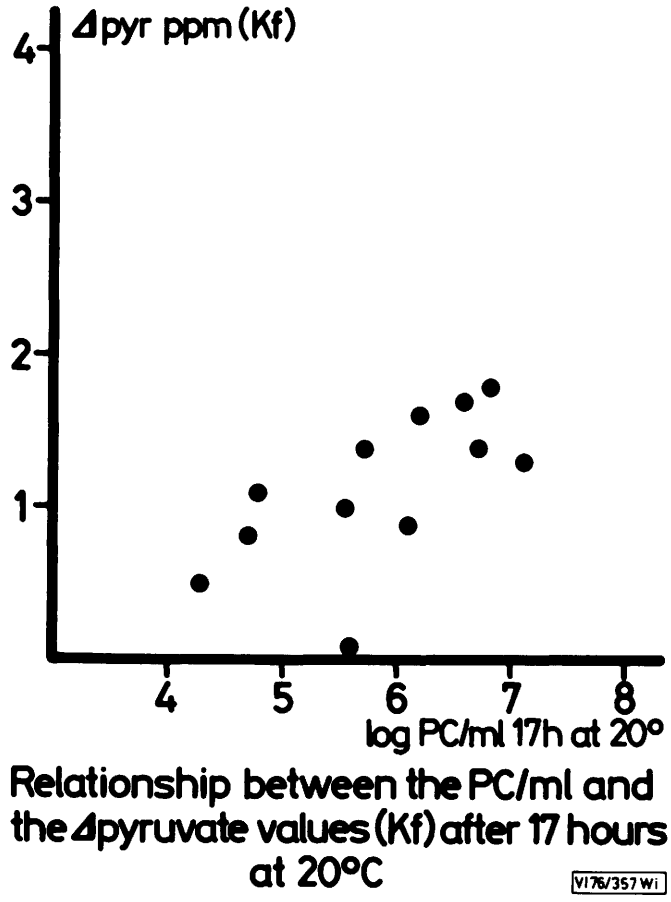
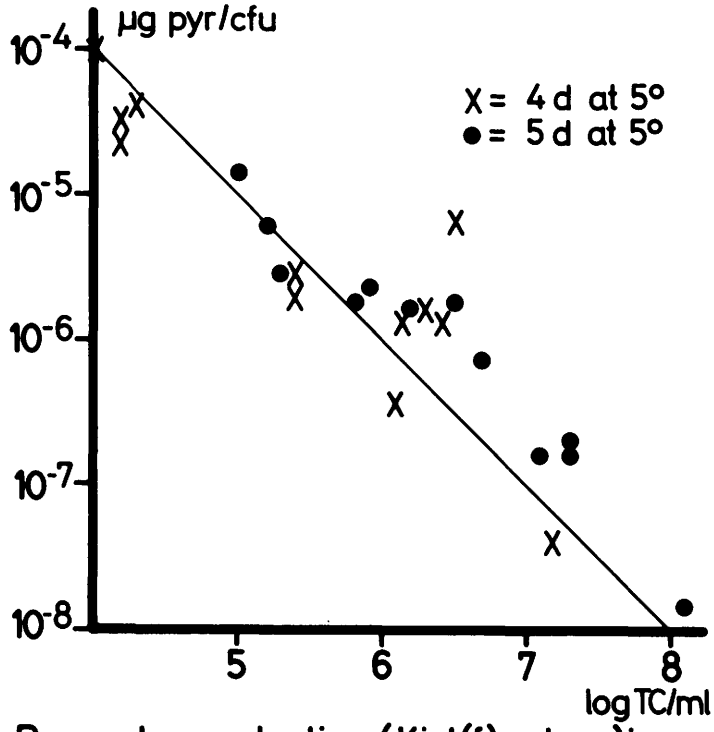


Fig. 11

6.3.1.3.1.4 Pyruvate values equivalent to one colony forming unit (CFU)

Storage of the samples at 5° to bring about increases in the total and psychrotroph counts was adopted to enable any relationships between counts and pyruvate to be studied under conditions relevant to bulk tank storage. From the results the amount of pyruvate produced per colony forming unit (CFU) has been calculated over the range of TC observed after 4 and 5d storage at 5°. Fig.12 shows that in accordance with the findings of Tolle & Heeschen (1975) pyruvate production/CFU is related to the total count and decreases as the total count increases. The reason for this is not clear but may involve rates of multiplication, diffusion gradients, the varied nature

of the microfloras or utilization of metabolites. The results expressed as CFU/0.1 ppm pyruvate produced in relation to the total count are shown in Table 7. After storage for 4d at 5° a mean log count of 5.41/ml gave a mean Δ pyruvate value of 1.34 ppm (Kf), i.e. 26,300 CFU produced 0.1 ppm pyruvate. After storage for 5d at 5° corresponding values for log total count/ml and mean Δ pyruvate were 6.37/ml and 3.0 ppm, or 78,000 CFU producing 0.1 ppm pyruvate.



Pyruvate production (Kiel(f) values) in relation to the number of colony-forming units

VI 76/373W1

Fig. 12

TABLE 7. Pyruvate production in relation to the number of colony-forming units (cfu) in milk stored at 5°

Sample	4d at 5° Increase in TC	Δ pyr (ppm)	Cfu giving 0.1ppm pyr	5d at 5° Increase in TC	Δ pyr (ppm)	Cfu giving 0.1ppm pyr
A	9×10^3	0.9	10^3	9.5×10^4	1.3	7.0×10^3
B	2.55×10^5	1.1	2.3×10^4	1.6×10^6	2.6	6.0×10^4
C	2.4×10^6	2.7	9.1×10^4	3.0×10^6	6.4	5.0×10^4
D	1.2×10^6	1.4	8.3×10^4	4.5×10^6	4.0	10^5
E	1.2×10^4	0.5	2.4×10^3	6.9×10^5	1.9	3.5×10^4
F	3.5×10^6	2.8	1.25×10^5	1.9×10^7	4.1	4.5×10^5
G	2.0×10^6	2.9	6.7×10^4	2.0×10^7	6.0	3.5×10^5
H	2.25×10^4	1.4	1.6×10^3	7.2×10^5	2.6	2.8×10^4
J	3.0×10^4	0.1	3.3×10^4	1.8×10^5	0.8	2.3×10^4
K	1.2×10^4	0.6	2.0×10^3	1.5×10^5	1.2	1.25×10^4
L	1.7×10^7	1.0	1.6×10^6	1.2×10^8	2.1	5.5×10^6
M	1.2×10^6	0.7	1.72×10^5	1.3×10^7	2.7	5.0×10^5
Mean	2.6×10^5	1.34	2.6×10^4	2.3×10^6	3.0	7.8×10^4

TC = total count

After storage at 20° for 17h, a mean log total count of 6.30/ml gave a mean Δ pyruvate value of 1.1 ppm (Kf), with 181,000 CFU producing 0.1 ppm pyruvate.

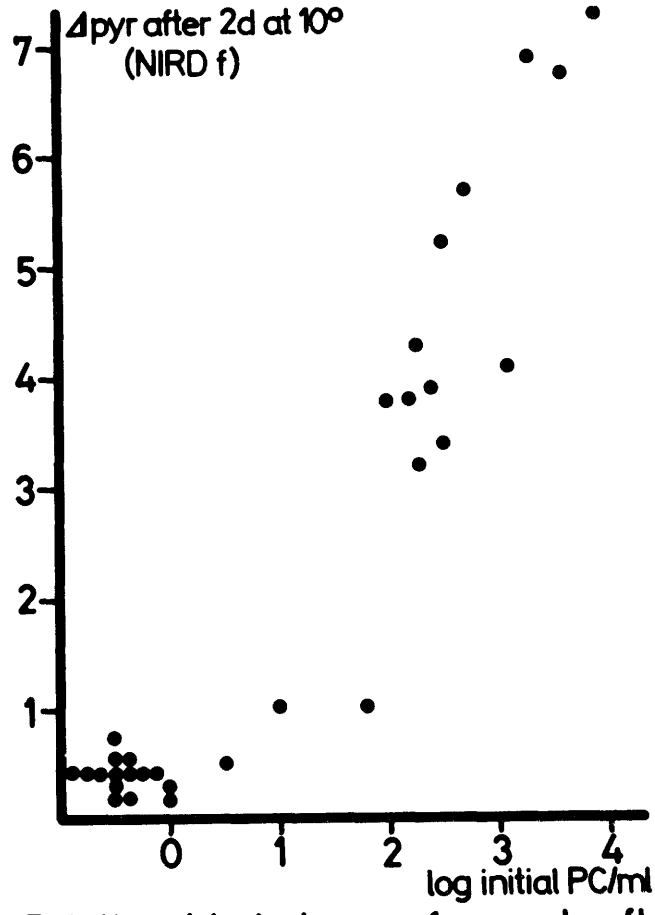
6.3.2 Pasteurized milks

The findings of this work are based on results obtained at NIRD only.

6.3.2.1 Initial biochemical and bacteriological status

Neither the initial pyruvate values nor the initial total or psychrotrophic counts were related (Table 8).

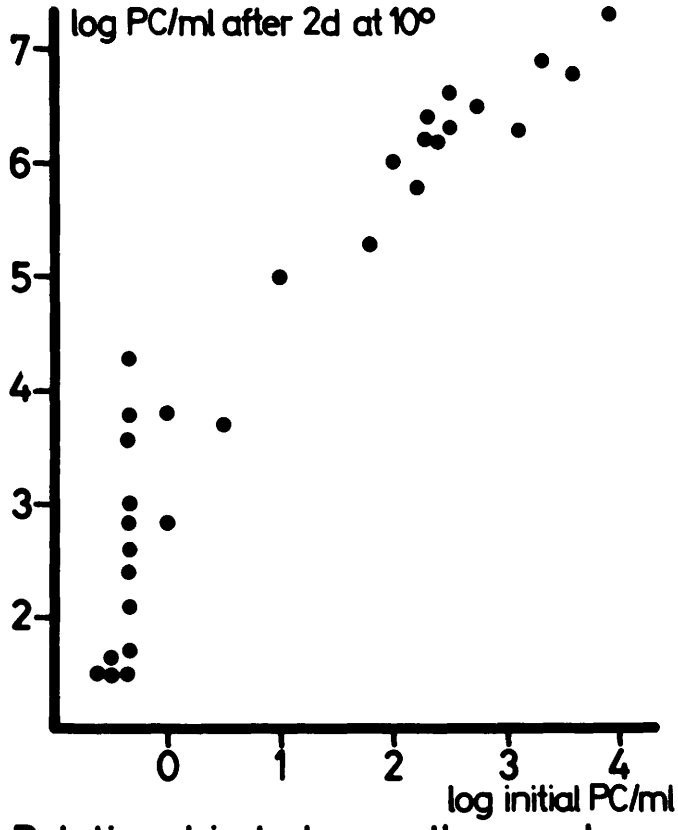
The initial pyruvate values and the initial total counts were poorly related to the total counts after storage at 10° (Table 8). However milks with initial psychrotroph counts of >100/ml, indicative of post-pasteurization contamination, all had Δ pyruvate values of >3 ppm after storage for 48 h at 10° (Fig.13), and the psychrotrophs had multiplied to give counts all of which were $>5 \times 10^5$/ml (Fig.14).



Relationship between Δpyruvate after 2 days at 10°C and the initial psychrotroph count of pasteurized milk VI76/372 Wi

Table 8. Colony counts and pyruvate values for pasteurized milk samples initially and after storage at 10° for 2 days

		Initial Status			2 days at 10°	
Dairy	Sample	Log TC/ml	Log PC/ml	Pyruvate NIRD(f)	Log Δ PC/ml	Δ Pyruvate NIRD (f)
	A	4.11	3.6	1.25	6.8	6.75
	B	3.57	2.7	1.1	6.5	5.7
	C	3.40	2.3	0.9	6.2	4.3
	D	3.54	2.5	0.75	6.3	5.2
A	E	3.73	3.1	1.25	6.3	4.1
	F	3.41	2.3	1.5	6.4	3.2
	G	3.51	2.2	1.1	5.8	3.8
	H	3.38	2.4	0.8	6.2	3.9
	J	4.58	3.9	1.6	7.3	7.3
	K	4.15	3.3	1.6	6.9	6.9
	L	3.72	1.0	1.8	5.0	1.0
	M	5.11	2.5	1.4	6.6	3.4
	N	4.68	1.8	1.25	5.3	1.0
	O	5.11	<0	1.4	4.3	0.4
B	P	5.11	2.0	1.4	6.0	3.8
	Q	4.30	<0	1.5	<4.0	0.4
	R	3.08	<0	1.2	<3.0	0.2
	S	3.67	<0	1.6	<3.0	0.4
	T	3.20	0	1.2	<3.0	0.2
	U	4.79	<0	1.3	<4.0	0.5
	C1	3.36	<0	1.6	1.7	0.4
	C2	3.32	<0	1.5	<2.0	0.2
	C3	3.28	0.5	1.4	3.7	0.5
	C4	3.46	<0	1.4	2.4	0.3
C	C5	3.46	<0	1.4	3.0	0.4
	C6	2.76	<0	1.5	<2.0	0.5
	C7	3.41	<0	1.3	<2.0	0.4
	C8	2.85	0	1.5	3.8	0.3
	C9	3.11	<0	1.9	<2.0	0.4
	C10	3.00	<0	1.9	2.7	0.7

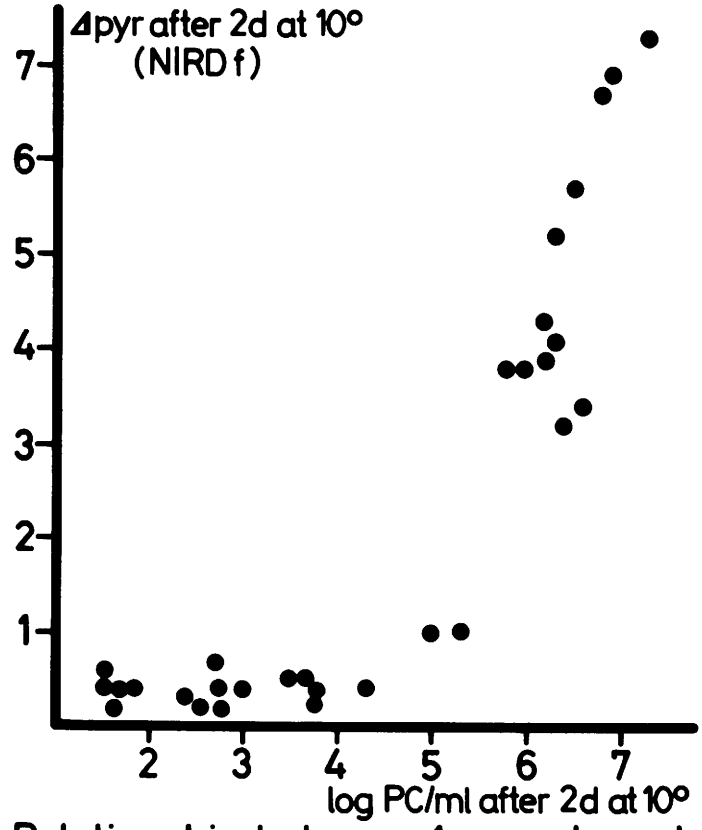


Relationship between the psychrotroph count of pasteurized milk initially and after 2 days at 10°C VI76/371Wi

Fig. 14

6.3.2.2 After incubation at 10° for 2 days

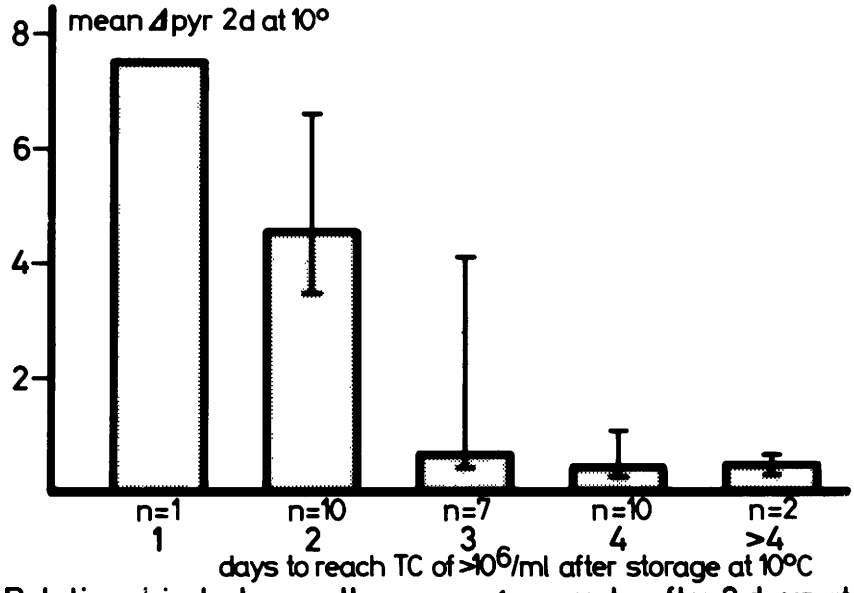
After storage at 10° for 2 days all samples with total and psychrotroph counts of $< 2 \times 10^5$/ml had Δ pyruvate values of < 2 ppm (Fig.15). Conversely Δ pyruvate values of > 3ppm corresponded to psychrotroph counts of $> 10^6$/ml (with one exception), and there was a relationship between Δ pyruvate values and counts. The histograms in Fig.16 show that where mean Δ pyruvate values were < 1 ppm after 2 days at 10°, the total count of the milks did not exceed 10^6/ml for at least 3 days (Table 9).



Relationship between Δpyruvate and the psychrotroph count of pasteurized milk after 2 days at 10°C

VI76/370 W1

Fig. 15



Relationship between the mean Δpyruvate after 2 days at 10°C and the number of days taken to give total counts exceeding 10^6/ml in pasteurized milk after storage at 10°C

VI76/369 W1

Fig. 16

Table 9. Relationship between mean Δ pyruvate after 2 days at 10° and number of days for total counts of pasteurized milk to exceed 10^6/ml after storage at 10°

Days at 10° to reach TC >10^6/ml	Mean Δ Pyruvate 2 days at 10°	No. of samples
1	7.5	1
2	4.4 (3.4-6.5)	10
3	0.6 (0.4-4.0)	7
4	0.4 (0.2-0.8)	10
>4	0.4 (0.3-0.5)	2

6.3.2.3 Comparison of Kiel and NIRD pyruvate results

Table 10 shows that although there was good agreement between initial fluorimetric pyruvate values, there was discrepancy between these values and those obtained colorimetrically (Kiel C). This difference which has also been observed with raw milks (Page 128 of this report) will doubtless disappear when the methods are standardised.

After storage at 10^{o} for 2 days, there were large differences between some of the NIRD (f) and Kiel (F) Δ pyruvate values, but Kiel (C) and Kiel (f) Δ values agreed well. Some of the factors which may affect markedly but inconsistently the results at higher levels of pyruvate have since become evident: eg. pyruvate values are increased by slow thawing of deep frozen samples as compared with rapid thawing, or holding samples for 2 h or more at room temperature as compared with storing them in ice while awaiting analysis.

6.4 Conclusions

6.4.1 Raw milks

6.4.1.1 The agreement between the absolute pyruvate values obtained by the three different methods was not good. This was probably due to a number of factors, especially inadequate preservation of samples and inadequate standardisation of analyses. These difficulties should be easily overcome. The methods were in better agreement if increases in pyruvate (Δ values) after storage of the samples at 5^{o} or 20^{o} were considered.

6.4.1.2 The samples were of everyday collected refrigerated bulk tank milk and initially had low total bacterial counts ($<10^{5}$/ml). After prolonged storage at 5^{o} the increases in total and psychrotrophic counts showed considerable variation which neither the initial counts nor the initial pyruvate values (by any of the three methods) predicted.

TABLE 10. Comparison of the pyruvate values obtained by Kiel and NIRD during the second trial of pasteurized milks, both initially and after storage at 10° for 2 days

Dairy	Sample	Initial Pyruvate Kiel C	Initial Pyruvate Kiel F	Initial Pyruvate NIRD F	Absolute Pyruvate after 2 days at 10° Kiel C	Absolute Pyruvate after 2 days at 10° Kiel F	Absolute Pyruvate after 2 days at 10° NIRD F	Δ Pyruvate after 2 days at 10° Kiel C	Δ Pyruvate after 2 days at 10° Kiel F	Δ Pyruvate after 2 days at 10° NIRD F
	A	2.25	1.75	1.25	3.6	2.9	8.0	1.35	1.15	6.75
	B	2.4	1.75	1.1	3.3	2.6	6.8	0.9	0.85	5.7
	C	1.6	0.9	0.9	3.1	2.55	5.2	1.45	1.65	4.3
	D	1.55	0.8	0.75	3.2	2.5	6.0	1.6	1.7	5.2
A	E	1.95	1.15	1.25	3.15	2.5	5.4	1.2	1.35	4.1
	F	1.95	1.3	1.5	2.95	2.45	4.7	1.0	1.15	3.2
	G	2.1	1.4	1.1	3.15	2.55	4.9	1.05	1.15	3.8
	H	2.0	1.45	0.8	2.85	2.4	4.7	0.85	0.95	3.9
	J	2.0	1.55	1.6	6.1	5.45	8.9	4.1	3.9	7.3
	K	2.0	1.55	1.6	5.15	4.25	8.5	3.15	2.7	6.9
	L	2.3	1.95	1.8	3.05	2.25	2.8	0.75	0.3	1.0
	M	1.85	1.7	1.4	4.25	3.45	4.8	2.4	1.75	3.4
	N	1.8	1.5	1.25	2.5	1.75	2.2	0.7	0.25	1.0
	O	1.85	1.6	1.4	2.45	1.75	1.8	0.6	0.15	0.4
B	P	2.0	1.7	1.4	5.2	4.85	5.2	3.45	3.15	3.8
	Q	1.8	1.6	1.5	2.5	1.8	1.9	0.7	0.2	0.4
	R	1.3	1.3	1.2	1.95	1.4	1.4	0.65	0.1	0.2
	S	1.85	1.7	1.6	2.6	1.85	2.0	0.75	0.25	0.4
	T	1.25	1.35	1.2	1.95	1.4	1.4	0.7	0.05	0.2
	U	1.6	1.5	1.3	2.5	1.8	1.8	0.9	0.3	0.5
	C1	1.9	1.6	1.6	2.35	1.8	2.0	0.45	0.2	0.4
	C2	1.8	1.6	1.5	2.3	1.7	1.7	0.5	0.1	0.2
	C3	1.8	1.5	1.4	2.4	1.6	1.9	0.6	0.1	0.5
C	C4	1.85	1.55	1.4	2.4	1.65	1.7	0.55	0.1	0.3
	C5	1.9	1.55	1.4	2.4	1.7	1.8	0.5	0.15	0.4
	C6	1.85	1.6	1.5	2.4	1.6	2.0	0.55	0	0.5
	C7	1.9	1.6	1.3	2.45	1.7	1.7	0.55	0.1	0.4
	C8	2.1	1.7	1.5	2.7	2.15	1.8	0.6	0.45	0.3
	C9	2.4	2.0	1.9	3.0	2.5	2.3	0.6	0.5	0.4
	C10	2.5	2.0	1.9	3.1	2.6	2.6	0.6	0.55	0.7

6.4.1.3 Pyruvate values increased after storing the milks at 5^{o} for 4d but increases were not closely related to increases in total or psychrotroph bacterial counts. The relationship was little or no better after a further 24h during which time both the pyruvate levels and the counts increased.

6.4.1.4 With three milks known to have been produced under good hygiene conditions, increases in pyruvate and total counts were small after storage at 5^{o} for 5d and 20^{o} for 17h.

6.4.1.5 The confirmation that pyruvate increases with bacterial growth at 5^{o} suggests that further studies would be worthwhile. Refrigerated bulk tank milks with higher initial colony counts, and alternate day collected milks should be included to determine whether initial pyruvate values are more informative for such milks and whether, after storage, increase in pyruvate correlates better with increase in bacterial numbers, with the aim of detecting those raw milks liable to undergo rapid deterioration at refrigeration temperatures and which would therefore not withstand cold storage at the processing dairy. Optimum conditions for pyruvate production, e.g. time and temperature of pre-incubation need further study. Milk production conditions on the farms should be evaluated in detail. Such studies would reveal whether increase in pyruvate is related to particular sources or degrees of contamination.

The results from a further 11 samples tested subsequently indicate that a marked increase in pyruvate after pre-incubating daily collected refrigerated tank milk for 24h at 18^{o} would detect a high proportion of milks which, after storage for 4 days at 5^{o}, would have psychrotroph counts of $>10^{6}$/ml and in which undesirable changes might therefore occur during cold storage.

6.4.2 Pasteurized milks

The results indicate that storage of pasteurized milks at 10^{o} for one or more predetermined periods, eg. 2d, preceded and followed

by the determination of pyruvate would be a rapid means of measuring the actual and potential quality of the milk. It may be that temperatures other than 10^{o} would be equally or more suitable for this purpose.

6.5 References

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6.6 APPENDIX

6.2.5 Biochemical Analyses

6.2.5.1 Pyruvate estimations

6.2.5.1.1 Manual colorimetric method used at NIRD

This method based on the method used for the determination of pyruvate in blood (Czok & Lamprecht, 1970).

Reagents

1 M Perchloric acid	- 70 % AR diluted 1 in 7.
Carbon Tetrachloride	-
0.7M tripotassium phosphate buffer	- 160 g/L
Tris buffer	- 6.05 g/L. 32 % HCl to pH 7.4
NADH (Boehringer 15297)	- 5 mg/10 ml. Tris
LDH (Boehringer 15371 - 5 mg/ml)	- 0.08 ml/10 ml Tris
Pyruvate solution (Boehringer 15685)	- 0.123 g/100 ml (1000 ppm)

Glass distilled water was used to make up the solutions.

Method

The samples were tested on the same day as they were plated. 4.0 ml milk + 4.0 ml perchloric acid were centrifuged at 3,000rpm for 10 min. The supernatant was extracted, 1.5ml carbon tetrachloride was added and the tube was shaken to extract the fat. 4.0ml of the upper aqueous layer was pipetted off into another tube and 4.0ml 0.7M tripotassium phosphate buffer added to bring the pH to 7.4. After mixing and filtration, the filtrate was used for the pyruvate determination. The spectrophotometer was set at 340nm and zeroed against air. 2.0ml filtrate and 0.2ml NADH solution were placed in the cuvette, mixed and the optical density reading noted. LDH (0.02ml) solution was added, and the optical density again after 10 min. The difference between the two readings, ΔE, was obtained.

Preparation of the Standard Curve

The standard solution was diluted 1:20 to give 50 ppm of pyruvate. Suitable volumes of this solution were added to bulk tank milk to increase the pyruvate levels by 0, 1.25, 2.5, 5.0, 7.5 and 10.0 ppm. The ΔE values were obtained, and the final pyruvate concentration, C, determined according to the formulae given below.

$$c = \frac{MW}{\varepsilon \times d \times 10} \times \frac{V}{v} \times F \times \Delta E \times 10$$

where ε = 6.22 cm^2/μ mole at 340 nm.
d = 1.0cm
V = total volume in cuvette (2.22ml)
v = volume of test solution (2.00ml)
F = dilution factor
MW = 88

The formula for the dilution factor $F = \frac{\left((4.0 \times 1.032 \times 0.87) + 4.0\right)}{4.0} \times \frac{8}{4} = 3.80$

where 1.032 = densitiy of milk
0.87 = water content of milk

The values for the density and the water content of the milk were adjusted according to the addition of pyruvate solution.

The standard curve was obtained by plotting ΔE against pyruvate concentration. The pyruvate values for the milk samples were derived by reading off the ΔE values on the standard curve.

6.2.5.1.2 Colorimetric Determination of Pyruvate using the Auto-Analyser II at Kiel

See chapter 3.2.2.2.

6.2.5.1.3. Fluorometric Determination of Pyruvate using the Auto Analyser II

Reagents

Sodium citrate (5%) (Merck 6448)	50g/L	Made as required
" " + Brij.	0.5ml/L	" " "
Tris Buffer (Merck 8382 AR) to pH 7.6 with 32% HCl	(0.3M) adjusted 36.3g/L	" " "
Tris Buffer (0.1M) pH 9.6	12.1g/L	" " "
Sodium Hydroxide (IN) (Merck 6498 AR extra pure) + 0.1M Titriplex III (Merck 8431)	40g/L 30ml/L	" " "
$NADH_2$ Stock solution	0.05g/100ml tris pH 9.6	" " "
(Sodium Salt-Merck 24543)		Stored in fridge

Enzyme Working Solution

$NADH_2$ Stock solution	7.0ml	(Made up daily (Kept in an insulated (box
LDH (from rabbit muscle) (Boehringer 15371)	0.5ml	
Tris pH 9.6	100ml	
Pyruvate Stock solution	0.128g/100ml	Made up weekly. Stored in fridge.

Working Standards for Pyruvate Estimation

Stock Solution (ml)	Distilled water (ml)	Pyruvate ppm
0.10	100	1.0
0.25	100	2.5
0.50	100	5.0
0.75	100	7.5
1.00	100	10.0

0.42ml milk are diluted with 0.8ml sodium citrate solution and segmented with 0.23ml air, then dialysed against 1.0ml tris buffer (pH 7.6, air segmented) + 0.23ml enzyme working solution. The dialysate passes through a water-bath at 37° to give the optimum reaction conditions. The addition of 0.6ml sodium hydroxide stabilises the base-line.

Procedure

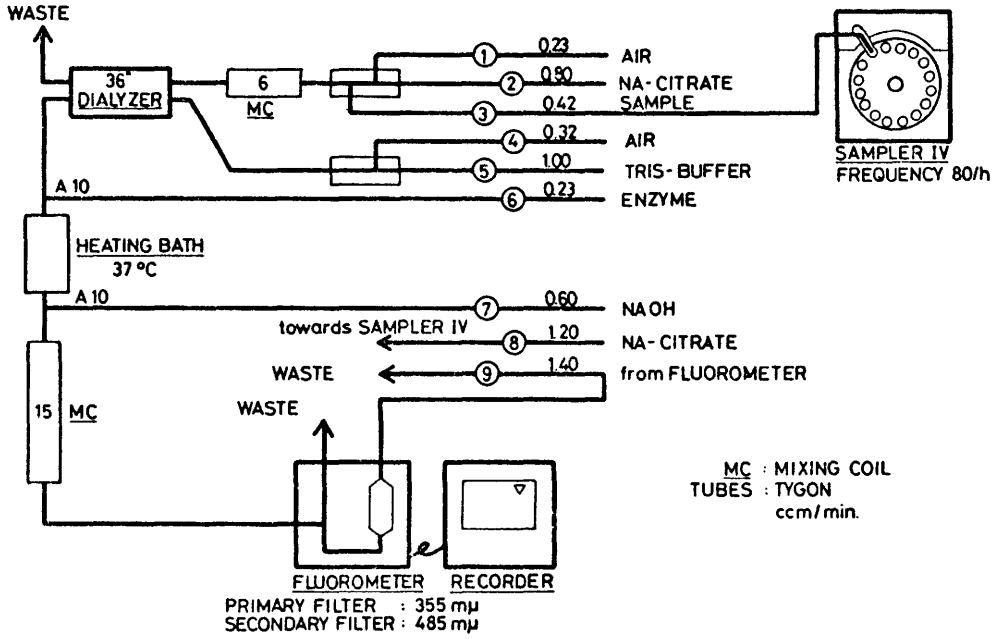
The system is calibrated at the start of each day's operation. The reagents are aspirated through the system followed by three of the 10ppm standards with two sodium citrate washes in between. Using the blank control, the peak given by the first 10ppm standard is adjusted to 5 scale divisions. The baseline for the reagents is then set to 95 scale divisions using the Full Scale Record control. The other two high standards and intervening washes serve to check that these adjustments are correct. All five standards are then run in ascending order of concentration followed by two washes. Next the 2.5ppm standard, two washes, five arbitrary milk samples in duplicate (the order chosen at random), two washes and the 2.5ppm standard are run. The peaks given by the five standards are used to plot a standard curve on a chart reader. This standard curve is used to check the reproducibility of the 2.5ppm standards and the milk samples.

Following the routine calibration, milk samples to be analysed are run through, with a 2.5ppm standard after every tenth sample.

When all the samples have been run, distilled water is pumped through the system to clean the tubes.

Examination of Raw Milk Samples

Sixteen samples, which had been preserved by the addition of an equal volume of 1M Perchloric Acid, were despatched from NIRD weekly. After centrifugation at 2900 rpm for 10 min the clear supernatant was analysed for pyruvate by both methods. The standard pyruvate solutions were also diluted with an equal volume of perchloric acid.



ENZYMATIC PYRUVATE DETERMINATION IN THE AUTO-ANALYZER SYSTEM II

74/024 W.

6.2.5.1.4 Fluorimetric Determinationof Lactate using the Auto Analyser II

Reagents

Sodium citrate (5%) (Merck 6448 AR)	50g/L
Tris buffer (0.1M) (Merck 8382 AR) (adjusted to pH 7.6 with 32% HCl)	12.1g/L

Hydrazine - Glycine Buffer

Hydrazine hydroxide (N_2H_5OH.80%)	270.0ml
Glycine	36.0g
Distilled water	1.6L
0.1M Titriplex III (Merck 8431)	30.0ml (adjusted to pH 9.5)

Lactic Acid Enzyme Solution

NAD	0.69
LDH (from Rabbit Muscle - Boehringer 15371)	2.0ml
Tris buffer	100ml
(Made up daily)	

Lactate Stock Solution (1000ppm)

L (+) Lactate - Lithium salt	0.107g
Distilled water	100ml

Working Standards for the Lactic Acid Estimations

Stock solution (ml)	Distilled Water	Lactate (ppm)
1.0	100	10
3.0	100	30
5.0	100	50
10.0	100	100
20.0	100	200

The flow diagram is identical to the one for the pyruvate estimation.

7. FINAL STATEMENTS

Milk - the most complete single food - is drawn twice a day in thousands of farms and on its long way to the consumer is extremely sensitive to bacterial deterioration. Saprophytic (non - pathogenic) microbes in raw milk and milk for consumption are the limiting factors for keeping quality and thus of the greatest importance for the consumer as well as for the dairy industry. Dependant on growth conditions, saprophytic bacteria give rise to biochemical changes in milk which may be described by the terms of glycolysis, proteolysis and lipolysis and which characterize metabolic processes in the degradation of carbohydrates, proteins and fats, respectively. Accordingly, the growth of these bacteria in milk is associated with changes which - in relation to the number, species and activity of microbes - have an adverse effect on the organoleptic quality of the product and finally causes its deterioration. It is not surprising that the highest number of microbial analyses in a single food area are probably done on milk. Because many farm milk supplies are stored at refrigeration temperatures assessment of the psychrotrophic microflora is an urgent objective. Current methods for examining the bacteriological quality of milk are unsuitable for these micro-organisms and consequently new, automated and accurate methods are urgently needed.

The principles of microbial spoilage indicate, that their are two ways, by which it can be measured:

1. The quantitative and qualitative estimation of the microbial flora as the causing factor and

2. The determination of key compounds of the microbial catabolism, indicating the actual or potential state of deterioration.

There is no doubt, that the first way for many reasons is difficult to realise and that under practical conditions the information obtained is of limited value. As regards the keeping quality it is primarily not the absolut number of bacteria but rather their biochemical activity which is of interest.

The present study:

"Objectivation of the Bacteriological and Organoleptic Quality of Milk for Consumption"

was based on the question, whether the keeping quality of market milk can be assessed by the determination of pyruvate. Pyruvate is a central substance in microbial catabolism and appeared to be suitable for indicating the state of deterioration of milk and - after preincubation of the sample - the activity of the contaminating bacteria.

Like all enzymatic methods the enzymatic determination of pyruvate has the advantages of excellent specificity; high sensitivity; small demand for laboratory cost per specimen; simplicity which makes it suitable for routine work.

A detailed chapter of this study deals with the principles of bacterial catabolism in milk and the position of pyruvate as a key compound within it even under psychrotrophic conditions.

In the following chapters results from experimental work done in Kiel and in Reading, including the exchange of samples between both, are described. In the context of practical application they can be summarized as follows:

1. Automated sampling:

   This together with an efficient preservation is an indispensable prerequisite for any testing procedure. Because pyruvate is in solution samples can be taken automatically and accurately by special equipment.

2. Preservation of samples:

   This can be done with formaldehyde up to 24 hours, but with trichloroacetic acid in a more precise way for a period of five days.

3. Standardization of the analytical method

The agreement between results from Reading and Kiel have continued to improve but is still not adequate. One reason may be the different techniques (manual and automated) used by the authors. The rapporteurs, however, are convinced, that this problem will be easily solved using the same technical procedure when standards (samples and reagents) are available.

4. Interval of milk-collection and pyruvate

When raw milk is stored under refrigerated conditions a continuous increase of pyruvate occurs, dependant on the number and activity of contaminating microorganisms. An increase of pyruvate (and therefore degradation of the milk) can be measured even in those cases where there is no increase in the total number of bacteria.

5. Bacterial numbers and pyruvate

The amount of pyruvate produced per colony-forming unit (cfu) depends on the total number of microorganisms present in milk and whether they are actively growing or not. The higher the total number of organisms, the lower is the amount of pyruvate produced per cfu. Thus the pyruvate measurement will provide the desirable differentiation in the lower ranges of bacterial numbers better than in the higher ones. Also more pyruvate is produced if the bacteria are actively multiplying; pyruvate is a more reliable indicator of deteriorative changes than is bacterial numbers.

6. Influence of the raw milk quality on the keeping quality of pasteurized milk

There is evidence, that the bacteriological quality of raw milk immediately before heat-treatment influences the organoleptic quality of the pasteurized product: the higher the bacterial numbers in the raw milk the higher the pyruvate-level and the shorter the keeping period of the pasteurized milk after heat-treatment.

7. Keeping quality of pasteurized milk and pyruvate-difference-value

Within the context of this paper this is the most important aspect. From the joint study done in Kiel and Reading it can be concluded, both from a scientific basis and from experimental results that the pyruvate-difference-value (pyruvate level after preincubation minus the level before incubation) indicates the keeping quality of refrigerated pasteurized milk earlier and more certainly than any other test available.

The aim of this study to make a prelimenary assessment of the pyruvate method has in particular indicated its potential as a test for pasteurized milk.

Further work should involve the following items:

- Standardization of the method (manual and automated) including the development of standard solutions in a biological matrix;
- International comparison tests;
- Confirmation of the results obtained concerning the pyruvate keeping quality test;
- Elaboration of pyruvate threshold values for raw and pasteurized milk under different conditions of storage and processing;
- Elaboration of a draft for directives concerning the quality requirements of milk for consumption.

The forementioned projects will require at least one further year of collaborative studies.

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No. 1	Credit to agriculture I. France, Belgium, G.D. Luxembourg	February 1976	F
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