A STUDY OF FACTORS AFFECTING THE ACTIVITY OF LACTIC ACID-PRODUCING CULTURES IN CHEESEMAKING

A Thesis submitted to the
University of Glasgow for the
Degree of Doctor of Philosophy
in the Faculty of Science

by

ROBERT JOHN MONTEITH CRAWFORD

The West of Scotland Agricultural College,
Auchincruive,
Ayr.

September, 1959.

ACKNOWLEDGMENT

I am grateful for the opportunity and facilities made available to me to carry out this study by the Governors and Principal of The West of Scotland Agricultural College. I thank Professor D. Murray Smillie in whose department the work was carried out for his help and encouragement. My thanks are also due to Professor J.W. Howie of the University of Glasgow for guidance and criticism and in particular for suggesting a study of the acid-producing ability of logarithmic phase cultures. I also thank members of staff of the Milk Utilisation Department of the College for advice and assistance with various determinations.

CONTENTS

SECTION ONE

INTRODUCTORY AND HISTORICAL

INTRODUCTORY AND HISTORICAL

Cheese is one of the oldest, simplest and most nourishing foods known to man. In its widest definition cheese is the curd of milk which has been coagulated by enzymic or other means and thereafter separated from the liquid whey to form a more or less solid mass. Cheesemaking has been practised since very early times as a means of converting a perishable foodstuff into a form which is less likely to deteriorate. In early times very little milk was produced during the winter months when food for the animals was scarce and the preparation of cheese during the summer when milk was available was an essential part of food gathering. Since grass is still the cheapest food for milking animals the main cheesemaking season remains the spring and summer months.

Cheese was, according to legend, first made accidentally by an Arab merchant. The merchant is said to have set out on a long journey over the desert carrying his supply of milk in a pouch made from the stomach of a sheep. During the course of

the day the heat of the sun warmed the milk which was acted on by the rennet enzyme present in the lining of the pouch and as a result there was formed from the milk a semi-solid mass called curd and a liquid, whey. At the end of his journey the merchant was sustained by both the curd and whey which had been formed. It may well be that cheese had its origin in some such manner.

Whatever its exact origin, cheese goes back to great antiquity. Reference to cheese preparation and use is contained in the Bible. The art of converting milk into a stable food was probably brought to Europe from Asia long before the Christian era and to Great Britain during the Roman Conquest.

At present some four hundred varieties of cheese are listed in the U.S. Department of Agriculture Handbook (141) indicating perhaps that the advice of Oliver (125) has gone unheeded. Oliver said that a cheesemaking system consisted of a number of practices based on certain principles which were applied to bring about definite results and which could be reproduced from day to day simply by repeating the system and its conditions. Such a system could be named after its originator or after the district in which it was first adopted. This authority expressed the opinion that once a name had

become associated with the cheese by a particular method all produce so made in any part of the world should bear that name. Only in this way could endless confusion be avoided. At that time - 1894 - he reported that upwards of ninety varieties of cheese were made in different parts of the world.

Sanders (141) considers that there are probably in the region of eighteen distinct types of cheese which may be classified on the grounds of degree of solidity and the agents responsible for producing the particular characteristics of flavour and appearance.

A classification on this basis would be as follows: -

1. Very hard.
   Ripened by bacteria, e.g. Parmesan.
2. Hard.
   (a) Ripened by bacteria, without 'eyes', e.g. Cheddar.
   (b) Ripened by bacteria, with 'eyes', e.g. Gruyère.
3. Semi-soft.
   (a) Ripened by bacteria, e.g. Brick.
   (b) Ripened by bacteria and surface micro-organisms, e.g. Limburger.
   (c) Ripened principally by moulds in the interior, e.g. Stilton.

4. Soft.

(a) Ripened, e.g. Camembert.

(b) Unripened, e.g. Cottage.

The two varieties of cheese made most extensively in Great Britain are Cheddar and Cheshire. The former is also manufactured extensively in overseas countries but the Cheshire variety is almost exclusively confined to England. The variety native to Scotland - the Dunlop - is a cheese in many ways similar to Cheddar.

The development of cheesemaking techniques in relation to the use of lactic-acid-producing cultures.

The village of Cheddar, situated at the southern foot of the Mendip Hills in the county of Somerset, has given its name to a variety of cheese prized for its full flavour, smooth texture and long-keeping character. One will perhaps more fully appreciate the part played by micro-organisms in the earlier stages of cheese manufacture if the development of the Cheddar and the Dunlop varieties are reviewed.

Reference to Cheddar cheese in early times was made by Sheldon (149) who stated that the variety was popular during the reign of Queen Elizabeth I (1558-1603) and could be traced back to three centuries earlier. In 1655, Samuel Hartlib (71)

described Cheddar cheese as the best in England. In his book on cheese Burdett (21) quotes a letter written in the early seventeenth century by Viscount Conway to Lord Poulett in which the writer described the scarcity of supplies of this variety which resulted in the cheese being ordered before they were made.

Cheesemaking was practised in Scotland in the same period but in this case a variety was made in which there was very little fermentation - the so-called 'sweet milk' cheese. The main dairying area in Scotland at that time and until the early nineteenth century was the County of Ayr. Aiton (1) recording the state of the agricultural industry in Ayrshire in 1811 described two types of cheese, one the English variety and the other the native variety Dunlop or Ayrshire. The native Scottish variety takes its name from the village and parish of Dunlop in north Ayrshire. The controversy over the originator of the variety exemplifies the mystery associated with the early history of many cheese varieties. In an early statistical account of the Dunlop parish written by the parish minister the credit for introducing this system of cheesemaking was given to a servant girl who fled to Ireland during the persecution and who introduced a 'new' system of cheesemaking on her return in 1688. Aiton

(1) doubted this explanation and pointed out that excellent cheese of similar type had already been made in the area for some considerable time. It has also been reported that this variety of cheese was named after a local landowner, Mrs. Dunlop of Dunlop House, who sheltered Barbara Gilmour on her return to Scotland.

An excellent description of the manufacture of Dunlop cheese was given by Smith (160) in 1810. The following excerpt shows the extreme simplicity of the method:-

"the rennet is always applied to the milk as soon as it can be conveyed to the pail. When the milk is coagulated it is cut with a knife, put into a drainer, and pressed down with a moderate weight. This operation is repeated three times at short intervals about three hours after, it is returned to the pail and hagged with a knife of a different kind, as small as possible. It is then put into the vat, and pressed down with weight varying from half a ton to a ton, for sixteen hours, the cheese clothes being frequently changed."

To complete the process the cheese was scalded, pressed for sixteen to twenty-four hours and taken to the curing loft where it was turned three times a week. At that time the Dunlop cheesemaking process was essentially one in which there was very

little fermentation - hence the term 'sweet-milk cheese'. Aiton (1) described English cheese as being more pronounced in flavour - he was probably referring to the Cheddar variety.

The secret of making Cheddar cheese was held solely by the makers in Somerset for many centuries. With the interchange of knowledge which took place during the nineteenth century, cheesemakers outside the county of Somerset began to practice the method and were very successful. In Scotland by this time- the early nineteenth century - the farmers of the counties of Wigtown and Kirkcudbrightshire were becoming aware of the profit to be made from making cheese and they were enthusiastic about the Cheddar variety because of its superior keeping characteristic. At this time the popularity of the Dunlop variety began to decline; the cheese was very perishable and it is probable that considerable quantities of the cheese deteriorated rapidly due to the very 'sweet' condition of the curd.

Around the middle of the nineteenth century the method for the making of Cheddar cheese had been widely adopted in Scotland. At that time the advice of some of the well-known Somerset cheesemakers was being received by the farmers in South-west Scotland. In 1854, (103) Harding of Marksbury in Gloucestershire - an authority on the Cheddar variety -

addressed a meeting of Ayrshire farmers in these words: -

"Cheese is made in the dairy yonder, Where A is feeding his kine on broad clover, tares and rye grass; or where B, on the edge of the moor, is making what was almost desert bloom as a rose with the varied crops of a first year's cultivation; or yonder again where C and D are managing old carse farms in the groove made generations ago. I will take the milk from any of them and make the same cheese anywhere. Cheese is not made in the field, or in the byre, or even in the cow - it is made in the dairy."

This opinion probably evoked much discussion since it had long been held that only the pastures of Somerset were able to produce milk of suitable properties for the manufacture of high-class Cheddar cheese. Harding declared his abounding confidence in the Cheddar system of manufacture but his ideas on the merits and fundamental principles involved were very vague as indeed were those of other cheesemakers at that time. It was appreciated that the salient feature of the system was the development of acid in the curd formed on the addition of the rennet enzyme to milk impregnated with sour whey retained from the previous day's cheesemaking. Harding and his contemporaries did not know why it

was that acid whey played such a vital part in the process. From our present knowledge we can explain the action by the fact that the whey abounded in bacteria capable of producing lactic acid and when added to the fresh milk hastened the formation of the curd and cheese.

In the meantime the scale of cheesemaking operations were changing. Until the middle of the nineteenth century cheesemaking was a relatively simple practice carried out on a small scale on individual farms. In 1851 (52) the first cheese factory was established in Oneida County, U.S.A., and proved so successful that within a few years several factories had been established in the area. In 1870 the first British factory was opened in Derbyshire (52). During the next ten years there was considerable expansion and numerous small co-operative factories were established. This movement was only partially successful. Much cheese of inferior quality was produced due to lack of expert knowledge on how to deal with the larger volumes of milk. Two developments during the late years of the century were to prove of great value in raising the standard of the finished cheese.

In the year 1890 the Danish scientist Storch (166) used a selected strain of bacteria in souring cream for buttermaking. This - the first scientific

work on the subject of bacterial fermentations used in the dairy industry - was to prove of great importance. Although the first pure culture was used in the manufacture of butter, the knowledge gained therefrom was soon to find application in the cheesemaking industry. It should be recorded that in the same year similar reports of the use of selected bacterial cultures were published in Germany (189) and the U.S.A. (28). Prior to, and for some considerable time after Storch's discovery various methods were used to bring about the required amount of souring essential to success in the cheesemaking process. These were threefold;

a. Natural souring caused by the growth and acid production of the natural bacterial flora of the milk and chiefly controlled by adjustments in the temperature of the milk (50).

b. Addition to the milk of sour whey or buttermilk. Many of the more shrewd cheesemakers followed the practice of obtaining whey from a neighbouring farm enjoying a successful period of cheese manufacture (103).

c. Addition of a home-made 'starter'. It was a common practice to allow a portion of milk to coagulate and then use the clotted milk as a means of promoting souring in the milk used for cheesemaking (165).

The second development of great value to the cheesemaker was the introduction of a method of determining the amount of souring in the preparation of cheese. In 1891 Lloyd (100,101) established a test by means of which the amount present in milk or other material could be measured, thereby putting the process of souring on a scientific basis for the first time. By his work over the last decade of the 19th century, Lloyd contributed greatly to the knowledge and practice of cheesemaking. The test which he introduced remains to this day one of the most important aids in the manufacture of first quality cheese.

Discoveries are not always accepted and put into practice immediately. The practice of using pure strains of bacteria in cheese manufacture was, however, adopted by many cheesemakers within a few years of the original work of Storch (166). Culture starters were first used in cheese manufacture in south-west Scotland following an investigation (23) begun in 1895 into the cause of a discolouration common to much of the cheese made in the area at that time. A committee set up to investigate the fault reached the conclusion that the proliferation of the contaminating organisms causing the defect should be checked by (a) thorough cooling of the evening milk and (b) the use

of a vigorous pure culture to start the fermentation in the mixed milk in the morning when cheesemaking was commenced. The success which followed extensive trials carried out in the area did much to establish the practice of using pure culture starters. Scott (145) reported that the introduction of pure cheese cultures took place in 1903 but this must only refer to the introduction in a particular area of Great Britain. Fleischman (49) writing in 1896 in U.S.A. mentions the use of pure cultures for the souring of cream - the cultures being obtainable from experimental stations - but indicates that the addition of 0.75 to 2.0 per cent of sour whey to the milk was the most common method of procuring the necessary souring in the production of American cheese at that time. In 1900 Smith (161) discussing the various means of setting up a fermentation by the use of sour whey, lactic ferment - a commercial culture preparation - and pure cultures, suggested that the pure culture practice was only in the experimental stage and time alone would prove whether the use of these cultures would in fact improve the general quality of cheese. At that time the practice adopted in most British dairies was the addition of sour whey to the milk if the fermentation was slow to start naturally. Smith (161) suggested that the use of

so-called lactic ferment and pure cultures was only another means to the same end, with the advantage that instead of working with the same 'breed' of bacteria in the milk and whey all season a pure 'breed' could be introduced at intervals when required. An advocate of the use of selected starter bacteria in cheese manufacture was Decker (44) who in 1905 strongly recommended the use of a commercial culture of lactic acid bacteria. Stevenson (165) reported that by 1911 the use of pure cultures had been extensively adopted by both buttermakers and cheesemakers in Great Britain. It would seem that the general adoption of these souring agents must have become very rapid after a certain point since the same author writing two years earlier in 1909 (163) stated that while considerable interest was being shown in the use of pure cultures of bacteria a difference of opinion existed as to their value in the manufacture of butter and cheese. This difference of opinion was due in the main to a marked inconsistency in the results obtained by their use because of the lack of knowledge regarding the nature and properties of the cultures. At a later date - 1919 - Walker-Tisdale and Woodnutt (187) mentioned the possibility of using naturally prepared culture as an alternative to the pure form but one of these authors a year

later (186) strongly advocated the use of the latter type.

During the past thirty-nine years cheesemaking operations have again changed markedly. On the one hand there has been a decided decline in the amount of cheese made on farms, while on the other, there has been an increase in the size of the factory units. The size of manufacturing unit is of importance both from the biological and economic points of view. This increase in the size of unit has been accomplished by a greater degree of mechanisation. Larger units made possible the provision of better equipment for culture preparation operated by more highly trained personnel. The change in the scale and knowledge of cheesemaking has resulted in the care of starters changing from simple largely uncontrolled methods employing little equipment to laboratory controlled techniques involving specially designed apparatus. This change has been one of necessity since from the economic viewpoint the cheesemaking process must be of the shortest possible duration consistent with the production of a high quality product and this is only possible if the fermentation process is of uniform speed from vat to vat and from day to day.

Two developments during this period have had a profound effect on the manufacture of cheese. The

first of these has been the almost universal adoption of the practice of pasteurising the milk used for the manufacture of cheese. Pasteurisation of milk, performed by heating to temperatures in the region of 70°C destroys bacteria capable of producing acid during the cheesemaking process. When milk is drawn from a healthy udder it contains few bacteria but after it has been exposed to contamination from the atmosphere of the byre and equipment used in the dairy it is liable to contain a greatly increased number of bacteria, some of which are harmful to cheese manufacture others capable of producing lactic acid. The adoption of pasteurisation as a pre-manufacturing treatment has coincided with a stricter control of the bacteriological quality of milk. Great emphasis is now placed on the production of milk containing few bacteria.

With the elimination of the naturally-occurring souring bacteria of milk either due to the hygienic measures adopted on farms or because of the heat treatment to which milk is subjected in the cheese factory the role of the lactic acid bacteria used during cheese manufacture has become more decisive.

Apart from increase in unit size - creamery and equipment - and the adoption of milk pasteurisation there have been few fundamental changes in cheesemaking techniques over the past half century. Details of the cheesemaking process used in 1908 (103,164) are shown in comparison to those of a present-day factory in table 1.

Larger amounts of acid-producing bacteria are now added to the milk in an attempt to shorten the time of manufacture. Today the general practice in British factories is to use in the region of 1 per cent of culture and allow the milk to sour - this part of the process is known as the 'ripening' period - before the addition of the rennet enzyme. The process adopted by New Zealand cheesemakers on the other hand varies considerably from the British counterpart for the manufacture of Cheddar cheese. Larger amounts of culture are used and rennet is added to the inoculated milk without allowing a ripening period. This technique which is used to produce Cheddar cheese similar in property to that produced by the British method has been modified and developed over the years. Whitehead (191) described the process used in New Zealand in 1943 which is shown in table 2.

TABLE 1

Cheesemaking timetable: 'Scottish' Cheddar

	Farmhouse and small factory 1908 (103,164)			Scottish factory 1953		
Milk treatment	Milk temperature adjusted to 84 - 86°F after being held in vat overnight			Milk pasteurised and run into vats at 86°F		
Starter: type and amount	Pure culture, whey or natural ¼ - ½ per cent			Pure culture ½ - 1½ per cent		
	Time (h-min)	Temp. (°F) x	Titratable acidity (% lactic acid)	Time (h-min)	Temp. (°F) x	Titratable acidity (% lactic acid)
Starter added	0.00	84 - 86	0.16	0.00	86	0.16
Rennet added	2.00	84 - 86	0.17 - 0.18	1.00 - 1.15	86	0.18
Curd cut	2.45	84 - 86	0.13	1.45 - 2.00	86	0.13
Maximum scald	3.45	95 - 102		2.45 - 3.00	102 - 104	
Whey run	5.00			3.45 - 4.00		0.30
Curd milled	7.00			5.30 - 5.45		0.70
Curd salted	7.10			5.40 - 5.55		0.80
Curd hooped	7.30	78 - 80	0.85	6.00 - 6.15	78 - 80	0.90

x The Fahrenheit scale of temperature is normally used in cheesemaking

TABLE 1(a)

Temperature equivalents for use in conjunction with cheesemaking timetables in this thesis

°C	°F
12.0	53.6
15.0	59.0
25.0	77.0
30.0	86.0
31.1	88.0
37.0	98.6
38.0	100.4
39.0	102.2
40.0	104.0
42.0	107.6
46.0	114.8
47.0	116.6
49.0	120.0

TABLE 2

Cheesemaking timetable: 'New Zealand' Cheddar (191)

Milk Treatment	Milk pasteurised and run into vats at 85 - 88°F		
Starter: type and amount	Pure culture 1½ per cent		
Process record	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)
Starter added	0.00	88	
Rennet added	0.30	88	
Curd cut	1.00	88	0.12
Maximum scald	2.00	98 - 102	
Whey run (beginning)	2.45		0.16
Whey run (end)	3.05		0.23
Curd milled	5.05		0.75
Curd salted	5.55		0.90
Curd hooped	6.25		

Since 1943 the method has been further modified (197) so that by the addition of more starter the ripening period is eliminated. The basis of the cheesemaking process is little altered from that used in the early years of the century.

During the past six years the attention of the cheesemaking industry has been focused on work carried out in the United States of America and Australia on the introduction of a rapid method for the manufacture of Cheddar cheese. The original 'short-time' method developed in the U.S.A. by Walter, Sadler, Malkames and Mitchell (188) had as its primary object a curtailment of the time of manufacture. In addition, however, the adoption of the method paved the way for further mechanisation of the cheesemaking process by eliminating cheddaring, milling and dry salting of the curd, a novel feature of the method being the salting and hooping of the curd in the whey.

In Australia, Czulak, Hammond and Meharry (36) while remaining convinced of the possibility of complete mechanisation of the cheesemaking process, modified the American method (188) to make use of conventional cheesemaking equipment. It should be explained that common to Cheddar cheese in all countries there is an important procedure carried

out known as 'cheddaring'. This term is used to describe the piling of the curd particles after the whey has been removed. Through the action of pressure, temperature and acid the curd particles gradually lose their identity and mat together. In the method of Czulak et al. (36) the cheddaring time is reduced from the normal 1½ hours to some 45 minutes. The cheddaring period which begins with the matting of the curd particles and continues with the turning and piling of blocks of consolidated curd ends in the milling of the curd.

An essential feature of the American and Australian methods was the use of normal starter cultures in conjunction with a heat- and salt-resistant culture of Streptococcus durans. A comparison of both methods is given in table 3.

In a trial in Scotland Crawford (31) reported that the cheese made by the Australian method were slow to mature and develop the typical flavour and other characteristics of Cheddar cheese. In this trial a strain of Lactobacillus bulgaricus was used as the heat-resistant organism in a comparison with the Str. durans culture originally used. Considerable

TABLE 3

Features of the American and Australian 'short-time' methods for Cheddar cheese

	American (188)	Australian (36)
Starter: Normal	1.5%	2.0%
: Str. durans	0.75%	0.75% - 1.0%
Ripening	1 hour	-
Maximum cooking temperature	115 - 120°F	108 - 117°F
Cheddaring	-	30 minutes
Salting	In whey	Dry
Acidity at salting	0.16	0.22
Hooping	Under whey Rectangular mould	Dry salted curd Any shape
Equipment	Normal vat with agitator, with special equipment for salting, hooping, etc.	As for normal cheddar
Time of making	4 hours	3 - 3½ hours

experimental work has been carried out in Australia (35,117,48) and by the author (unpublished data) in further attempts to improve the method.

Mechanisation and change of technique in cheese manufacture are only acceptable if the desirable features of a variety are unaltered. The following passage from a very early account of cheesemaking contains some very useful advice. Writing in 1794, Steele (162) described one of the causes of defects in butter and cheese in these words;

"The second cause of imperfection enumerated, was inattention and carelessness. This business has been so long carried on, and always in nearly the same manner, without at least any notable alteration in the fashion of doing it, in almost every place, that we are persuaded, people will scarcely be got generally convinced, that it is practicable there could be any variation made, and much less convinced of any profitable change; every one imagining they can do this business as well, if not much better than any of their neighbours; therefore they are at rest, entirely

well satisfied that the custom, so universal and well established by long continued use, is and must, of course, be the best, and only made, which could possibly be put in practice; and that there neither is occasion for, nor could there be any better manner invented, nor fallen upon, neither any farther improvement introduced upon the common practice."

It is with this statement - some hundred and sixty-five years old, but which could be equally well made at the present time - that it is of interest to pass from the background of chesse manufacture to consider in more detail the cultures of lactic acid-producing streptococci used in cheese manufacture and some of the factors affecting their growth and fermentation characteristics.

During the transition from farm - to factory - scale manufacture the reasons for slow acid production have been extensively investigated. Without doubt the most serious, widespread and insidious cause of slow acid production is bacteriophage attack on the starter bacteria. Depending on the intensity of the attack and the concentration of phage particles in the whey and curd, the result of the infection

may vary from a slight drag in acid development during the later stages of the making process to a complete cessation of acid production - the 'pack-up' described by the New Zealand workers. The prevention and control of slow-working arising from this cause have been greatly eased by systematic measures based on the results obtained over the years since Whitehead and Cox (196) demonstrated that many of the sudden mysterious failures in the activity of starter cultures were due to the development of bacteriophage which caused lysis of the starter bacteria and resulted in the starter bacteria being unable to produce sufficient amounts of lactic acid. Much of the work on the problem of maintaining active starter cultures and ensuring rapid acid production in cheese manufacture has been carried out in New Zealand where the problem appears to have been more serious than in Great Britain. The main reason lies in the extensive use of single-strain cultures of starter bacteria in that country. This type of starter culture has not been used extensively in Great Britain but experience gained over several seasons by the author proved that bacteriophage contamination of this type of culture caused great damage to its acid-producing abilities and made the provision of aseptic methods of starter preparation

a requirement of success.

When it was conclusively demonstrated by the New Zealand workers that the most serious aspect of bacteriophage infection in a factory was the ease with which the atmosphere of the cheese factory could become contaminated the development of effective control measures followed.

The first recommendation to be made was the establishment of specially designed and equipped premises for the preparation of starter cultures at a distance from the cheese factory. The adoption of bacteriological methods of control and the designing and use of equipment in which the milk for starter preparation was protected from airborne contamination during the post-pasteurisation cooling period, resulted in a large measure of success. The realisation that a cotton wool filter provided an effective barrier to bacteriophage was of fundamental importance. A second novel idea developed in starter control (198) was an arrangement by which the over-lapping lids of the containers which held the milk during treatment dipped into the water of the pasteurising bath thereby precluding an ingress of contaminated air into the milk containers during post-pasteurisation cooling. This 'water seal' principle has been employed in many of the more

recent forms of starter equipment.

Numerous other devices and modifications have been used (194,97) for the elimination of airborne infection of starter cultures during their preparation. A large measure of success has followed their introduction and it may now be said that phage-free starters may be prepared under the most severe conditions of airborne phage contamination by mechanical protection.

In recent years a further aid in the preparation of active starter cultures has been the introduction by Reiter (133) of a calcium-free milk powder which when reconstituted with calcium-free water provides a medium for starter bacteria. The elimination of calcium from the medium prevents the proliferation of any bacteriophage particles gaining access to it. A second feature of the material is its ability to inactivate a considerable amount of any phage contamination. There have been several reports (37,9,32) of the successful use of this material under commercial conditions and it promises to provide the cheesemaker with a simple but effective alternative to mechanical methods of protecting starters.

Successful cheese manufacture has been practised in many cheese factories where no precautions are

taken in the preparation of starter cultures. In these factories the starters are open to contamination during their preparation. Recently the reason for this successful action has been satisfactorily explained by investigational work carried out by the author. It would appear that under these conditions a starter culture frequently undergoes a preliminary phage action within the first few days in the factory. This is followed by 'secondary growth' (121) and a recovery of acid-producing ability of the culture while at the same time the active acid-producing strains become carriers of the phage. This state would seem to confer a resistance on the culture which enables it to work successfully for long periods of time. The development and use of laboratory prepared, 'phage resistant', 'phage carrying' and 'lysogenic' starters has been reported elsewhere (115,84,39). These artificially prepared cultures were successful over limited periods of time, the acid-producing ability of the culture usually failing due to attack by a fresh strain of phage. The regularity of acid production in cheese manufacture where unprotected starters are in use would appear to indicate a useful source of cultures for factories where the equipment required for mechanically prepared aseptic techniques is unavailable. A second important point about these phage-carrying

cultures which the author has found to be widespread on farm and in factory alike is that they undoubtedly constitute a natural reservoir of bacteriophage.

At the present time it may be said that the souring action necessary for successful cheese manufacture is brought about by starters prepared by the use of aseptic techniques, a special medium which prevents phage multiplication or by allowing the unprotected cultures to undergo a natural immunisation procedure in the factory.

It is true to say therefore that the industry has adapted itself to changes in unit size to meet the requirements of a mechanised process of manufacture which remains dependent on the biological process of fermentation for success.

Problems remain, however, in the production of active acid-producing cultures and some of these will be discussed below.

SECTION TWO

FUNCTIONS OF A CHEESE STARTER

FUNCTIONS OF A CHEESE STARTER

The term 'starter' used to describe a bacterial culture used in the manufacture of a dairy product is derived from the fact that the culture which is composed of lactic acid-producing bacteria starts a vigorous fermentation of lactose, the milk sugar. In the manufacture of cheese the fermentation fulfils several functions.

The first of these is to sour or 'ripen' the milk before the addition of rennet. According to Singleton (156) it was generally held at one time that the work of the starter was completed with the addition of rennet.

As 'ripening' progresses there is an increase in the bacterial numbers and a small increase in acidity. British cheesemakers generally 'ripen' the milk for periods of ½ to 2 hours before adding the coagulating enzyme rennet. In many cases the exact point at which to add the rennet is determined by a test in which the time of coagulation is determined when a measured volume of rennet is added to a portion of milk taken from the cheese vat. The point of renneting is vital since both under- and over-

'ripening' of the milk may result in serious abnormalities in the making procedure. For example, if rennet is added to the cheese milk before a sufficient number of cells of the culture have been formed there is a tendency for the cheesemaking process to be extended because of slow acid development. The reverse is true if the milk is 'ripened' for too long a time. A modified practice is followed in New Zealand where the cheesemaker adds a much larger amount of starter culture and rennets the milk immediately - table 2.

The increase in acidity of the milk due to the addition of the acid milk culture and the production of acid by the starter bacteria assists in the formation of a firm coagulum when rennet is added.

The <u>second</u> function of the starter is to produce acid in the curd particle at a rapid and uniform rate during the making process. After the milk coagulum formed by the rennet enzyme has been cut into small cubes there is a continual expression of whey from the curd particles. This exudation of whey is brought about by a combination of several factors, the first and most important being the rate and extent of acid production inside the curd particle. In combination with the action of acid production is

that of temperature inside the curd, the two factors being closely linked. The physical properties of the final cheese are in fact largely dependent on the amount of whey expressed from the curd particles. From the time when the milk congulum is cut the titratable acidity of the whey expressed from the curd increases from 0.12 - 0.14 per cent lactic acid to 0.25 - 0.30 per cent lactic acid in a period of two hours. During this time the temperature of the whey-curd mixture is raised from 30 - 40°C. It is during this period of rapid whey expression and increase in titratable acidity that the starter bacteria pass into the logarithmic phase of growth and the real vigour of the starter becomes most apparent. At this stage the cheesemaker prepares for the later part of the process - the cheddaring period - of some two hours - which culminates in the milling and salting of the curd when the titratable acidity of the whey has increased to 0.7 - 0.8 per cent lactic acid. Throughout the various stages of the process the skilled cheesemaker relies on determinations of titratable acidity, less commonly hydrogen ion concentration and in large measure on certain subjective tests to guide him in his manipulation and control of the making procedure. As a result of whey removal from the curd particles

the soft jelly-like material changes into a relatively firm mass, the control of this process constituting the chief art of the cheesemaker, determining as it undoubtedly does the final composition and quality of the cheese. If too much whey is retained in the curd, fermentation of the lactose will continue and result in the formation of an excessive amount of acid causing the cheese to become over-acid and sour. If too great a quantity of whey is expressed the cheese becomes hard and dry. Perhaps equally important with the amount of whey removed from the curd is the rate of such removal influencing as it does certain economic and biological considerations.

The third function of acid-producing starter cultures used in the preparation of cheese is that of restricting the growth of undesirable contaminant bacteria present in the milk and curd. Small numbers of such bacteria are found in cheese made under normal conditions but they seldom give rise to extensive defects. The action of the starter bacteria is largely responsible for ensuring that the contaminants are not provided with conditions suitable for multiplication. As early as 1898, Campbell (23) commented on the restraining action of starter bacteria on the growth of other organisms. He

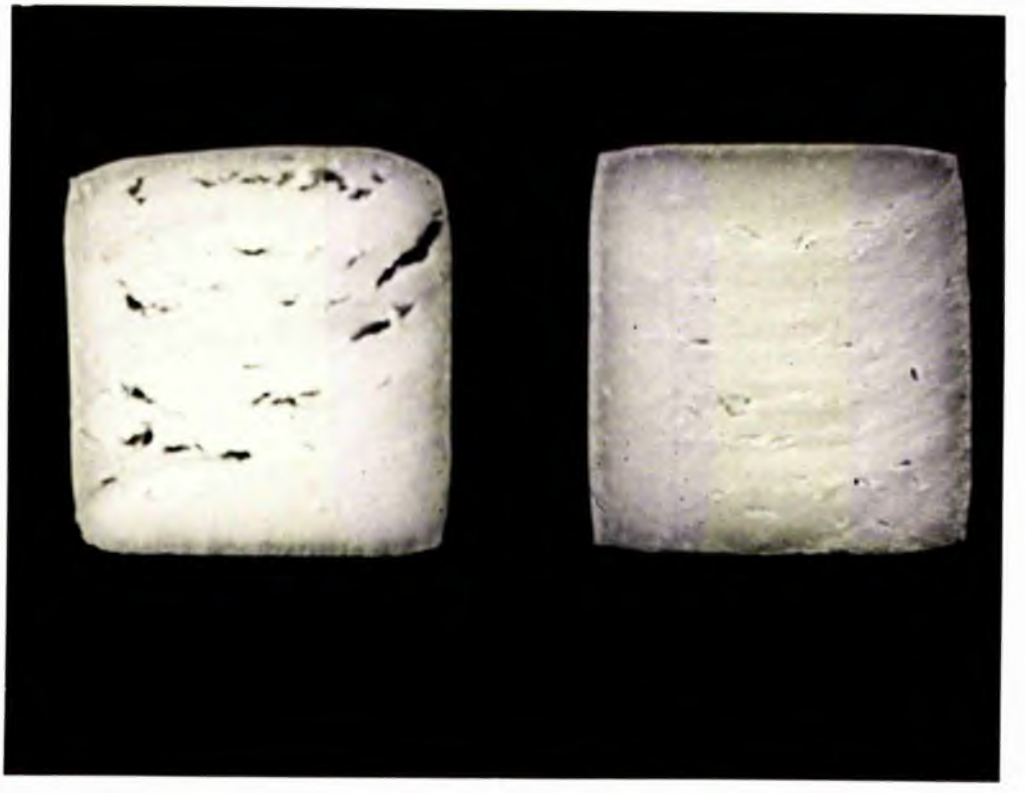
reported that when a starter was added to milk containing undesirable bacteria, its growth was so rapid that multiplication of the undesirable organisms was checked.

When a starter culture is wholly or partially inactivated and the required amount of acid is not formed during the cheesemaking process, the hydrogen ion concentration remains high and suitable for the multiplication of many contaminant bacteria. In these circumstances multiplication and fermentation of these organisms may result in the development of serious faults in the cheese.

The result of the formation of an abnormally small amount of acid in cheese is shown in plate 1. A cheese made from milk in which acid production was normal is compared with that prepared from milk to which penicillin was added to produce a concentration of 0.15 i.u./ml.

The texture of the cheese made from the penicillin-free portion of milk is normal in appearance, whereas the low-acid cheese has a very open texture due to the formation of large amounts of gas by contaminants of the coliform group. A second fault in the low-acid cheese was a severe off-flavour.

Whitehead (190) carried out work on the influence of bacteria of the coliform group on Cheddar cheese



B A

PLATE 1

A comparison of the texture of cheese made from normal milk and milk containing penicillin.

A- normal, B- penicillin

and while concluding that many of these organisms had a deleterious effect on the cheese flavour, open texture did not result when an active culture of lactic streptococci was used as starter even though large numbers of coliform organisms were present in the milk. Marshall (108) and Harrison (70) concluded from earlier work that the addition of starter to unpasteurised milk containing large numbers of such bacilli resulted in quality improvement in the resulting cheese.

Whitehead (190) did not experience any cases of * 'floating curd', a condition not uncommon in cases of severe coliform contamination and concluded, as did Harrison (70) and Marshall (108) that the starter had a very marked influence in restricting the growth of such contaminants.

The <u>fourth</u> function of the starter is to assist in the curing of the cheese. Curing is the term used to describe the part of the process of cheese manufacture which follows the milling, salting, hooping and pressing of the highly acid curd.

* A description of the condition when the curd becomes so buoyant due to the formation of gas that it rises to the surface of the whey instead of settling on the bottom of the vat.

When cheese is taken from the press and placed in a room where the temperature is controlled between 10 and 16°C at a relative humidity of 85°R.H. and held there for several months - the curing period - a complex process takes place within the cheese. Bacteriologists have associated certain organisms with various stages of curing while chemists have related changes in cheese composition to the development of properties of cheese during this period. Both series of studies indicate that the characteristics of mature cheese are due to the action of bacterial and non-bacterial enzymes. The main changes which take place during curing are in the flavour and physical condition of the cheese. Characteristic flavour of mature cheese is a combination of several odours and tastes and is still the subject of intensive scientific investigation. During the curing period the physical condition of the cheese changes from being tough and pliant to soft and plastic. This change is associated mainly with changes in the protein of the cheese through the combined effect of acidity, moisture, salt (sodium chloride), rennet and bacterial action. During the curing period the protein of the cheese is converted through a series of stages involving the formation of proteoses and peptones to amino-acids at the same time becoming soluble in water, estimates of the amount of the

water-soluble materials being used to indicate the extent of maturation.

The process of casein breakdown begins with the addition of rennet, undergoes a second major change through the formation of lactic acid in the curd and young cheese and continues due to bacterial and enzymic action throughout the curing period.

Lactose is quickly destroyed in the cheese, van Slyke and Bosworth (181) showing that this material was usually destroyed in two weeks. Other workers (105,171) have confirmed this opinion. Considerable attention has also been given to the formation of volatile acids in cheese. Hiscox (75) reported that the production of volatile acids took place at a similar rate to the production of flavour in cheese. Dahlberg and Kosikowsky (41) were unable to find such a direct relationship but later (42) reported a possible relationship between volatile acidity and flavour intensity.

Calbert and Price (22) drew attention to the relationship of diacetyl to the flavour of Cheddar cheese. Diacetyl is formed from lactose or citric acid by the action of certain starter bacteria and has long been recognised (179) as the material responsible for the flavour in butter.

van Slyke and Price (183) were of the opinion

that the typical flavour of Cheddar cheese was directly affected by the products of the fermentation of lactose, the initial formation of lactic acid creating a background to the cheese flavour which developed during the curing period.

Two factors influencing the amounts of soluble salts held in cheese are the amount of moisture retained by the curd and the acid development during the manufacturing process.

It is obvious that the action of starter bacteria in ensuring a normal maturation of the cheese is a vital one through the production of acid necessary for the preliminary change in the protein, secondly since the acid production regulates the amount of moisture in the cheese, an important factor in determining the rate of curing of cheese and thirdly in the production of compounds from lactose and citric acid which may well contribute to the formation of the typical flavour of cheese.

SECTION THREE

LACTIC ACID FERMENTATION

LACTIC ACID FERMENTATION

Milk which is freshly drawn from the cow shows considerable variation in acidity, calculated as lactic acid (titratable acidity) and hydrogen ion concentration. van Slyke and Baker (180) determined the pH values of some three hundred samples of freshly drawn milk and found them to vary from 6.5 to 7.2 with 80 per cent under 6.76. McInerney (106) reported that the titratable acidities of fresh milk from individual cows in two herds varied from 0.10 to 0.22 per cent lactic acid. Milk with high solids - not- fat and ash contents had a high titratable acidity, and vice versa. The natural acidity of freshly drawn milk which is expressed as per cent lactic acid is due in the main to the phosphates and casein of the milk. Robinson and Samson (138) suggested that the main cause of natural acidity in milk was the presence of inorganic phosphates and Rice and Markley (136) concluded that the casein and phosphates together were responsible for most of the acidity of fresh milk.

As opposed to natural acidity, the process of souring results in developed acidity. Although

living organisms were observed in sour milk in the early part of the eighteenth century, it was not until 1837 that it was suggested that living organisms were the cause of the souring. The idea that this change was brought about by living cells found little support until Pasteur (131) proved that lactic acid-producing bacteria did in fact exist. In the year 1873 Lister (98) succeeded in isolating one of these forms and so provided incontrovertible evidence of their importance. Prior to the discovery that micro-organisms played an important part in fermentations the change of lactose to lactic acid - discovered by Scheele in 1780 - was thought to result from a change in the casein.

Lactic acid, CH_3-CHOH-COOH, is a non-volatile, odourless acid which mixes with water, alcohol and ether in all proportions. The transformation of lactose to lactic acid takes place in the manner described below. Lactose, a polysaccharide, is first hydrolysed to glucose and galactose. From this point a succession of steps follow through glyceric aldehyde and pyruvic aldehyde to lactic acid. The transformation is not quantitative and secondary products including various acids, aldehydes and alcohol are generally formed. The nature and amount of these subsidiary products varies with the

organism causing the fermentation.

Lactic acid is known in three isomeric forms dextro d, laevo l and intermediate i which are specific to some types of bacteria. Some organisms produce only one form while other species produce both d and l forms, the amount of each type determining the amount of the third isomer which results from interaction of the d and l acids. The production of a single isomeric form appears to be constant for a given organism but where both d and l forms are produced, the amount of these isomers are affected by the environmental conditions under which the organisms are growing.

When milk sours, through the action of bacteria, either present as contaminants or added in pure culture, there is an increase in the titratable acidity of the milk. The acidity increases under suitable temperature conditions, precipitation of the casein occurring at an acidity of 0.5 to 0.6 per cent lactic acid at the iso-electric point pH 4.6. The titratable acidity readings which the cheesemaker obtains during the manufacture of cheese are total acid determinations and do not differentiate between true lactic acid and other acids. Knudsen (92) suggested that when a single-strain culture of Streptococcus cremoris and a mixed-strain starter

growing in milk produced the same titratable acidity but a different hydrogen ion concentration different acids were produced. Czulak (33) reported that cultures of Streptococcus diacetilactis produced the same titratable acidity in milk as cultures of Str. cremoris but the pH was higher.

Hastings, Evans and Hart (73) demonstrated the part played by enzymes in the fermentation of lactose by a culture of Streptococcus lactis. They showed that an increase in the amount of acid produced by this organism in milk occurred after the cells of the culture had been destroyed.

With many lactic acid-producing species of bacteria the acid production continues after coagulation of the casein of the milk until the accumulation of acid prevents further growth of the organisms. The maximum amount of acid produced in milk varies widely with different species. For example, the most common components of cheese starters, Str. cremoris and Str. lactis have a final titratable acidity in milk of from 0.75 to 0.95 per cent lactic acid whereas the lactobacilli commonly produce acid until 2 to 2.5 per cent lactic acid is formed.

Lactose is present in milk to the extent of some 5 per cent but it is rarely, if ever, the limiting factor in acid production. Under normal conditions

there is a considerable amount of lactose remaining unfermented after the final acidity is reached. van Slyke and Bosworth (182) determined the amount of lactose used in the fermentation of milk by cultures of Str. lactis and Aerobacter aerogenes and found that 22 per cent of the total lactose content was utilised, 88.5 per cent of this amount being converted to lactic acid.

SECTION FOUR

EVALUATION OF THE ACID-PRODUCING PROPERTIES OF STARTER CULTURES

EVALUATION OF THE ACID-PRODUCING PROPERTIES OF STARTER CULTURES

The fermentation of lactose by cheese starters is of importance to the laboratory worker and cheesemaker alike. Since the amount of acid formed and its rate of production vary considerably from strain to strain it is essential that a simple but effective method of evaluating these properties be available. Total amount of acid produced by a starter in milk is a less variable property than the rate of production. Cultures may produce the same amount of acid in a long incubation period but the amount of acid formed in the first six hours of inoubation at or near the optimum growth temperature may vary considerably.

The two main components of starters used in the production of Cheddar cheese are Streptococcus cremoris and Streptococcus lactis. These organisms are homo-fermentative in action - they ferment lactose to produce lactic acid and only traces of other substances. This being so the fermenting capacity of cells of these organisms may be more easily determined than

those of the hetero-fermentative organisms, Leuconostoc dextranicum and Leuconostoc citrovorum frequently found in cheese starter or the citrate - utilising homo-fermentative organism Streptococcus diacetilactis.

Cultures of Str. lactis and Str. cremoris ferment lactose to produce lactic acid and traces of acetic (46,65) and propionic acids (65). These trace compounds are not of sufficient quantity to be considered independently in determinations of the vigour of a culture or of its suitability for use in cheese manufacture.

Baker, Brew and Conn (12) estimating the amount of lactic acid which was produced by individual cells of Str. lactis, found the hourly cell rate to be a very variable property ranging from 5×10^{-10} mg to 10×10^{-10} mg for a vigorous culture. The rate of acid production tended to decrease as coagulation point was approached. Matuszewski and Supinska (112) and Matuszewski (111) provided further estimates of the fermenting capacities of cells of starter bacteria. The hourly cell rate of acid production by thirteen strains of Str. lactis was found to vary from 4.91×10^{-10} mg to 24.4×10^{-10} mg (112). These workers suggested a relationship between the cell size and acid production rate. Matuszewski (111) determined the fermentation rate of a strain of Str. lactis

and one of Str. cremoris. At temperatures from 28 to 30°C, Str. cremoris produced acid at an hourly cell rate of 24.4 x 10^{-10} mg. In the case of the strain of Str. lactis the rate was lower at 15.7 x 10^{-10} mg for this temperature range. For both strains, the rate increased at the higher temperature of 38 to 40°C while the opposite was found to be true at 18 to 20°C.

In the determination of the activity of starters for practical purposes, it is unnecessary to ascertain the individual cell rate of acid production. The real object of a test for determining starter activity is to find how the particular culture will behave under practical conditions where rapid acid production is vital to the process. For some considerable time it was thought that the titratable acidity of a starter when ready for use at the end of a 16 - 18 hour propagation period was an indication of the well-being and acid-producing properties of the culture. It is now well known that such a reading at this point bears little relation to the subsequent action of the culture in the vat, although starters at this stage vary in titratable acidity from 0.7 to 0.95 per cent lactic acid.

Sherman and Hodge (151) found that fast-growing strains of Str. lactis produced a lower final acidity

in milk than slow-growing strains.

The first attempt to provide a reliable test for determining the vigour of cheese cultures was described by Whitehead and Cox (196) in 1932. While the test was a means of providing a useful comparison between the vigour of a number of cultures on the same day it was less reliable because of variations in the milk medium in comparing the same cultures on different days. Basically the test consisted of following the normal cheesemaking steps on a small scale in a jar or bottle and determining the titratable acidity at various stages. The test has fallen into disuse because of its being too elaborate for everyday application under commercial conditions.

A second type of test used to determine the vigour of a starter culture was described by Anderson and Meanwell (5). This test consists of determining the titratable acidity of sterilised milk inoculated with 1 per cent starter and maintained for 6 hours at 30 or 37°C. The test is simple, requires a minimum of titrations and gives a reasonable indication of a starter's power to produce acid at the required rate. Storrs and Anderson (169) defined the 'activity' of a starter as determined in this test as the difference between the titratable acidity at the beginning of the test and that at the end multiplied by ten. In this

test a fast starter had an 'activity' of 3 i.e., it developed 3 per cent lactic acid under the conditions of the test. A somewhat similar test varying in detail proposed by Johns and Berard (88). Sterile separated milk was incubated for 2 hours at 30°C and a further four hours at 38.8°C, acidity titrations being carried out at hourly intervals. Babel (8) determined the titratable acidity of sterile reconstituted separated milk inoculated with 1 per cent starter after various periods of incubation and under various test conditions.

The term 'starter quotient' was defined by Hlynka and Hood (76) who compared the acid-producing ability of thirteen starters in a test in which pasteurised whole milk was incubated with 3 per cent of culture and incubated at 36.6°C. Titratable acidities were determined on inoculation and after 1, 2, 3, 4 and 5 hours incubation. The starter 'quotient' was calculated by dividing the first titratable acidity of 0.23 per cent lactic acid or over by the titratable acidity obtained two hours later provided the second reading did not exceed 0.56 per cent lactic acid. This result expressed as a simple ratio with decimal omitted for convenience was termed the 'quotient' of the test culture. This method of expressing the vigour of

a starter was based upon the observation by Baker et al. (12) that titratable acidity in a milk culture under defined conditions increased geometrically. It was claimed that the method allowed of a more accurate expression of true rate of acid production during the active growth phase and that the measurement of starter activity was independent of the amount of inoculum used in the test. The test periods used in other methods include the lag phase of growth - the period when cell multiplication and activity is low.

The use of a standard milk medium was proposed by Horrall and Elliker (79) who described a test based on the inoculation of sterile reconstituted separated milk with 3 per cent starter, and the use of a 3½ hour incubation period at 37.8°C At the end of the incubation period the titratable acidity was determined and expressed as the 'activity factor' of the culture. Horrall and Elliker (79) claimed for their test the advantages of using a standard milk medium and of obtaining a result in a short time.

Golding and McCorkle (58) stressed the need for standardising techniques and media in testing the vigour of starters. These workers pointed out that a standard milk medium was required before a strict comparison could be made between the results obtained

in the laboratory tests and in cheesemaking. In the test described by them weighed amounts of starter and pasteurised reconstituted skim milk were mixed and incubated at 30°C for 6 hours. The difference between the titratable acidity of the fermented milk and the unfermented reconstituted skim milk was recorded as the gain in lactic acid and was a measure of the acid produced by the starter. In the test of Golding and McCorkle (58) the amounts of milk and starter inoculum were measured by weight rather than volume. This departure from general practice was made because it had been found that when a series of inocula were measured by pipette the weights of the individual inocula varied by as much as 10 per cent.

In the opinion of the author the practice of measuring the amount of inoculum by weight is open to criticism. Tests to determine the acid-producing ability of starter bacteria are frequently carried out in conditions where contamination with airborne bacteriophage may occur. When the starter inoculum is measured volumetrically and subsequently transferred by pipette the exposure of culture and milk to contamination is at a minimum. On the other hand the time required for the accurate determination and adjustment of the weight of inoculum will expose the

culture to the risk of contamination by airborne bacteriophage for a much increased period. A second criticism is that few cheesemaking creamery laboratories are equipped with a balance of suitable accuracy for weighing the materials.

A test based on a different principle was proposed by Leber (95) who described the use of a dye reduction test as a means of determining the vigour of starters. In his test 9 ml quantities of reconstituted separated milk were inoculated with 1 ml of starter culture following the addition of 1 ml of a 0.005 per cent solution of resazurin. During an incubation period at 36.6°C examinations weremade at intervals for dye reduction. Leber (95) claimed that the rate of acid production of the test cultures was related to the time taken for complete reduction of the dye. Johns (87) criticised the test on the ground that the rate of change of the dye was not in fact comparable with the rate of acid production while Golding (56) also has pointed out that dye reduction tests using resazurin or methylene blue were unsuitable for judging starter activity.

Golding (56) suggested a test in which it was possible to relate starter activity to the rate of coagulation of milk on the addition of rennet. He inoculated milk with a starter and after 2½ hours at 30°C determined the rate of coagulation of milk

on the addition of rennet. In essentials this test is similar to the so-called rennet test which many cheesemakers in Britain use as a guide in determining the point at which they should add rennet to the cheese milk. It is well known that the result of this test varies very considerably with the composition of the milk and so a standard medium would be a first essential to the use of the method in making accurate comparisons of starter activity.

Wright and Tramer (202) have criticised the method of determining the activity of cheese starters in which pasteurised whole milk is inoculated with culture and incubated for a period before the determination of titratable acidity. They reported that certain starters were classified as slow acid-producing strains in the medium due to the bacterial cells being carried up into the cream layer following agglutination. The addition of agar-agar or rennet to the milk prevented the rise of fat and enabled the cultures to produce normal amounts of acid. Increase in activity was also obtained by heat treatment to destroy the 'agglutinin' or by homogenisation.

By these findings they explained the discrepancy between results obtained in laboratory activity tests and those obtained with certain starters in cheese

manufacture.

The present position with regard to the testing of cheese starter activity in New Zealand is very much changed from the time when the 'vitality' test of Whitehead and Cox (196) was introduced. In the twenty-seven years since that time extensive work has been carried out on the control of bacteriophage and at present most cheesemakers use several tested single-strain cultures maintained in a central laboratory for a number of years. These cultures have been propagated daily, the fastest sub-strains being reselected at regular intervals. It is interesting to note that the selection of the most active sub-strain is made by taking the sub-strain which coagulates sterile milk in the shortest time at a temperature of 37°C (193). The New Zealand workers contend that since they use only twelve or so established strains, activity tests in the creamery are to some extent superfluous. However, true that may be the position is very different in Gt. Britain where cheesemakers obtain starters from many sources and require to compare the acid-producing ability of the new culture with those already in use.

TABLE 4

Methods for determining the rate of acid production by starter cultures

Test	Medium and heat treatment	Amount of inoculum (per cent)	Temp. of incubation (°C)	Time of incubation (h)	Result expressed as
Whitehead & Cox (196)	Whole milk pasteurised	0.5 -	30 - 37	6	Starter 'vitality'
Anderson & Meanwell (5)	Wholemilk sterilised	1.0	30 - 37	6	Starter 'activity'
Johns & Berard (88)	Skim milk sterilised	1.0	30 - 38.8	6	Acid production in given time
Hlynka & Hood (76)	Whole milk pasteurised	3.0	36.6	6	'Starter quotient'
Horrall & Elliker (79)	Reconstituted skim milk sterilised	3.0	37.8	3.5	'Activity factor'
Golding & McCorkle (58)	Reconstituted skim milk pasteurised	1.0	30.0	6	Acid production unit time
Leber (95)	Reconstituted skim milk raw	10.0	36.6		
Golding (56)	Whole milk pasteurised				

The use of tests to determine the acid-producing ability of cheese cultures has largely been confined to testing laboratory 'mother cultures'. Seldom has the activity of the 'bulk' starter - the culture used in cheese manufacture - been determined before use. One factory (114) adopted the practice of testing all starters before use in the manufacturing process. This practice was discontinued as it was thought that the twenty-four hour refrigeration period between the end of the propagation period and use in the vat tended to reduce the activity of the cultures.

The possibility of using a culture of known activity is discussed in section six of this thesis.

SECTION FIVE

CHARACTERISTICS OF MEMBERS OF THE TRIBE STREPTOCOCCEAE USED IN CHEESE STARTERS

CHARACTERISTICS OF MEMBERS OF THE TRIBE STREPTOCOCCEAE USED IN CHEESE STARTERS

The bacteria which are mainly employed in the preparation of starters for use in the manufacture of most British cheese including Cheddar and Dunlop are organisms which ferment lactose to produce lactic acid essential to the process.

These bacteria belong to the tribe Streptoococceae of the family Lactobacteriaceae. The tribe contains homo- and hetero-fermentative groups. Homo-fermentative organisms of interest to cheesemakers belong to the genus Streptococcus (Rosenbach) (140) while the hetero-fermentative types have been given various generic names; Betacoccus (Orla-Jensen) (128), Streptococcus (60) Lactococcus (14) and Leuconostoc (van Tieghem (184), Hucker and Pederson (82)). The hetero-fermentative organisms do not produce large quantities of acid but are frequently associated with the homo-fermentative organisms in commercial cultures used

extensively in cheese manufacture.

Differentiation of a number of bacteria of the same genus into groups to which the label 'species' is given is in the main an arbitrary one. This is no less true of members of the genus Streptococcus than of any other bacteria. Some authorities think of the homo-fermentative streptococci as consisting of one species Streptococcus lactis and its several varieties. Perhaps more support is given to the division of the homo-fermentative lactic group into at least two distinct species. Str. lactis and Streptococcus cremoris. Adherents to this second method of classification have then to decide on a variety or specific rating for the organisms of this group which utilise citrate.

HOMO-FERMENTATIVE ORGANISMS

Streptococcus lactis

Streptococcus lactis was isolated, studied and described by Lister in 1873 (98) and 1878 (99). The name given to the organism by Lister was Bacterium lactis and although many of diagnostoc techniques used at present in differentiating the members of this tribe were unknown at that time Sherman (150) considers that the organisms isolated and described by Lister were similar to the typical lactis strains of later studies.

Distinctive features. Growth takes place at temperatures of 10 and 40°C but not at 45°C. Litmus is acidified and completely reduced before the milk medium coagulates. Ammonia is formed from peptones and amino-acids. The organism is able to tolerate 4.0 but not 6.5 per cent sodium chloride. Growth occurs at pH 9.2 but not 9.6. Belongs to serological group N.

Streptococcus cremoris

The general concensus of opinion is to regard this organism as a species distinct from Str. lactis which it resembles in several properties. Str. cremoris was defined as a new species in 1919 by Orla-Jensen (128) and is accepted as such by Bergey (16) although other workers (201) prefer to regard this organism as a variant of Str. lactis. There is no proof that such a variation has been or can be brought about under carefully controlled laboratory conditions. Knudsen (92) considered that most starter bacteria were varieties of Str. cremoris (Orla-Jensen (128)). The original pure culture starter - No. 18 - used by Storch (166) in 1890 was later considered to be a strain of Str. cremoris.

Distinctive features. Growth of the organism takes place at 10 but not at 40°C. The organism

does not produce ammonia from peptones or amino-acids. Growth does not take place at pH 9.2 or in the presence of 4 per cent sodium chloride. Cultures of Str. cremoris may be further distinguished from those of Str. lactis by their inability to ferment maltose and dextrin. The author has, however, found the sugar reactions to be less reliable for typing purposes than the other characteristics mentioned above.

Streptococcus diacetilactis

In addition to the well-defined species described above, the streptococcus noted for the production of carbon dioxide, volatile acids, acetylmethylcarbinol and diacetyl are of interest to the cheesemaker. This organism which has been given various names by different workers is able to utilise citric acid to produce fermentation products similar to the hetero-fermentative organisms described below.

The position of this organism within the serological group N presents considerable ground for theorising.

Bergey (16) lists several species in an appendix to the genus Streptococcus and among these appear several variant forms which have been isolated from dairy products. van Beynum and Pette (177) isolated from cream and butter an organism which produced

diacetyl and small amounts of acetymethylcarbinol in milk. To this organism they gave the specific epithet aromaticus. These workers described a second variant form of streptococcus to which they applied the specific epithet citrophilus on account of its power to utilise citric acid. In the same year, 1936, the Polish workers Matuszewski, Pijanowski and Supinska (113) isolated an organism of similar properties from fermenting vegetative material to which they gave the name Streptococcus diacetilactis. At a later date, 1939, Karnad (91) applied the specific epithet diacetyl aromaticus to strains of citrate-fermenting streptococci. The two opposing views on the ranking of these strains have been presented by Krishnaswamy and Babel (93) on the one hand and by Swartling (172) on the other. The former group of workers suggested that the citrate-fermenting strains were entitled to rank as varieties within the lactic group of streptococci and should therefore be named Streptococcus lactis var. aromaticus. This designation was in accordance with the method of classification proposed by Hammer and Baker (63). Swartling (172) carried out an extensive investigation into the biochemical and serological properties of citric acid- fermenting streptococci and reached

several conclusions. From the tests carried out and the descriptions provided by other authors he concluded that the streptococci described in the early literature which were marked by their ability to produce d- lactic acid in milk, CO_2, volatile acids and C_4 compounds from citric acid constituted a taxonomic unit should have the specific epithet diacetilactis in preference to that of citrophilus, which was descriptive but not specific. This specific epithet must also be preferred to the lactis aromaticus of Joshi and Ram Ayyar (90) and diacetyl aromaticus of Karnad (91) forms contrary to the recommendations for botanical nomenclature made by Buchanan, St. John-Brooks and Breed (20). Swartling turned down the suggestion of Bergey (16) that Str. diacetilactis was another name for bacteria having the properties of Str. cremoris (Orla-Jensen). The author is in complete agreement with this view. On no occasion has the author found a typical strain of Str. cremoris which gave a positive Vosges-Proskauer reaction - indicating the formation of acetylmethylcarbinol - or which produced carbon dioxide. The difference between these organisms is further emphasised by the early reference (116) to the lack of flavour of butter made from cream soured with pure culture starters of Str. cremoris. Str. diacetilactis produces large amounts

of the flavour compounds whereas only a few strains of Str. cremoris do so and only when incubated under abnormal conditions of low temperature (104).

Distinctive features. Strains of this organism grow at 10°C and generally at 40°C but not at 45°C. Czulak (33) noted the production of gas in milk media by this organism when incubation was continued beyond the clotting point of the milk. Hydrolysis of arginine is a variable property. Belongs to serological group N. Produces acetylmethylcarbinol and diacetyl from citric acid.

HETERO-FERMENTATIVE ORGANISMS

Leuconostoc dextranicum and Leuconostoc citrovorum are found as usual components of many general-purpose commercial starters. This type of starter is used more extensively in Great Britain, U.S.A. and Canada than in New Zealand where single-strain cultures of Str. lactis and Str. cremoris have been generally adopted. Hansen, Bendixen and Theophilus (68) and Sherwood (154) have reported that many of these hetero-fermentative organisms are able to cause open texture in cheese due to the production of gas in the fermentation of sugars or in the breakdown of citric acid. Many cheesemakers are of the opinion that starters containing these organisms impart a more

desirable flavour to Cheddar cheese. While this has not been proved, the regularity with which the opinion has been expressed seems to indicate that there may well be some basis for the assertion. Evans (46) and Hucker and Marquardt (81) reported that the addition to bulk milk of a pure culture of hetero-fermentative organisms resulted in an improvement of the flavour of the cheese obtained. The Swedish workers Thomé and Pollack (174) have reported on the role of the 'aroma bacteria' in Swedish Herrgård cheese which is a hard-pressed variety cured for a period similar to that of Cheddar. It was found that during curing this type of starter formed a larger amount of amino-acids than a streptococcus culture free from aroma bacteria. These Swedish workers expressed the view that the aroma bacteria contributed to the aroma of the ripened cheese. Whitehead (193) has expressed the opinion that the members of this group play little part in the development of the so-called characteristic flavour of Cheddar cheese. Loftus Hills (102) offered the view of several practical cheesemakers in Australia that the use of mixed-strain starters containing these organisms resulted in the development of a more aromatic early flavour in Cheddar cheese.

Leuconostoc dextranicum (82)

The follwoing synonyms are listed for this organism: Laotococcus dextranicus (Beijerinck (14)), Betacoocus bovis (Orla-Jensen (128)), Betacoccus cremoris (92), Streptococcus paracitrovorus (Hammer (60)).

Distinctive features. The optimum growth temperature is between 21 and 25°C. Acid production by this organism in milk is slow but finally brings about coagulation of the protein. Carbon dioxide is formed during the fermentation of sugars and the breakdown of citric acid. Slime is produced in rapidly growing cultures in sucrose media.

Leuconostoc citrovorum (82)

Synonymous is Streptococcus citrovorus of Hammer (60).

Distinctive features. The optimum temperature is between 20 and 25°C. Very little acid is produced in milk. Coagulation seldom results from the growth of this organism in milk. Slime is not produced in sucrose media.

OTHER STREPTOCOCCI

Streptococcus durans

A recent development in the manufacture of Cheddar cheese has been the introduction of the 'short-time'

method briefly described above (188,36). In this method a thermodurio starter is used in conjunction with the conventional starter containing the organisms described above. Walter et al. (188) who first described this method selected as their thermodurio organism a non-haemolytic strain of Streptococcus durans.

In 1935, Sherman and Wing (152) described a haemolytic non-pathogenic organism which they had isolated from dried milk. They were of the opinion that this organism was distinct from other haemolytic streptococci, many of which were pathogenic, and was in fact a new species for which they suggested the name Streptococcus haemothermophilus. Two years later in 1937 the same workers (153) advocated that this new specific epithet be withdrawn in favour of the epithet durans. In suggesting this change they pointed out that fuller investigation of the characteristics of the organism had shown that it bore no relation to the species thermophilus. Furthermore resistance of the organism to heat and to alkaline media had been confirmed.

Distinctive features. Growth takes place at 10 and 45°C but seldom above 50°C. Withstands 60°C for 30 minutes. Acidifies milk and causes curdling. Gas is not formed in milk. Inability to ferment

mannitol is suggested (146) as a means of distinguishing this organism from other members of the enterococcal group e.g. Streptococcus faecalis. Ammonia is produced from peptone. This organism is able to withstand 2, 4 and 6.5 per cent sodium chloride, pH 9.6 and 0.01 to 0.1 per cent methylene blue. Generally beta-haemolytic but cultures used in cheesemaking (188) are non-haemolytic variants. Serologically conformant with Lancefields group D.

Streptococcus thermophilus

The search for rapid acid-producing heat-tolerant organisms for use in the 'short-time' method for Cheddar cheese manufacture is being actively carried out and one recent introduction has been the use of a culture of Streptococcus thermophilus by Czulak and Hammond (35) in Australia where the method is being used on a commercial scale. Given below are the main features of this species so named by Orla-Jensen in 1916 (127).

Distinctive features. Growth does not take place below 20°C. The optimum temperature is between 40 and 45°C. The organism withstands 65°C for 30 minutes but is unable to grow in the presence of 2 per cent sodium chloride, at pH 9.6 or in media containing 0.01 and 0.1 per cent methylene blue. Ammonia is not formed from peptones or amino-acids. The serological

position of this organism is undecided: there is some evidence (147), however, to suggest that it belongs to a new serological group.

COMBINATIONS OF STREPTOCOCCI USED AS CHEESE STARTERS

Starters used in cheesemaking may be divided into three types: -

Single-strain

Multi-strain

Mixed-strain

Single-strain

As the name suggests, starters of this type consist of only one strain of organism. The term is almost universally restricted to cultures of the non-aroma, non-gas-producing group N streptococci.

Multi-strain

This form of starter consists of more than one strain of starter bacteria. The strains may be of the same species or of two species having similar characteristics, for example, Str. cremoris and Str. lactis. This term is used by the author for combinations of non-aroma, non-gas-producing group N streptococci.

Mixed-strain

Starters of this type include the general purpose

commercial starters and consist of a mixture of acid- and aroma-producing organisms. Such starters generally contain Str. cremoris and / or Str. lactis, the homo-fermentative organism Str. diacetilactis and / or members of the genus Leuconostoc.

SECTION SIX

CONDITIONS OF PROPAGATION AS FACTORS IN THE ACID-PRODUCING ACTIVITY OF STARTER CULTURES

CONDITIONS OF PROPAGATION AS FACTORS IN THE ACID-PRODUCING ACTIVITY OF STARTER CULTURES

Successful cheese manufacture is dependent on the use in their most active condition of starter cultures of high acid-producing ability. Many factors act singly or in combination in the preparation of cultures possessing the required rate of acid production.

In normal practice, cheese starter cultures are prepared by incubating for a period of fourteen to eighteen hours heat-treated milk previously inoculated with 1 - 2 per cent of a twenty-four-hour old culture at 21 - 22°C. Such conditions of amount of inoculum, temperature and duration of incubation are generally accepted as the most suitable for the transfer of starter cultures of the type used in British cheese manufacture, where the organisms Streptococcus lactis, Streptococcus cremoris and Streptococcus diacetilactis are used either singly or in combination.

The optimum temperature for growth and acid production by these organisms is in the range

29 - 33°C (8,16,57,40). The incubation temperature used in daily transfer is, therefore, considerably below the optimum. It is probable that the system of daily transfer presently adopted owes its origin to the method of working in the commercial cheese factories. It is convenient for the creamery personnel to inoculate milk with the culture of lactic acid bacteria and leave it unattended at suitable temperature overnight. It must be assumed that by trial and error it was found that the temperature 21 - 22°C provided a suitably active culture. The cheesemaker relies on the physical condition of the culture as a guide to the activity of the starter and at this temperature a 1 per cent inoculum coagulates the milk within the overnight period.

Conditions of starter control in cheese factories are now very much improved but a considerable number of factories are still without suitable equipment for the efficient control of the temperature of the inoculated milk. In these factories the person in charge of starter control must guess at the rate at which the inoculated milk will cool during the overnight incubation period in the uncontrolled temperature of the starter room and after deciding whether the night will be cold or warm he adjusts the temperature of the starter milk to 25 - 27°C

or 20 - 23°C. This system must always be one of chance and while the practice is becoming less common it is all too prevalent.

The question of the over-ripening of starter cultures has been debated before (88,64,57) and to some degree, has remained unanswered. The activity of a culture depends to a large extent on the number of viable organisms present in the culture and on the vigour of the cells. Johns and Berard (88) have reported that the numbers of organisms present in milk inoculated with 1 per cent culture was at a maximum by the tenth hour of incubation at 21 - 22°C and remained relatively constant for the next six hours. The maximum acidity brought about by the cellular activity was not, however, reached until after thirteen hours incubation. This is in agreement with the opinion of Knudsen (92) that the number of viable cells reached a maximum at the time of coagulation of the milk medium and from that moment bacteria began to be weakened in acid-producing ability. Johns and Berard (88) have stated that over-ripening did not affect the subsequent rate of acid development over a six hour period of incubation. These workers brought about over-ripening by extending the incubation time. Golding, McCorkle and Milich (59) investigated several factors affecting starter bacteria by means of a starter activity test modified

on that of Anderson and Meanwell (5). In this study they examined the acid production rate of three cultures incubated at different temperatures for varying periods and reached the conclusion that an incubation temperature of 21 - 22°C produced starter cultures of higher activity than did a temperature of 30°C. In this work twelve- and sixteen-hour incubation periods were used, and from the tables published there appears to be no difference in the activity of the starters although the titratable acidity of the twelve-hour cultures was always lower than that of the sixteen-hour culture. Hammer and Baker (64) have noted that cultures prepared at 21.1°C could be used after 30 hours without showing an increase in the time required for coagulation of milk inoculated with the culture. Johns and Berard (88) reported that a longer than usual incubation period did not decrease the rate of acid production of starters. Babel (11) advocated the use of an incubation temperature of 22°C for daily transfer of starter cultures in order to maintain a balance between the citrate-fermenting and lactic acid-producing streptococci, a theory applicable only to mixed-strain cultures.

Most studies of the effect of cultural conditions on starter bacteria have been made on commercial mixed-strain cultures. Little work has been done to

investigate the effect of propagation conditions on the acid-producing ability of single-strain cultures of Str. lactis, Str. cremoris and Str. diacetilactis which are the main component acid-producing strains of mixed-strain starters.

I TEMPERATURE OF PROPAGATION

In order to substantiate the claim of Golding et al. (59) that 21 - 22°C provided the optimum temperature for propagating cultures the work described below was undertaken.

EXPERIMENTAL

Materials and methods

Test cultures. The following single-strain starter cultures received as named strains from various sources or isolated by the author from commercial cultures and identified by the accepted diagnostic reactions (16,119) were chosen:

Str. lactis; strains 712, 926, 507, 818, A 11, C 2, C 6, C 10, ML3, FC/54 J/5.

Str. cremoris; strains 508, 607, 806, IP5, 924, A 12, C3, ML1, FC/55 J/2.

Str. diacetilactis; strains DRC 1, DRC 2, 176.

Milk. 100 ml quantities of sterile separated milk were used. The milk in Erlenmeyer flasks, plugged with cotton wool, was autoclaved at 10 lb/sq. in. pressure for 10 minutes following a steaming

at 100°C for 30 minutes.

Method 1. Eight flasks of sterile separated milk were each inoculated with 1 per cent of an eighteen-hour-old separated milk culture of the test culture prepared by the normal bacteriological method of starter control (199). After the contents of the flasks had been mixed, four flasks were incubated at 22°C (±1°C) the remaining four being incubated at 30°C (±1°C). Prior to inoculation the temperature of the milk was adjusted to the incubation temperature. After eighteen hours incubation the milk which was now coagulated was mixed by shaking and an 'activity' test (5) in duplicate was carried out on each of the eight cultures. The test consisted of the addition of 1 per cent of the culture to 100 ml of sterile separated milk followed by incubation at 30°C for 5½ hours. At the end of the test period the titratable acidity was determined by titrating a 10 ml portion with N/9 sodium hydroxide (NaOH) solution using 1 ml of a 0.5 per cent phenolphthalein solution as indicator. In some of the tests the hydrogen ion concentration was determined at the end of the six-hour period by means of a Marconi pH meter used in conjunction with a glass electrode and calomel reference electrode. In these latter determinations the results are expressed to the nearest 0.1 pH unit.

A further series of experiments was carried out

with a selection of cultures which had been used extensively in commercial cheese manufacture. The experimental technique was similar, but a wider range of temperature was adopted viz., 20, 22, 30 and 37°C, duplicate flasks of inoculated milk being incubated. The lowest temperature in the range is near the minimum temperature for growth of the lactic streptococci while the highest, 37°C is in the maximum growth range of Str. cremoris.

Method 2. Twelve of the above-mentioned test cultures which had been widely used in commercial cheese manufacture were transferred daily over an eight-day period in 10 ml amounts of sterile separated milk inoculated with 0.1 ml culture and incubated at three test temperatures, 22, 30 and 37°C. After each transfer the titratable acidity of the cultures was determined and after the eight transfers the acid-producing ability of the final culture was tested by the method of Anderson and Meanwell (5) described above.

RESULTS

Acid-producing ability of cultures after one transfer at various temperatures. Full details of the results obtained with four representative cultures, two of Str. cremoris and two of Str. lactis are presented in tables 5 - 8 while a summary of the results of all the tests are shown in table 9. The figures given

in table 9 are the mean of eight values. The results of the experiments with thirteen different cultures, representative of three species of starter bacteria, indicated that a propagation temperature of 30°C affected the acid-producing ability of the next transfer of the cultures at their optimum growth temperature, the acid production of the strains of Str. cremoris being particularly reduced by propagation at the higher temperature. One test culture - 607 - suffered a 35 per cent reduction in the amount of acid produced in a six-hour test in milk compared with the culture propagated at 22°C. Determinations of pH made at the same time confirmed this result. In all cases there was a close agreement between the titratable acidity and pH results. One strain of Str. lactis suffered an 11 per cent reduction in the amount of acid produced in the test period after propagation at 30°C but other strains were less seriously affected. In the series of tests in which a wider range of propagation was used a similar result was obtained. An examination of tables 10 - 16 shows that propagation temperatures of 30 and 37°C are prejudical to the preparation of active culture of the lactic streptococci. In all cases the acid-producing ability of cultures propagated at 37°C was lower than that of the same cultures propagated at 22°C. The amount of reduction in activity resulting

TABLE 5

A comparison of the acid-producing properties of a culture propagated at 22 and 30°C. Titratable acidity (per cent lactic acid) and hydrogen ion concentration of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: Str. cremoris No. 806

	Propagation temperature (°C)			
Culture	22		30	
	% lactic acid	pH	% lactic acid	pH
1	0.46	4.7	0.43	4.9
	0.47	4.8	0.40	4.9
2	0.45	4.8	0.41	4.9
	0.44	4.8	0.42	4.9
3	0.47	4.8	0.39	5.0
	0.45	4.8	0.41	5.0
4	0.43	4.8	0.41	5.0
	0.48	4.8	0.38	5.0
Mean	0.46	4.8	0.41	5.0

TABLE 6

A comparison of the acid-producing properties of a culture propagated at 22 and 30°C. Titratable acidity (per cent lactic acid) and hydrogen ion concentration of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: *Str. cremoris* No. 607

	Propagation temperature (°C)			
Culture	22		30	
	%lactic acid	pH	%lactic acid	pH
1	0.41	5.0	0.33	5.3
	0.43	5.0	0.30	5.4
2	0.43	5.0	0.30	5.4
	0.41	5.0	0.31	5.4
3	0.42	4.9	0.35	5.3
	0.44	4.9	0.33	5.3
4	0.44	4.9	0.33	5.3
	0.42	5.0	0.34	5.3
Mean	0.43	4.95	0.32	5.3

TABLE 7

A comparison of the acid-producing properties of a culture propagated at 22 and 30°C. Titratable acidity (per cent lactic acid) and hydrogen ion concentration of milk maintained at 30°C for 2½ hours after inoculation with 1 per cent culture

Test strain: *Str. lactis* No. 712

	Propagation temperature (°C)			
Culture	22		30	
	% lactic acid	pH	% lactic acid	pH
1	0.41	5.0	0.38	5.1
	0.42	5.0	0.38	5.1
2	0.45	4.9	0.40	5.1
	0.44	5.0	0.39	5.1
3	0.40	5.0	0.45	4.9
	0.44	5.0	0.41	5.0
4	0.43	4.9	0.40	5.1
	0.43	4.9	0.40	5.1
Mean	0.43	5.0	0.40	5.1

TABLE 8

A comparison of the acid-producing properties of a culture propagated at 22 and 30°C. Titratable acidity (per cent lactic acid) and hydrogen ion concentration of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: *Str. lactis* No. 507

Culture	Propagation temperature (°C)			
	22		30	
	%lactic acid	pH	%lactic acid	pH
1	0.45	4.8	0.46	4.9
	0.46	4.8	0.47	4.8
2	0.45	4.9	0.44	4.8
	0.45	4.8	0.45	4.9
3	0.47	4.8	0.47	4.9
	0.47	4.8	0.46	4.9
4	0.47	4.9	0.45	4.9
	0.42	4.9	0.40	5.0
Mean	0.46	4.85	0.45	4.9

TABLE 9

A comparison of the acid-producing properties of cultures propagated at 22 and 30°C. Titratable acidity (per cent lactic acid) and hydrogen ion concentration of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test culture	Strain of organism	Propagation temperature (°C) 22 % lactic acid	pH	30 % lactic acid	pH	Reduction in activity (%)
FC/55J/2	Str. cremoris	0.45	-	0.42	-	21
806	Str. cremoris	0.46	4.8	0.41	5.0	12
A 12	Str. cremoris	0.56	-	0.43	-	30
1P5	Str. cremoris	0.61	4.4	0.49	4.6	25
924	Str. cremoris	0.43	5.0	0.39	5.2	10
508	Str. cremoris	0.39	5.2	0.32	5.4	22
607	Str. cremoris	0.43	4.9	0.32	5.3	35
FC/55J/4	Str. lactis	0.53	-	0.51	-	4
507	Str. lactis	0.46	4.8	0.45	4.9	2
926	Str. lactis	0.43	5.0	0.41	5.1	2
712	Str. lactis	0.43	5.0	0.40	5.1	7
818	Str. lactis	0.41	5.2	0.37	5.3	11
A 11	Str. lactis	0.385	-	0.36	-	6.5

* mean of eight values

from one transfer at 37°C varied from the complete inactivation of Str. cremoris strains E8 and ML1 to a drop of 32 per cent in the activity of Str. lactis strain ML3 and Str. diacetilactis strain 176. When an incubation temperature of 30°C was used the activity of the resulting cultures was lower in all cases although not to the same degree. The effect of a 30°C incubation temperature was less severe than that of 37°C. The majority of the strains of Str. cremoris were more severely affected than strains of Str. lactis. The reaction of the limited number of strains of Str. diacetilactis was somewhat variable, indicating an inconsistency in resistance to high propagation temperatures.

The use of an incubation temperature of 20°C did not result in any significant change in the activity of the cultures.

The effect of continued daily transfer of starter cultures at various temperatures. Strains of Str. lactis were less affected by high propagation temperatures than were the test strains of Str. cremoris. Two exceptions were found however. Strain C2, of the lactis group, was unable to grow at 37°C whereas Str. cremoris strain C3 grew normally in the propagation period, coagulated the milk and produced the usual amount of acid in the milk medium. At the

TABLE 10

A comparison of the acid-producing properties of a culture propagated at various temperatures. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: Str. cremoris C1

Culture	Propagation temperature (°C)			
	20	22	30	37
1	0.415	0.395	0.34	0.24
	0.41	0.37	0.32	0.245
2	0.42	0.375	0.31	0.25
	0.41	0.415	0.32	0.25
Mean	0.415	0.39	0.32	0.245

TABLE 11

A comparison of the acid-producing properties of a culture propagated at various temperatures.

Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: Str. cremoris C3

Culture	Propagation temperature (°C)			
	20	22	30	37
1	0.39	0.405	0.37	0.28
	0.41	0.40	0.39	0.29
2	0.42	0.395	0.36	0.28
	0.41	0.40	0.355	0.26
Mean	0.41	0.40	0.37	0.28

TABLE 12

A comparison of the acid-producing properties of a culture propagated at various temperatures. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: *Str. lactis* ML3

Culture	Propagation temperature (°C)			
	20	22	30	37
1	0.415	0.405	0.36	0.31
	0.405	0.42	0.39	0.30
2	0.42	0.415	0.37	0.31
	0.41	0.405	0.38	0.31
Mean	0.41	0.41	0.38	0.31

TABLE 13

A comparison of the acid-producing properties of a culture propagated at various temperatures. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: Str. lactis C2

Culture	Propagation temperature (°C)			
	20	22	30	37
1	0.43	0.40	0.38	0.23
	0.415	0.395	0.39	0.23
2	0.385	0.38	0.40	0.22
	0.40	0.405	0.385	0.215
Mean	0.41	0.395	0.39	0.225

TABLE 14

A comparison of the acid-producing properties of a culture propagated at various temperatures. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: *Str. diacetilactis* DRC1

Culture	Propagation temperature (°C)			
	20	22	30	37
1	0.51	0.36	0.33	0.28
	0.36	0.36	0.33	0.275
2	0.35	0.36	0.32	0.28
	0.34	0.34	0.345	0.27
Mean	0.355	0.355	0.33	0.275

TABLE 15

A comparison of the acid-producing properties of a culture propagated at various temperatures.

Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain: Str. diacetilactis DRC2

Culture	Propagation temperature (°C)			
	20	22	30	37
1	0.34	0.32	0.27	0.25
	0.35	0.34	0.28	0.25
2	0.31	0.34	0.28	0.27
	0.33	0.34	0.27	0.25
Mean	0.33	0.335	0.275	0.255

TABLE 16

The acid-producing ability of cultures propagated at various temperatures. Titratable acidity (per cent lactic acid)[x] of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Test strain	Organism	Propagation temperature (°C)			
		20	22	30	37
C1	Str. cremoris	0.415	0.39	0.32	0.245
C3	Str. cremoris	0.41	0.40	0.37	0.28
ML1	Str. cremoris	0.30	0.32	0.28	0.205
E8	Str. cremoris	0.39	0.40	0.345	0.215
ML3	Str. lactis	0.41	0.41	0.38	0.31
C2	Str. lactis	0.41	0.395	0.39	0.225
C6	Str. lactis	0.295	0.29	0.265	0.255
C10	Str. lactis	0.41	0.435	0.415	0.315
DRC1	Str. diacetilactis	0.355	0.355	0.33	0.275
DRC2	Str. diacetilactis	0.33	0.335	0.275	0.255
176	Str. diacetilactis	0.39	0.42	0.37	0.31

x — Mean of four values

end of the eight day test period the activity test made on this culture showed that although growth was normal the acid-producing ability had been impaired by transfer at high temperature. A propagation temperature of 30°C had little effect on the acid-producing properties of strain C2. When the propagation temperature was raised to 37°C however, the culture was inactivated after two transfers - table 17. The titratable acidity of four test cultures - two of Str. lactis and two of Str. cremoris are presented in table 18. It will be seen that there is little difference between the titratable acidities on daily transfer of the cultures propagated at 30 and 37°C over the eight-day period. After two transfers at 37°C the cultures of Str. cremoris were inactivated and the titratable acidity readings shown in table 19 are those of the uninoculated milk medium. The titratable acidity readings of the Str. lactis strains propagated at 37°C are lower than the corresponding transfers at 22 or 30°C. An examination of the acid-producing ability of the cultures after the eight-day transfer period is shown in table 20. The activity of strains C2, C10 and ML3 of the lactis group was similar after propagation at either 22 or 30°C. Only one strain of Str. cremoris retained its

TABLE 17

The effect of a propagation temperature of 37°C on the growth of strains of Str. lactis and Str. cremoris as judged by the titratable acidity (per cent lactic acid) at the end of each twenty-four hour incubation period

Test strain	Organism	Daily transfer Titratable acidity (% lactic acid)							
		1	2	3	4	5	6	7	8
C2	Str. lactis	0.61	0.19	0.17+	0.18+	0.18	0.18	0.18	0.18
C3	Str. cremoris	0.74	0.64	0.71	0.66	0.73	0.83	0.82	0.66

+ Titratable acidity of sterile separated milk: 0.17 and 0.18 (per cent lactic acid)

TABLE 18

Titratable acidity (per cent lactic acid) of twenty-four hour old milk cultures propagated at various temperatures

Test strain	Organism	Propagation temp. (°C)	Daily transfer Titratable acidity (per cent lactic acid)						
			1	2	3	4	5	6	7
C1	Str. cremoris	22	0.87	0.90	0.99	0.96	0.91	0.96	0.91
		30	0.89	0.94	0.93	0.96	0.99	0.91	0.90
		37	0.67	0.19	x0.18	0.18	0.18	0.18	0.18
C13	Str. cremoris	22	0.84	0.82	0.96	0.95	0.88	0.90	0.90
		30	0.92	0.92	0.93	0.97	0.97	0.93	0.93
		37	0.67	0.20	0.18	x0.17	0.17	0.18	0.18
C6	Str. lactis	22	0.88	0.83	0.87	0.87	0.87	0.91	0.88
		30	0.86	0.94	0.96	0.97	0.93	0.92	0.91
		37	0.81	0.76	0.77	0.74	0.65	0.65	0.68
ML3	Str. lactis	22	0.88	0.89	0.96	0.96	0.87	0.86	0.99
		30	0.94	0.90	0.96	0.97	0.94	0.94	0.95
		37	0.83	0.67	0.78	0.74	0.76	0.78	0.75

x Titratable acidity of sterile separated milk: 0.18 and 0.17 (per cent lactic acid)

TABLE 19

Rate of acid production of single-strain starters of Str. cremoris propagated for eight daily transfers at various temperatures. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent culture

Propagation temperature (°C)	Starters							
	C 1	C 3	C 13	E8	HP	K	KH	ML1
22	0.49	0.47	0.38	0.54	0.43	0.46	0.54	0.35
30	0.42	0.42	0.31	0.36	0.30	0.40	0.43	0.35
37	x 0.18	0.22	0.18	0.18	0.18	0.18	0.19	0.21

x Titratable acidity of sterile separated milk: 0.18 (per cent lactic acid)

TABLE 20

Rate of acid production of single-strain starters of Str. lactis propagated for eight daily transfers at various temperatures

Propagation temperature (°C)	Starters			
	C2	C6	C10	ML3
22	0.39	0.38	0.51	0.54
30	0.40	0.32	0.48	0.54
37	x 0.18	0.25	0.30	0.30

x Titratable acidity of sterile separated milk: 0.18 (per cent lactic acid)

activity after propagation at 30°C.

DISCUSSION

It has been confirmed that the optimum temperature for the propagation of active acid-producing starter cultures of the lactic streptococci is in the range 20 - 22°C where a 1 per cent inoculum is used and an incubation time of 18 to 24 hours is allowed. The reaction of starter bacteria to change in propagation temperature is essentially a characteristic, firstly of species and secondly, of individual strain. With few exceptions the activity of strains of Str. cremoris were seriously affected by either one or eight transfers. The results presented in table 9 show that the amount of acid produced in milk in a 5½ hours period of incubation at 30°C by test strains of Str. cremoris and Str. lactis propagated for one transfer at 30°C was lower than that produced by the same strains propagated at 22°C by from 10 - 35 and 2 - 11 per cent respectively. Exceptions to the general specific reaction were noted. Growth temperature is particularly critical with certain strains. One strain of Str. lactis suffered no loss of activity after propagation at 30°C but was completely inactivated at a propagation at 37°C. The lowering of acid-producing ability on propagation at 30°C did not appear to be related to the normal rate of acid production of the test strain. Test strains 1P5, 806 and A12 which were naturally fast acid-producers

were affected to the same extent as the slow acid-forming strains 924, 508 and 607 - table 9. The effect of increasing the propagation temperature from 22°C to either 30 or 37°C was similar for one or eight transfers.

Only a limited number of strains of Str. diacetilactis were examined but the reactions of the test strains suggested an inconsistency in reaction. The extensive study carried out by Swartling (172) on cultures which he classified as Str. diacetilactis showed a very considerable variation in many of the type reactions of this species with respect to growth at 40°C and hydrolysis of arginine, reactions of considerable value in the differentiation of Str. lactis and Str. cremoris.

While it has long been known that Str. cremoris is unable to grow at 40°C the use of a test based on the reduction of acid-producing ability of the cultures on propagation at 30°C, as compared with propagation at 22°C is of possible value in typing unknown strains of the lactic streptococci. One difficulty associated with such a test is the need for working conditions where freedom from airborne bacteriophage contamination is ensured. A second requirement is that of considerable replication since as is shown in tables 5 - 8 a considerable variation in individual activity tests results was obtained in carefully controlled

experiments carried out by an experienced worker using standarised equipment.

The need for close supervision of the temperature of propagation of starter cultures is clearly demonstrated. A low temperature is less likely to give trouble in subsequent activity than a high one. The significance of temperature control in a starter preparation has become more important in recent years since two new methods of starter control were developed. In both of these methods - the system described by Lewis (97) for the preparation of laboratory and bulk starter and that described by Jones (89) for the propagation of laboratory cultures, heating and cooling of the milk takes place at a predetermined rate. Recently the author has found that failures of cheese starters were caused by the temperature of the cooled milk being too high at inoculation and throughout the incubation period. The harmful effect of high incubation temperature may not show in the first transfer but if continued a lowering in activity may result.

The results show conclusively that temperature of propagation is a very important factor in determining the rate of acid production of starter cultures.

II. REFRIGERATION OF MATURE CULTURES BEFORE USE

Starter cultures are used in cheese manufacture or for retransfer when fourteen to twenty-four hours old. In some cases, however, 'bulk' starter not immediately required for cheesemaking may be held at a low temperature after being prepared before use. The influence of this treatment on starter cultures was determined in a series of experiments described below.

EXPERIMENTAL

Materials and methods

Test cultures. The following starter cultures were used;

Str. cremoris; single-strain cultures FC/54 J/5, FD/53 J/1 and FD/53 J/5.

Mixed-strain starters; 91 and FD/54 A.

Milk. 100 ml quantities of sterile separated milk were used. Freshly separated milk in Erlenmeyer flasks, plugged with cotton wool, was autoclaved at 10 lb/ sq. in. pressure for 10 minutes following a steaming at 100°C for 30 minutes.

Method. Four flasks of sterile milk were each inoculated with 1 per cent of an eighteen-hour-old separated milk culture of the test strain prepared in the normal manner at 22°C. After the contents of the flasks had been mixed the flasks of inoculated

milk were incubated at 22°C (±1°C) for eighteen hours. Two of the flasks were then subjected to an activity test (5) while the remaining two flasks were placed in a refrigerator thermostatically controlled at 4°C for twenty-four hours, after which time an activity test (5) was carried out using the same batch of sterile separated milk as that used at twenty-four hours. Prior to carrying out the test the temperature of the refrigerated cultures was raised to 22°C. At the end of the incubation period of the activity test titratable acidity and hydrogen ion concentration were determined.

RESULTS

The results obtained with each culture are detailed in tables 21 - 25. An examination of table 21 shows that the reaction of the test cultures to storage at 4°C when tested on different occasions varied considerably. In test number 1 the culture stored for twenty-four hours after the completion of the propagation period had a very much lower activity than the fresh culture. A similar result was found in tests 2 and 5. On the three other occasions when tests were made there did not appear to be any signficant difference in the rate of acid production by the cultures. In table 22 it will be seen that on two occasions the refrigerated culture

TABLE 21

A comparison of the rate of acid production in milk of fresh and refrigerated starter. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent starter

FD/53J/5: single-strain starter of Str. cremoris

Test	Age of culture (h) 18 % lactic acid	pH	42 % lactic acid	pH
1	0.455	-	0.37	-
2	0.470	5.20	0.38	5.45
3	0.45	-	0.445	-
4	0.465	-	0.45	-
5	0.40	5.40	0.325	5.40
6	0.455	5.20	0.455	5.30

TABLE 22

A comparison of the rate of acid production in milk of fresh and refrigerated starters. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent starter

Culture 91 Mixed-strain

Test	Age of culture (h) 18 % lactic acid	pH	42 % lactic acid	pH
1	0.64	-	0.535	-
2	0.50	5.2	0.505	5.2
3	0.455	-	0.500	-
4	0.575	-	0.525	-
5	0.515	5.0	0.515	4.9

TABLE 23

A comparison of the rate of acid production in milk of fresh and refrigerated starters. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent starter

FC/54J/5; single-strain starter of Str. cremoris

Test	Age of culture (h) 18		42	
	% lactic acid	pH	% lactic acid	pH
1	0.53	5.10	0.54	5.10
2	0.44	5.20	0.47	5.10
3	0.55	-	0.435	-
4	0.48	5.00	0.49	5.20
5	0.52	5.40	0.56	5.00
6	0.485	-	0.475	-
7	0.57	4.90	0.47	5.30
8	0.51	5.05	0.48	5.00

TABLE 24

A comparison of the rate of acid production in milk of fresh and refrigerated starters. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent starter

FD/53J/1: single-strain starter of Str. cremoris

Test	Age of culture (h) 18		42	
	% lactic acid	pH	% lactic acid	pH
1	0.47	-	0.38	-
2	0.435	5.20	0.38	5.45
3	0.46	-	0.43	-
4	0.43	-	0.40	-
5	0.425	5.30	0.43	5.30

TABLE 25

A comparison of the rate of acid production in milk of fresh and refrigerated starters. Titratable acidity (per cent lactic acid) of milk maintained at 30°C for 5½ hours after inoculation with 1 per cent starter

Culture FD/54A. Mixed-strain

Test	Age of culture (h) 18		42	
	% lactic acid	pH	% lactic acid	pH
1	0.525	-	0.480	-
2	0.520	5.05	0.515	5.15
3	0.615	-	0.590	-
4	0.560	-	0.485	-
5	0.540	5.00	0.500	5.10
6	0.51	5.00	0.530	5.00

TABLE 26

The effect of prolonged refrigeration at 4°C on the acid production by a starter culture. Titratable acidity (per cent lactic acid) and hydrogen ion concentration after 5½ hours incubation at 30°C of milk inoculated with 1 per cent starter

FC/54J/1: single-strain starter of Str. cremoris

Series		Age of culture (h) 18	42	66
A	% lactic acid	0.53	0.54	0.49
	pH	5.10	5.10	5.20
B	% lactic acid	0.44	0.47	0.41
	pH	5.20	5.10	5.30

TABLE 27

The effect of prolonged refrigeration at 4°C on the acid production by several starters. Titratable acidity (per cent lactic acid) and hydrogen ion concentration after 5½ hours incubation at 30°C of milk inoculated with 1 per cent starter

Starter	Age of culture (h) 18		118	
	% lactic acid	pH	% lactic acid	pH
FC/54 J/1	0.52	4.95	0.305	5.80
FD/53 J/1	0.46	5.25	0.305	5.85
FD/53 J/5	0.46	5.25	0.310	5.90
91	0.59	4.90	0.340	5.65
FD/54 A	0.44	5.30	0.345	5.70

TABLE 28

A comparison of the bacterial count and 'activity' of cultures 18, 42 and 66 hours-old in sterile separated milk on inoculation and after 6 hours incubation at 30°C

PC/54 J/1: single-strain starter of *Str. cremoris*: series 1.

Stage of test	Age of culture (h) 18 % lactic acid	18 pH	18 Bacterial count (per ml)	42 % lactic acid	42 pH	42 Bacterial count (per ml)	66 % lactic acid	66 pH	66 Bacterial count (per ml)
On inoculation	0.225	6.4	$3.5_6 \times 10^6$	0.225	6.4	$2.5_6 \times 10^6$	0.225	6.4	$2.5_6 \times 10^6$
After 6 hours incubation	0.530	5.1	$290_6 \times 10^6$	0.540	5.1	$330_6 \times 10^6$	0.490	5.2	$260_6 \times 10^6$

TABLE 29

A comparison of the bacterial count and 'activity' of cultures 18, 42 and 66 hours-old in sterile separated milk on inoculation and after 6 hours incubation at 30°C

FC/54 J/1: single-strain starter of Str. cremoris: series 2.

Stage of test	Age of culture (h) 18 % lactic acid	pH	Bacterial count (per ml)	42 % lactic acid	pH	Bacterial count (per ml)	66 % lactic acid	pH	Bacterial count (per ml)
On inoculation	0.18	6.4	5×10^6	0.18	6.4	4×10^6	0.18	6.4	4×10^6
After 6 hours incubation	0.44	5.2	316×10^6	0.47	5.1	366×10^6	0.41	5.3	250×10^6

was less active than the fresh culture but in the remaining three tests this reaction was reversed. A considerable variation in reaction to refrigeration is shown by the other test starters - tables 23 - 25.

The effect of prolonging the time of refrigeration from twenty-four hours to forty-eight and ninety-six hours is shown in tables 26 and 27. The acid-producing ability of a culture refrigerated at 4°C for forty-eight hours after the end of the propagation period was lower than that of the culture when 18 hours old. A refrigeration period of ninety-six hours after propagation reduced the activity still further. The viable bacterial count of samples of milk withdrawn from the test flasks on inoculation at the end of the incubation period of the test was established by the pour-plate method (200). Tables 28 and 29 give the results of these tests.

There was no marked difference between the viable bacterial count of the cultures when freshly propagated or when inoculated after refrigeration. The bacterial counts at the end of the test period were also similar.

DISCUSSION

Storage of mature milk starters at 4°C for a period of twenty-four hours did not appear to have any excessive damaging effect on the activity of the cultures. One culture, FD/53J/5, appeared to be

affected more severely than the others by this treatment. On four of the six tests with this starter, activity of the refrigerated culture was appreciably less than that of the fresh eighteen-hour old culture.

The results obtained on testing the remaining starters were erratic. In some cases the activity of the refrigerated culture was lower and in other cases higher than the eighteen-hour old culture. An extension of the refrigeration time to forty-eight hours gave a lessening of the activity while further refrigeration extending to ninety-six hours resulted in very much reduced acid production with all of the test starters.

Where the cultures were maintained at low temperature, the time of holding became significant after forty-eight hours. In addition to the tests described it has been the practice over the past eight years to hold a large number of starter cultures of various types at 4°C for forty-eight hours after every five days of normal daily transfer. The transfer subsequent to the storage period has always resulted in coagulation of milk within the normal propagation time and there has been no indication that the activity of the cultures has been impaired by this treatment.

111. PREPARATION AND ACID-PRODUCING ABILITY OF LOGARITHMIC GROWTH PHASE CULTURES

Bacterial growth and cellular activity take place in a well established pattern when bacteria in the stationary phase of growth are introduced into a suitable growth medium. Following an interval of time - the lag phase - during which there is little multiplication in cell numbers but an increase in cell size and cellular activity (200) there occurs a period of vigorous multiplication and cellular activity. The onset and duration of the next phase of growth - the so-called logarithmic phase - depends on various factors. If the temperature of the growth medium is below the optimum for the particular organism then the logarithmic phase of growth is less distinct. In addition to temperature, concentration of nutrients, the pH of the medium and the formation of substances toxic to the bacteria all have an influence on the duration of the logarithmic phase of growth. On completion of the logarithmic phase of growth there is a period of very much reduced activity - the stationary phase - the rate of production of new cells is only as fast as the death of the old cells.

The work described in sub-sections 1 and 2 above has shown that an incubation temperature in the range 20 - 22°C is most suitable for preparing active acid-producing cultures when an incubation period of 18 - 24 hours is used. Cultures propagated in this manner retain their activity through a storage period at low temperature of at least twenty-four hours. Since it has been established (112) that cellular activity and cell production follow the same pattern a series of experiments were undertaken to establish the period of logarithmic growth of starter cultures by means of determinations of the acid production of the cultures in milk.

A. PREPARATION OF LOGARITHMIC PHASE CULTURES

EXPERIMENTAL

Materials and methods

Milk. Separated milk sterilised by steaming at 100°C for 30 minutes followed by 10 lb/ sq. in. pressure for 10 minutes was used in the experments described below.

Test cultures. The following fourteen starters were used;

Str. cremoris; strains A 12, FC/55 J/2, HDV 30 and HDV 23.

Str. lactis; strains A 11 and FC/54 J/5.

Multi-strain starters; 112 and A 11/12/14.

Mixed-strain starters; 91, HDV 5, HDV 4, HDV 18, 88 and 85/6.

Method. 1 litre of sterile separated milk was inoculated with 1 per cent of an eighteen-hour-old separated milk culture of the test starter prepared at 22°C in the conventional manner. The flask of inoculated milk was then incubated at the required temperature for the duration of the test period. Samples were withdrawn at intervals during the incubation period by sterile pipette and subjected to hydrogen ion concentration and titratable acidity determinations.

Logarithmic phase of growth at sub-optimum temperature. The procedure described above was carried out at 22°C.

Logarithmic phase of growth at optimum temperature. It has been shown (8,16,57,40) that the optimum temperature for growth and cellular activity of most starters is in the range 29 - 33°C. Furthermore the work described earlier in this section has shown that by incubating a starter at 30°C for eighteen hours its acid-producing properties are markedly impaired. In order to establish the onset and duration of the phase of logarithmic growth at the optimum temperature of the starter bacteria a series of tests was made at 30°C by the method described above.

RESULTS

Logarithmic phase of growth at sub-optimum temperature. The rate of acid production by a single-strain starter culture in milk at 22°C is shown in table 30. The most active period of acid development was found to be between the 8th and 11th hours of incubation. Hourly change in titratable acidity increased from 0.05 per cent in the ninth to 0.13 per cent in the eleventh hour of incubation.

Logarithmic phase of growth at optimum growth temperature. Acid production by a starter culture at optimum growth temperature is shown in figure 1. It will be seen that this takes place in a logarithmic manner between the 3rd and 7th hours of incubation. The acid production curves for four of fourteen test cultures representative of the various types and combinations of starters used in cheese manufacture are shown in figures 2 - 5. All follow essentially the same pattern, a slow increase in titratable acidity in the medium during the first three hours of incubation, a rapid increase in acidity for the next three hours followed by a marked reduction in rate of change of titratable acidity. With test cultures All and FC/55J/2, single-strain cultures of Str. lactis and Str. cremoris, the maximum titratable acidity was reached after 8 hours

TABLE 30

Rate of acid production by a 1 per cent inoculum of a starter in sterile separated milk at 22°C

Incubation period (h)	Titratable acidity (% lactic acid)	pH
0	0.15	6.6
2	0.15	6.6
4	0.16	6.5
6	0.21	6.3
8	0.26	5.9
9	0.31	5.6
10	0.42	5.3
11	0.55	4.9
12	0.65	4.7
13	0.67	4.6
14	0.69	4.5
25	0.80	4.3

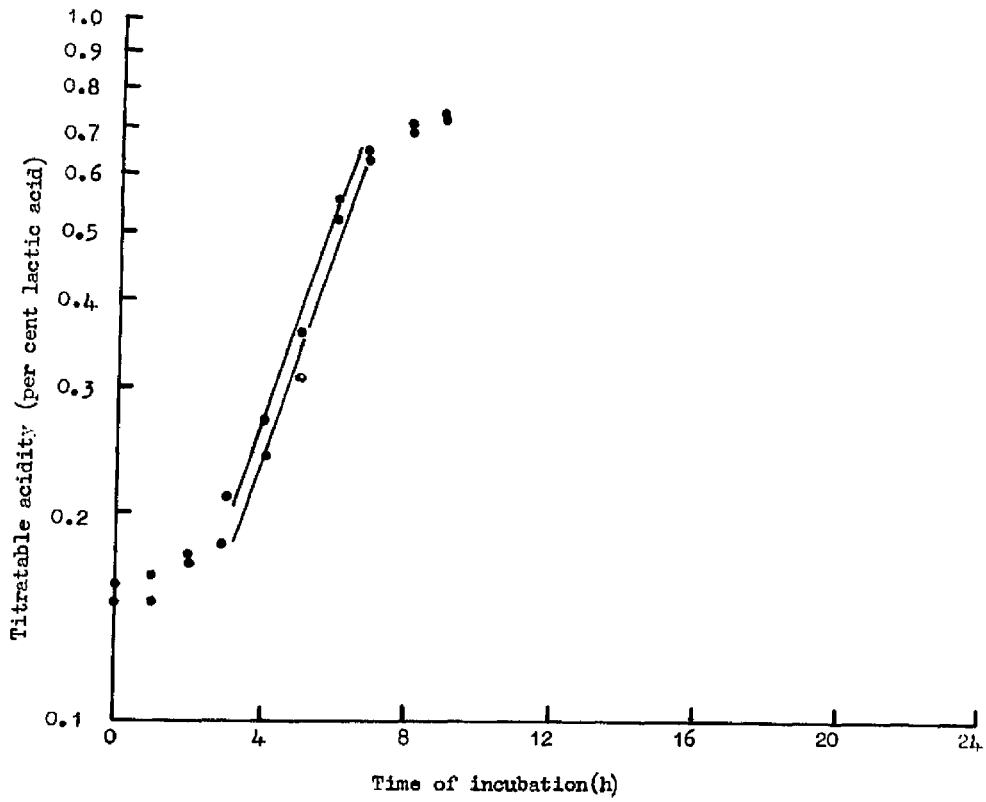


FIG. 1

Acid production by a starter at 30°C. Duplicate values. The vertical scale is logarithmic

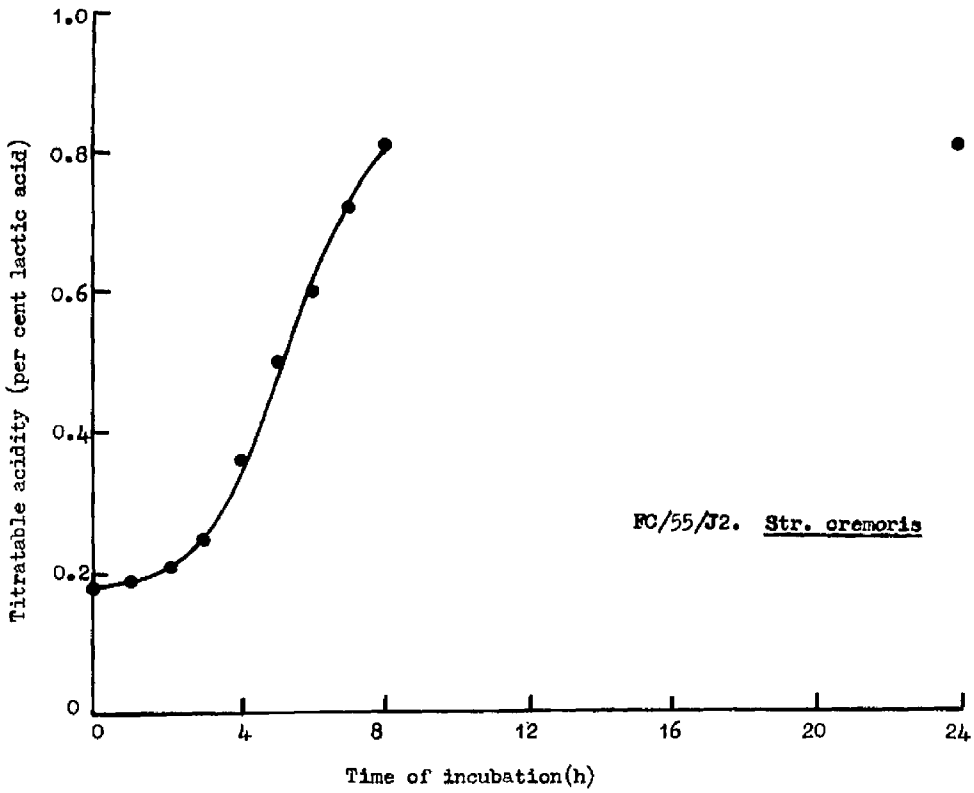


FIG. 2

Acid production by a starter at 30°C

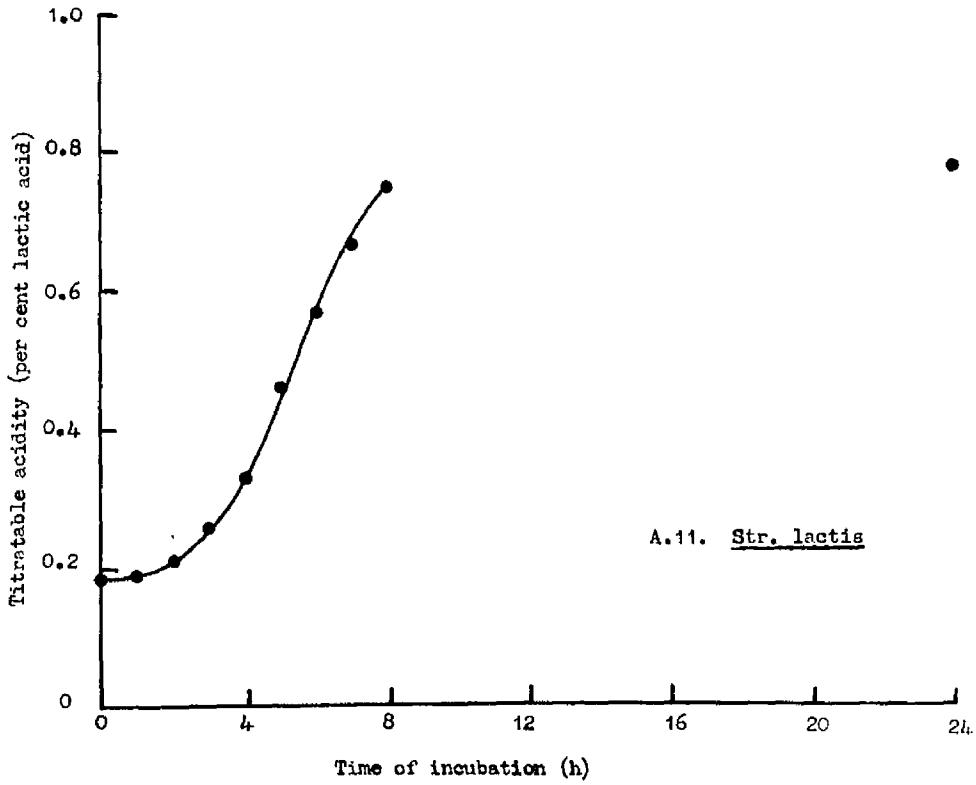


FIG. 3

Acid production by a starter at 30°C

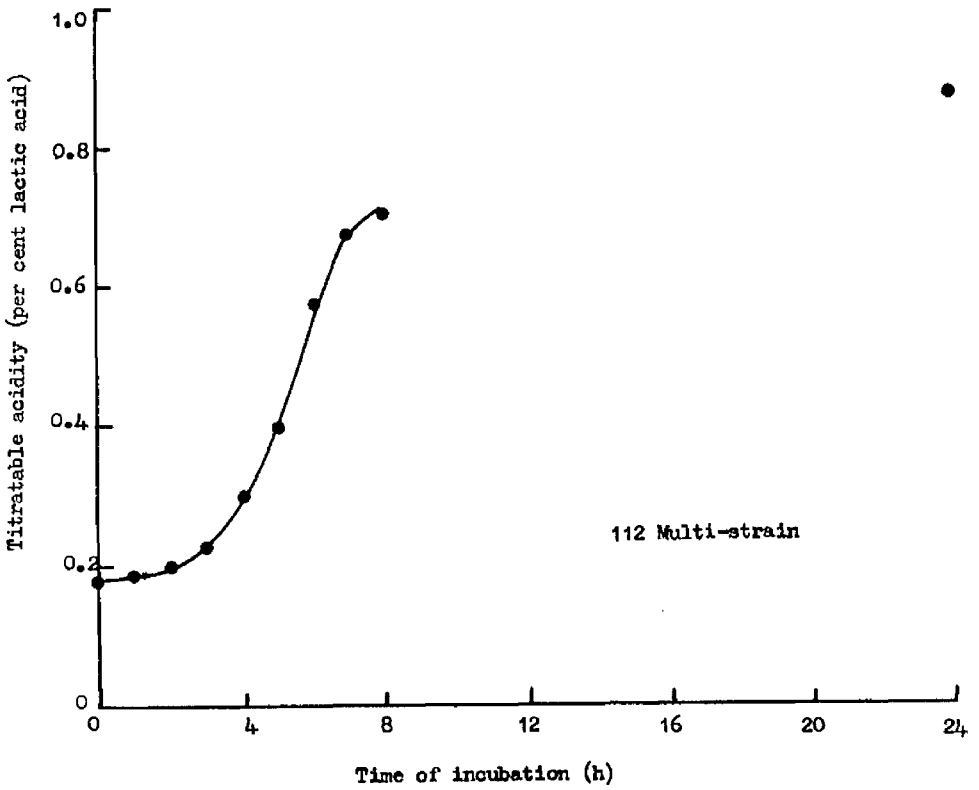


FIG. 4

Aoid production by a starter at 30°C

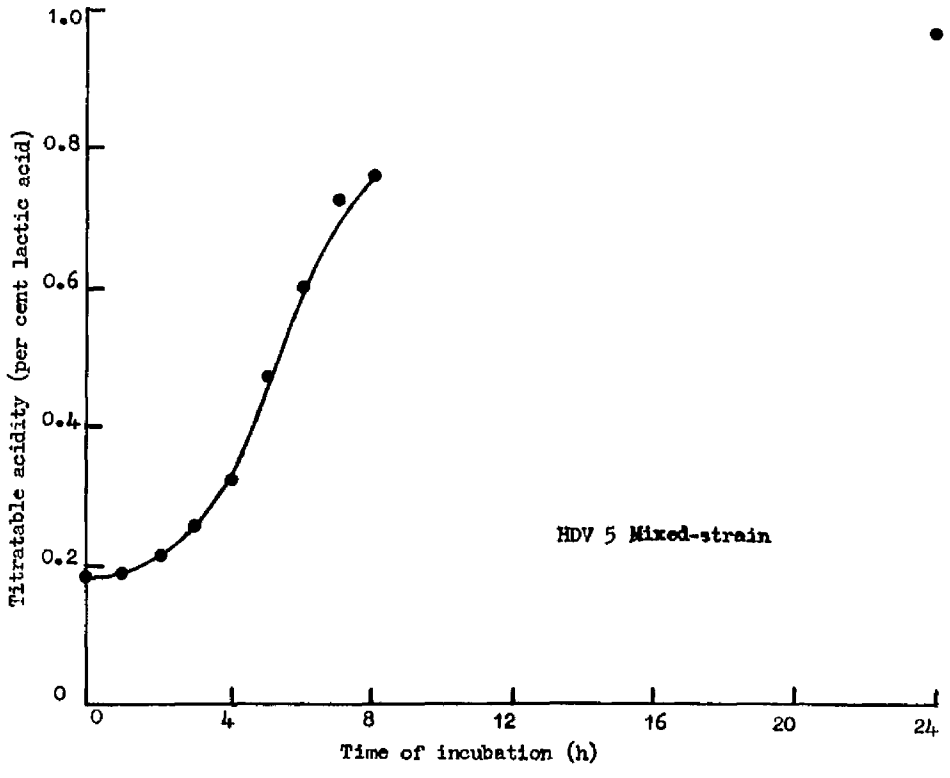


FIG. 5

Acid production by a starter at 30°C

incubation at 30°C. Final acidities were reached with the other cultures within the twenty-four hour incubation period. The results presented are similar to those obtained with other test cultures. Figure 6 shows a comparison of the acid production of a single-strain culture of Str. lactis at sub-optimum and optimum temperatures. There is a period of logarithmic cellular activity at each temperature.

That the rate of acid production is similar on different occasions and in different batches of milk is shown in tables 31 and 32. Results obtained in four tests made on different days with the same culture are presented. The maximum hourly increase in titratable acidity took place during the 6th hour of incubation. Rate of change in titratable acidity increased from the 3rd hour of incubation to the 6th hour and then began to decrease.

Determinations of hydrogen ion concentration established a similar pattern of activity.

Having established that the logarithmic phase of acid production took place between the 3rd and 7th hours of incubation when a 1 per cent inoculation was used at a temperature of 30°C for the majority of the cultures tested, it was decided to determine the rate of acid production of cultures retransferred into

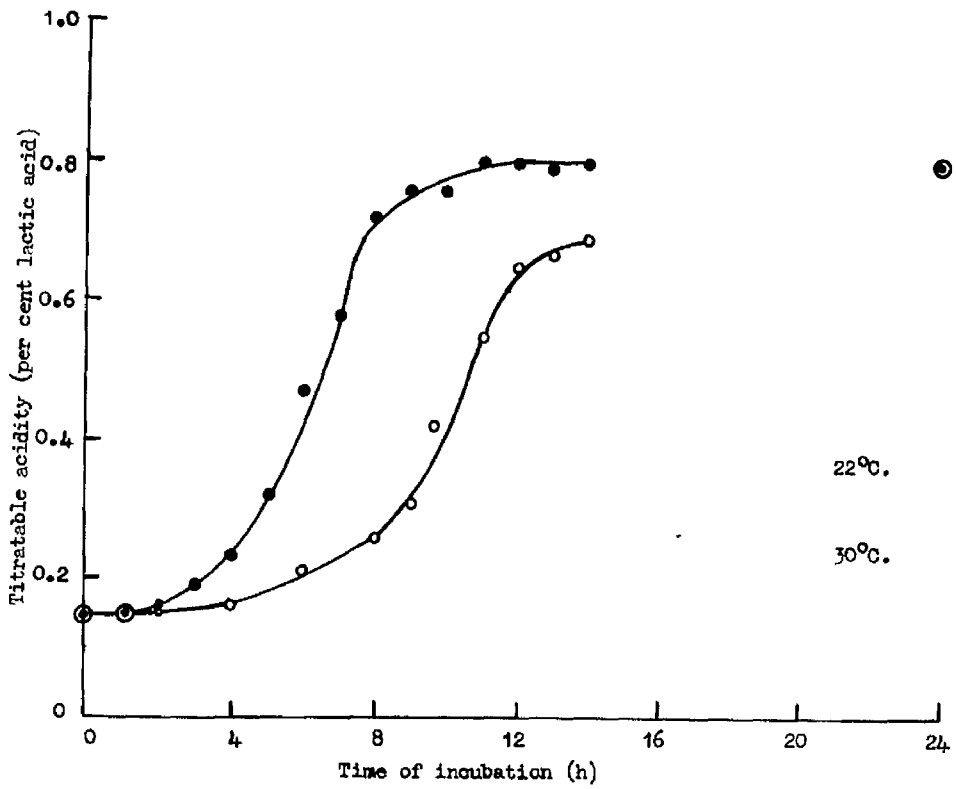


FIG. 6

A comparison of acid production by a starter culture in milk at sub-optimum and optimum growth temperatures

TABLE 31

Hourly increase in titratable acidity (per cent lactic acid) of milk incubated at 30°C after inoculation with 1 per cent of a starter

Hour of incubation	Test 1	2	3	4
1st	0.005	0	0	0
2nd	0.005	0.020	0.015	0.010
3rd	0.010	0.040	0.035	0.030
4th	0.060	0.060	0.050	0.040
5th	0.070	0.090	0.100	0.090
6th	0.210	0.200	0.160	0.150
7th	0.120	0.100	0.160	0.110
8th	0.070	0.060	0.040	0.140
9th	-	0.030	0.020	0.040

TABLE 32

Hourly increase in titratable acidity (per cent lactic acid) and change in hydrogen ion concentration of milk incubated at 30°C after inoculation with various amounts of starter FC/54 J/5

Hour of incubation	Amount of inoculum (%)					
	1		2		4	
	% lactic acid	pH	% lactic acid	pH	% lactic acid	pH
1st	0.0	0.0	0.01	0.1	0.02	0.1
2nd	0.01	0.2	0.02	0.2	0.04	0.3
3rd	0.03	0.1	0.05	0.2	0.06	0.3
4th	0.04	0.3	0.06	0.4	0.11	0.5
5th	0.09	0.4	0.16	0.6	0.22	0.4
6th	0.15	0.4	0.16	0.3	0.09	0.1
7th	0.11	0.5	0.02	0.2	0.04	0.2
8th	0.14	0.1	0.07	0.05	0.08	0.1
9th	0.04	0.1	0.03	0.1	0.0	0.1
10th	0.0	0.05	0.04	0.1	0.02	0.0
11th	0.04	0.05	0.03	0.1	0.03	0.1
12th	0.0	0.0	0.01	0.0	0.0	0.0
13th	0.0	0.05	0.0	0.0	0.0	0.0
14th	0.01	0.0	0.0	0.0	0.0	0.0

milk while in their logarithmic phase of growth.

B ACID-PRODUCING ABILITY OF LOGARITHMIC PHASE CULTURES

EXPERIMENTAL

(a) Milk culture tests

In all cases the sample taken during the logarithmic phase of acid production was subjected to an activity test of the type previously used in this work. Reference was always made to a control twenty-four hour old stationary phase culture prepared in the normal manner at 22°C.

Method

A litre portion of sterile separated milk was inoculated with 1 per cent of the test strain and incubated at 30°C. At intervals during the incubation period samples were withdrawn and titrated against N/9 sodium hydroxide (NaOH) using a 0.5 per cent solution of phenolphthalein as indicator and in some cases subjected to pH determination. Near the end of the logarithmic phase of activity a portion was withdrawn and used to inoculate a 100 ml quantity of sterile separated milk which was then incubated at 30°C for 6 hours prior to the titratable acidity and hydrogen ion concentrations being determined. A point near the end of the logarithmic phase of activity was considered to be preferable to one early in the

logarithmic period since it was thought unlikely that the harmful effect of extended incubation at 30°C discussed earlier would have exerted any measureable influence on the vigour of the cells, the numbers of which would then be at a high level.

RESULTS

In the first group of tests a 1 per cent inoculum was used and the logarithmic culture was tested for activity when 6 hours old. Results obtained with five test cultures are shown in table 33. The activity of the logarithmic phase culture of test strain HDV 4 was lower than that of a stationary phase culture prepared at 22°C in the conventional manner. Both methods of determination- titratable acidity and hydrogen ion concentration - gave results in agreement. Results obtained when logarithmic phase cultures were prepared in different batches of milk on different days using a test starter are shown in table 34. Young cultures of this test organism were in every case more active than stationary phase cultures.

In a second series of tests the rate of inoculation was increased and the activity of young cultures of the same and different age were tested by the method described above. The activity of a four-hour-old culture produced at 30°C by the use of a 4 per cent

TABLE 33

The amount of acid produced in milk during a 6 hour 'activity' test (5) at 30°C by 1 per cent inocula of stationary and logarithmic phase cultures of various starters

Starter	Phase of growth	Titratable acidity (% lactic acid)	pH	Age of culture (h)	'Activity' Titratable acidity (% lactic acid)	pH
HDV 4	Stationary	0.93	4.5	18	0.49	4.8
	Logarithmic	0.40	5.2	6	0.36	5.3
FC/54 J/5	Stationary	0.81	4.3	18	0.53	-
	Logarithmic	0.59	4.7	6	0.59	-
HDV 30	Stationary	0.91	4.4	18	0.49	4.8
	Logarithmic	0.51	4.8	6	0.495	4.8
112	Stationary	0.85	4.3	18	0.45	4.9
	Logarithmic	0.58	4.8	6	0.48	4.7
91	Stationary	0.91	4.5	18	0.395	5.1
	Logarithmic	0.52	4.9	6	0.42	4.9

TABLE 34

The amount of acid produced in milk during a 6 hour activity test (5) at 30°C by 1 per inocula of stationary and logarithmic phase cultures of a starter when tested on different days

FC/54: single-strain starter of Str. cremoris

Test	Phase of growth	Titratable acidity (% lactic acid)	pH	Age of culture (h)	'Activity' Titratable acidity (% lactic acid)	pH
1	Stationary	0.81	4.3	18	0.53	-
	Logarithmic	0.59	4.7	6	0.59	-
2	Stationary	0.80	4.3	18	0.52	4.8
	Logarithmic	0.57	4.6	6	0.55	4.6
3	Stationary	0.82	4.3	18	0.49	5.0
	Logarithmic	0.45	4.9	6	0.54	4.9
4	Stationary	0.84	4.3	18	0.47	5.1
	Logarithmic	0.47	5.2	6	0.54	4.9

inoculum was equal to that of a stationary phase preparation - table 35. Acid production by a five-hour-old culture produced using a 2 per cent inoculum was equal to that of the control while a culture of the same age produced by a 4 per cent inoculation had a very much higher activity than the stationary phase culture. The activity of logarithmic phase cultures D, E and F, six-hour-old cultures produced at 30°C by using 1, 2 and 4 per cent inoculations was higher than the control culture by 0.06 - 0.07 per cent lactic acid and 0.2 pH unit - table 35. The titratable acidity of these young cultures ranged from 0.47 to 0.66 per cent lactic acid. A comparison between the activity of six-hour-old logarithmic phase cultures produced by using 1, 2 and 4 per cent inoculations is shown in table 36. The vigour of the logarithmic phase cultures was in every case higher than that of the stationary phase cultures and were similar to each other. Titratable acidity determinations of the cultures showed a considerable difference between the cultures produced by the various methods at the end of the six hour propagation period. A similar comparison was made with other test cultures representative of the combinations of starter bacteria, the results are shown in tables 37 and 38. An examination of tables 36, 37 and 38 indicates that

TABLE 35

A comparison of the amount of acid produced in milk by a 1 per cent inocula of stationary and logarithmic phase cultures of a starter at 30°C in 6 hours

FC/54 J 5: single-strain starter of Str. cremoris

Phase of growth	Propagation conditions					Acid production	
	Age (h)	Temp. (°C)	Inoculum (%)	Titratable acidity (% lactic acid)	pH	Titratable acidity (% lactic acid)	pH
Stationary	18	22	1	0.80	4.3	0.47	5.1
Logarithmic							
A	4	30	4	0.39	5.3	0.47	5.1
B	5	30	2	0.45	5.1	0.45	5.1
C	5	30	4	0.57	4.9	0.54	4.9
D	6	30	1	0.47	5.2	0.54	4.9
E	6	30	2	0.61	4.8	0.53	4.9
F	6	30	4	0.66	4.8	0.54	4.9

TABLE 36

A comparison of the amount of acid produced in milk by a 1 per cent inocula of stationary and logarithmic phase cultures of a starter at 30°C in 6 hours

FC/54 J/5; single-strain starter of Str. cremoris

Phase of growth	Propagation conditions					Acid production	
	Age (h)	Temp. (°C)	Inoculum (%)	Titratable acidity (% lactic acid)	pH	Titratable acidity (% lactic acid)	pH
Stationary	18	22	1	0.82	4.3	0.49	5.0
Logarithmic							
A	6	30	1	0.45	4.9	0.54	4.9
B	6	30	2	0.60	4.6	0.60	4.8
C	6	30	4	0.67	4.5	0.56	4.8

TABLE 37

A comparison of the amount of acid produced in milk by a 1 per cent inoculum of stationary and logarithmic phase cultures of a starter at 30°C in 6 hours

Starter 112: multi-strain

Phase of growth	Propagation conditions					Acid production	
	Age (h)	Temp. (°C)	Inoculum (%)	Titratable acidity (% lactic acid)	pH	Titratable acidity (% lactic acid)	pH
Stationary	18	22	1	0.85	4.3	0.45	4.9
Logarithmic							
A	6	30	1	0.58	4.8	0.48	4.7
B	6	30	2	0.69	4.6	0.50	4.7
C	6	30	4	0.77	4.5	0.48	4.7

TABLE 38

A comparison of the amount of acid produced in milk by a 1 per cent inoculum of stationary and logarithmic phase cultures of a starter at 30°C in 6 hours

Starter No. 91: mixed-strain

Phase of growth	Propagation conditions					Acid production	
	Age (h)	Temp. (°C)	Inoculum (%)	Titratable acidity (% lactic acid)	pH	Titratable acidity (% lactic acid)	pH
Stationary	18	22	2	0.91	4.5	0.395	5.1
Logarithmic							
A	6	30	1	0.52	4.9	0.42	4.9
B	6	30	2	0.68	4.6	0.46	4.8
C	6	30	4	0.78	4.4	0.44	4.9

of the three rates of inoculation used, that of 2 per cent gave more active cultures than either 1 or 4 per cent inoculations. The activity of logarithmic phase cultures produced by using higher rates of inoculation is shown in table 39. An incubation period of 3 hours used in conjunction with a 5 per cent inoculum was insufficient to produce a logarithmic culture with acid-producing abilities equal to a stationary culture produced by 18 hours incubation at 22°C. By continuing the incubation for a further hour a culture possessing an activity greater than the control was produced. It is of interest to note that the hydrogen ion concentration of the milk at the end of the test period when cultures B and D were examined were different although the titratable acidity was the same.

A comparison was made of the bacterial numbers present in logarithmic and stationary phase cultures. The individual cell count of the cultures as determined by the direct microscopic count method is shown in table 40. The count is lower for the logarithmic phase cultures for each culture in duplicate tests carried out on different occasions in different batches of milk. It must therefore be assumed that since the number of cells added to the milk inoculated with logarithmic phase cultures was lower than that added when stationary phase cultures were used the vigour

TABLE 39

A comparison of the acid production by a 1 per cent inoculum of stationary and logarithmic phase cultures of a starter at 30°C in 6 hours

FC/54 J/5: single-strain starter of Str. lactis

Phase of growth	Propagation conditions					Acid production	
	Age (h)	Temp. (°C)	Inoculum (%)	Titratable acidity (% lactic acid)	pH	Titratable acidity (% lactic acid)	pH
Stationary	18	22	1	0.82	4.3	0.48	4.8
Logarithmic							
A	3	30	5	0.56	4.8	0.435	5.1
B	3	30	10	0.53	4.8	0.495	4.85
C	4	30	5	0.69	4.5	0.505	4.7
D	4	30	10	0.66	4.5	0.495	4.7

TABLE 40

Bacterial count of milk inoculated with 1 per cent of starter culture and incubated at 22 and 30°C

Culture	Incubation temperature (°C)	Incubation time (h)	Bacterial count (per ml) A	Bacterial count (per ml) B
A 12	22	18	460×10^6	630×10^6
	30	6	210×10^6	220×10^6
A 11	22	18	900×10^6	580×10^6
	30	6	670×10^6	460×10^6

of the cells of the young cultures must have been greater than those of the old cultures within the test period. The viable bacterial count (200) of six-hour old logarithmic phase cultures prepared by using various amounts of inocula is shown in table 41. The bacterial count of the cultures produced by the use of 1 and 2 per cent inocula of culture is similar. In two cases the count of the cultures prepared by using a 4 per cent inoculum is slightly lower than the others but no relationship was established between this and other propagation conditions. The titratable acidity and hydrogen ion concentration of milk incubated at 30°C after inoculation with 10 per cent of culture is shown for a number of test starters in table 42. A considerable variation exists between the amount of acid produced by the test cultures viz. from 0.34 per cent lactic acid and pH 5.7 of culture D48/5 and 0.37 per cent lactic acid and pH 5.3 in the case of culture 924 to 0.50 per cent lactic acid and pH 5.0 with test strain HDV23.

Having shown that the acid-producing ability of cultures used while in the logarithmic phase of growth was at least equal to that of stationary phase cultures in tests in milk it was considered desirable to test the activity of young cultures further in cheese manufacture.

TABLE 41

Bacterial numbers of logarithmic phase cultures produced at 30°C in a milk medium

Cultures	Propagation conditions					
	Age (h)	Temp. (°C)	Inoculum (%)	Titratable acidity (% lactic acid)	pH	Bacterial count (per ml)
A	6	30	1	0.50	4.9	750×10^6
B	6	30	2	0.57	4.7	720×10^6
C	6	30	4	0.66	4.5	500×10^6
A	6	30	1	0.57	4.6	450×10^6
B	6	30	2	0.65	4.5	380×10^6
C	6	30	4	0.72	4.4	320×10^6
A	6	30	1	0.52	4.9	430×10^6
B	6	30	2	0.64	4.6	500×10^6
C	6	30	4	0.68	4.4	450×10^6

TABLE 42

Titratable acidity (per cent lactic acid) and hydrogen ion concentration of milk maintained at 30°C for 3 hours after inoculation with 10 per cent of various starters

Starter	Titratable acidity (% lactic acid)	pH
HDV 18	0.48	5.0
HDV 5	0.47	5.1
HDV 4	0.48	5.0
HDV 23/5	0.43	5.2
85/6/4	0.43	5.2
H/55	0.44	5.2
HDV 5/L/C	0.49	5.0
HDV 30/1	0.48	5.0
HDV 30/2	0.46	5.0
HDV 23	0.50	5.0
123	0.46	5.0
85	0.48	5.1
121	0.48	5.1
112	0.49	5.1
90	0.45	5.3
122	0.48	5.1
176	0.33	5.6
D 48/5	0.34	5.7
924	0.37	5.3
HP	0.42	5.2

EXPERIMENTAL

(b) Cheesemaking experiments

Test cultures. Mixed-strain starters 91, 125, 126 and 129 were used in addition to multi-strain cultures 121 and 123.

Series A. A comparison was made between a stationary phase culture prepared in the conventional manner by incubation at 22°C for 18 hours and a logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3¼ hours at 30°C. The cheesemaking process was based on the method practised in Scotland and detailed in table 1. In this method 1 - 1½ per cent of starter is added to the milk and a ripening time of 1 - 2 hours is allowed before the addition of rennet. Full details of the experimental process are given in table 43. After the cheese had been pressed, a curing period of three months at 12 - 15°C was allowed before examination.

Series B. In this group of experiments the aim was to produce high quality Cheddar cheese by means of logarithmic phase cultures - tables 44 - 51.

RESULTS

Cheesemaking experiments confirmed the results obtained in milk culture tests with logarithmic phase

TABLE 43

A comparison of stationary and logarithmic phase starters in Cheddar cheese manufacture

Series A.1

Cheese process record

Milk: Whole (raw) 20 gallons
Starter: No. 122 Vat 1 - 18 hour-old stationary phase culture Titratable acidity 0.72, pH 4.4
Vat 2 - 3½ hour-old logarithmic phase culture Titratable acidity 0.52, pH 4.8
Amount of inoculum: 1½ per cent Amount of rennet: 1 oz Weight of curd: Vat 1, 23¼ lb, Vat 2, 23½ lb

Operation		Time (h-min)	Temp. (°F) x	Titratable acidity (% lactic acid)		pH
Starter added	Vat 1.	0.00	85		0.185	6.5
	Vat 2.	0.00	86		0.185	6.5
Rennet added	Vat 1.	1.45	87		0.205	6.3
	Vat 2.	2.00	87		0.205	6.3
Curd cut	Vat 1.	2.30	86		0.14	6.3
	Vat 2.	2.40	86		0.135	6.2
Maximum scald	Vat 1.	3.35	102		0.17	5.9
	Vat 2.	3.40	104		0.19	5.8
Curd settled	Vat 1.	4.05			0.20	5.7
	Vat 2.	4.10			0.22	5.7
Whey run	Vat 1.	4.35		from whey	0.25	5.5
				from curd	0.29	
	Vat 2.	4.15		from whey	0.23	5.7
				from curd	0.29	
Curd milled	Vat 1.	6.35			0.98	4.5
	Vat 2.	6.15			0.89	4.6
Curd salted	Vat 1.	6.45				
	Vat 2.	6.25				
Curd hooped	Vat 1.	6.55				
	Vat 2.	6.35				
Whey from press	Vat 1.	7.15				4.7
	Vat 2.	6.55				4.8

x The Fahrenheit scale of temperature is normally used in cheesemaking - several Celsius equivalents are shown in table 1(a)

TABLE 44

Process record of cheese manufacture using a logarithmic growth phase culture

Series B.1

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Mixed-strain No. 129, Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.55, pH 4.6
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 23 lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	86	0.17	6.4
Rennet added	1.30	86	0.185	6.3
Curd cut	2.10	86	0.125	6.2
Maximum scald	3.10	103	0.15	6.1
Curd settled	3.45		0.16	6.0
Whey run	4.40		0.27	5.8
Curd milled	6.25		0.68	
Curd salted	6.30			5.05
Curd hooped	6.45			

TABLE 45

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 2

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Multi-strain No. 121. Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.51, pH 4.6
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 22½ lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	83	0.17	6.4
Rennet added	1.35	86	0.185	6.3
Curd cut	2.18	85	0.125	6.2
Maximum scald	3.20	103	0.15	6.0
Curd settled	3.50		0.18	5.8
Whey run	4.25		0.27	5.7
Curd milled	6.05		0.71	
Curd salted	6.10			5.05
Curd hooped	6.25			

TABLE 46

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 3

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Mixed-strain No. 91. Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3¼ hours at 30°C. Titratable acidity (% lactic acid) 0.59, pH 4.8
Amount of inoculum: 1¼%
Amount of rennet: 1 oz
Weight of curd: 22½ lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	85	0.17	6.5
Rennet added	1.42	86	0.18	6.3
Curd cut	2.24	85	0.125	6.2
Maximum scald	3.29	102	0.15	6.1
Curd settled	3.59		0.16	
Whey run	6.29		0.30	
Curd milled	8.44		0.73	
Curd salted	8.50			5.1
Curd hooped	9.00			

TABLE 47

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 4

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Mixed-strain No. 125. Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.55, pH 4.8
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 22½ lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	87	0.17	6.5
Rennet added	1.42	86	0.185	6.3
Curd cut	2.25	85	0.125	6.2
Maximum scald	3.30	102	0.15	6.1
Curd settled	4.00		0.16	
Whey run	5.50		0.31	
Curd milled	7.55		0.71	
Curd salted	8.00			4.9
Curd hooped	8.10			

TABLE 48

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 5

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Mixed-strain No. 126. Logarithmic phase culture prepared by inoculating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.58, pH 4.7
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 23 lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	83	0.18	6.4
Rennet added	1.30	83	0.195	6.3
Curd cut	2.05	86	0.125	6.3
Maximum scald	3.05	102	0.15	6.2
Curd settled	3.35		0.16	6.1
Whey run	5.05		0.30	5.7
Curd milled	6.55			
Curd salted	7.05			
Curd hooped	7.15			5.1

TABLE 49

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 6

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Multi-strain No. 123. Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.55, pH 4.9
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 22½ lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	83	0.18	6.4
Rennet added	1.30	86	0.195	6.3
Curd cut	2.05	86	0.125	6.3
Maximum scald	3.05	102	0.155	6.2
Curd settled	3.35		0.165	6.1
Whey run	4.55		0.29	5.7
Curd milled	6.55		0.69	
Curd salted	7.05			5.15
Curd hooped	7.15			

TABLE 50

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 7

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Mixed-strain No. 91. Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.61, pH 4.9
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 23 lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	84	0.17	6.5
Rennet added	1.30	86	0.18	6.3
Curd cut	2.05	86	0.13	6.3
Maximum scald	3.05	102	0.15	6.15
Curd settled	3.35		0.16	6.1
Whey run	4.45		0.26	5.9
Curd milled	6.30		0.74	
Curd salted	6.40			5.3
Curd hooped	6.55			

TABLE 51

Process record of cheese manufacture using a logarithmic growth phase culture

Series B. 8

Milk: Whole (raw) 20 gallons
Type of cheese: Cheddar
Starter: Multi-strain No. 125. Logarithmic phase culture prepared by incubating sterile separated milk inoculated with 10 per cent of culture for 3½ hours at 30°C. Titratable acidity (% lactic acid) 0.59, pH 4.6
Amount of inoculum: 1½%
Amount of rennet: 1 oz
Weight of curd: 22½ lb

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid)	pH
Starter added	0.00	83	0.17	6.5
Rennet added	1.30	86	0.185	6.3
Curd cut	2.05	86	0.13	6.3
Maximum scald	3.05	102.5	0.15	6.1
Curd settled	3.35		0.16	6.05
Whey run	4.55		0.27	5.9
Curd milled	6.55		0.79	
Curd salted	7.05			
Curd hooped	7.15			5.2

cultures. The comparison of the activity of eighteen and three and a quarter hour-old cultures of starter 122 presented in table 43 shows that there was little difference in the rate of acid production of the two types of starter culture. The rate of acid development was very rapid with both cultures and a high titratable acidity was obtained at the hooping stage. While it is true that the titratable acidity at this stage was higher than that normally found in commercial cheesemaking the experiments proved that there was no danger of the acid production failing to reach the required level in the second transfer of the culture at high temperatures. Cheese made in series B were examined after eight weeks curing at 12 - 15°C when they were sold. The official grader for the purchasing body inspected the cheese and judged numbers 2, 3, 4, 5 and 6 in 1st. grade. No. 7 was faulted because of weak body. In all cases however the texture was close and the flavour was normal. It will be seen from tables 46 and 47 that the time of manufacture was longer than is desirable. Both cheese were made on the same day and tests carried out on the milk used in each vat indicated the presence of inhibitory materials in the milk which prevented normal acid development. Repeat experiments carried out with the same starters - tables 50 and 51 proved that the logarithmic phase cultures were normal in acid

production. A 1 per cent inoculum was used in the cheesemaking experiments. A faster rate of acid development could be obtained if required by using more starter - a practice not uncommon with conventional starters.

DISCUSSION

Starter cultures are used in cheese manufacture when in their stationary phase of growth after fourteen to eighteen hours incubation at 20 - 22°C. The work described above shows that it is possible to produce in a much shorter time starters equal in activity to those prepared in the conventional manner. Rice (134) quotes Toens and Baker (175) as having found that cultures possessing increased flavour could be produced by using large inocula at the optimum growth temperature of the starter bacteria.

It was shown in sub-section 1 that when a starter culture was incubated for twenty-four hours at 30°C there was a lowering of the acid-producing ability of the succeeding generation. In sub-section 3 describing the preparation and use of logarithmic phase cultures at 30°C it has been proved that the culture does not suffer damage in acid-producing ability until some time after six hours. Babel (10) has described the successful use of cultures propagated at 30°C for 8 hours and then held for 18 hours at

7.2°C before use. The rate of acid production of six hour logarithmic phase cultures is presented in table 33. With the exception of the first culture the titratable acidity and hydrogen ion concentration produced in milk at 30°C by a 1 per cent inoculum of the logarithmic phase cultures is equal to, and in most cases greater than that produced under the same conditions by control stationary phase cultures. One culture which did not behave in this manner was a slow acid-producing strain. That the production of logarithmic phase cultures with good acid-producing properties was possible on different days was shown by the results presented in table 34. In each case the logarithmic phase culture produced appreciably more lactic acid than the control stationary phase culture. The advantage of using young cells is due to the fact that when introduced into a new growth medium cell multiplication and activity proceed at a rapid rate without an initial lag period. This has been amply demonstrated by estimations of the bacterial numbers present in logarithmic and stationary phase cultures. In all tests the viable bacterial count of the young cultures was lower than that of mature stationary phase cultures prepared at 22°C.

Four-hour-old logarithmic phase cultures

prepared at 30°C by using a 4 per cent inoculum were equal in activity to stationary phase cultures but were less active than a five-hour-old culture prepared at 30°C with the same amount of inoculum, or six-hour-old cultures prepared by means of 1, 2 or 4 per cent inocula. The six-hour-old cultures produced by the various rates of inoculation were equal in activity although the titratable acidity of the parent cultures was different.

Cheesemaking experiments provided confirmation of the activity of logarithmic phase cultures. In these experiments it was shown that the acid-producing ability of a 3½ hour-old culture prepared at the optimum growth temperature of the starter was at least equal to that of a stationary phase culture. Cheese made with logarithmic phase cultures were of good quality.

Logarithmic phase cultures have two possible applications in commercial cheesemaking. Firstly, the method could be used to prepare starter in an emergency to supplement inadequate supplies or to replace the normal supply which had failed to grow due to antibiotic or bacteriophage contamination of the starter milk. Secondly, cheesemakers have long felt the need for a test which would tell them

the acid-producing properties of the bulk starter before its use in cheese manufacture. Since it is well known that the titratable acidity of mature stationary phase cultures bears no relation to the acid-producing activity of the starter, the cheesemaker only becomes aware of the activity of the culture after the cheese manufacture process has progressed to a fairly advanced stage in the first vat. Titratable acidity readings of the logarithmic phase cultures, however, have a value in indicating the activity of the cultures, especially if the low rate of inoculation is used. The titratable acidity at the end of the six-hour propagation time is equivalent to the titratable acidity obtained in laboratory activity tests. If the titratable acidity at this point is low the cheesemaker is able to add a larger amount of starter and conversely, if the starter is exceedingly active he is in position to decrease the amount of culture used.

The use of logarithmic phase cultures has several advantages viz. 1. the cheesemaker is able to produce a large quantity of starter at short notice, 2. the titratable acidity determination made on logarithmic phase cultures is a measure of the activity of the bulk starter - a property quite distinct from the titratable acidity of mature stationary phase cultures.

SUMMARY

Twenty-three single-strain cultures of the chief acid-producing organisms used in cheese starters were propagated at various temperatures. It has been shown conclusively that the optimum propagation temperature is in the range 20 - 22°C. The use of this temperature ensures the production of active acid-producing cultures.

The effect of propagating starters at high temperatures was shown to be largely a characteristic of species of organism. Strains of Str. lactis were much more able to withstand high propagation temperatures than were those of Str. cremoris. The possibility exists of using a test based on the lowering of acid-producing ability on high temperature incubation as a means of differentiating Str. lactis and Str. cremoris.

It is considered that the use of high incubation temperatures is a very important factor in the over-ripening of starters.

It has been shown that cultures propagated in the conventional manner at 22°C did not suffer material loss of acid-producing ability in the subsequent generation from refrigeration at 4°C for periods up to forty-eight hours before transfer.

Logarithmic phase cultures of starter organisms prepared at a propagation temperature of 30°C were

shown to have acid-producing properties at least equal to those of conventional stationary phase cultures as judged by rate of acid production in activity tests and in cheese manufacture. The use of logarithmic phase cultures provides the cheesemaker with a means of augmenting his supply of starter at short notice in addition to providing him with a culture of known activity.

SECTION SEVEN

FACTORS ACCELERATING THE PRODUCTION OF ACID BY STARTER BACTERIA

FACTORS ACCELERATING THE PRODUCTION OF ACID BY STARTER BACTERIA

I STIMULATION BY MICRO-ORGANISMS

Although the standard of bacteriological control exerted over the preparation of starter cultures used in cheesemaking is very much improved of late a large number of starter cultures become contaminated through faulty equipment or bad techniques. Various organisms have been shown to exert considerable influence on the growth of the lactic acid bacteria.

Petts and Lolkema (132) reported that the rate of increase of bacterial numbers and acid production of a strain of Streptococcus thermophilus was increased by a culture of Lactobacillus bulgaricus. The stimulatory effect was associated with the liberation of water-soluble and heat-stable substances not liberated by Str. thermophilus. Marshall (109) has reported that the presence of Bacillus subtilis in milk influenced the lactic acid fermentation. The effect of the presence of B. subtilis was stimulatory and in the earlier stages of fermentation the stimulus was greater with higher concentrations

of the organism. Cox and Whitehead (30) reported that B. subtilis appeared to stimulate the production of acid by lactic acid streptococci. Morgan and Curle (116) however, have noted that some strains of B. subtilis were able to cause slow acid production by starter cultures. Rice (135) obtained marked stimulation by the growth in milk of B. subtilis. The stimulation occurred during the logarithmic phase of growth with diminishing intensity as the maximum acidity was approached. Rice (135) was of the opinion that the growth of B. subtilis induced proteolysis which released a readily available source of nitrogen.

During the routine re-isolation of a starter culture by plating out on yeast-dextrose-agar and picking off colonies into sterile separated milk, one plate became contaminated with an airborne contaminant. The sparse growth on the plate was greatly stimulated near the contaminant colony. This natural occurrence appeared to be of interest and the following procedure was adopted for a further investigation.

EXPERIMENTAL

Isolation and identification of air organism

The colony was picked off into yeast-dextrose-broth which was incubated at 30°C until growth was

advanced in forty-eight hours. Following two further serial isolations the culture was examined for identification purposes. Details of the properties of the culture are shown below.

General characteristics of the organism. Rod shaped cells, occurring singly or in short chains.

Spore formation; the formation of spores was demonstrated by the technique of Schaeffer and Fulton (51). Spores were central to sub-terminal.

Motility; motility was demonstrated in hanging drop preparations of young cultures.

Air supply; the culture failed to grow in yeast-dextrose-broth with a seal of petroleum jelly or in an atmosphere of hydrogen using a McIntosh and Fildes jar (107).

Growth temperature; growth at 30°C was pronounced and was shown by the formation of a pellicle and turbidity which cleared.

Staining; young cells stained uniformly; gram positive.

Size; diameter of the cells was less than 0.9 micron.

Liquefaction of gelatine;	hydrolysis was slow to take place but was extensive after 7 days incubation at either 30 or 37°C.
Hydrolysis of starch;	growth of the culture on nutrient agar to which starch had been added brought about hydrolysis of the starch in close proximity to the surface colonies.
Reduction of nitrates;	nitrites were formed from nitrates.
Growth on potato slopes;	growth was luxuriant, wrinkled and changing from cream to brown on continued incubation.
Catalase;	catalase was produced.
Voges-Proskauer reaction;	acetylmethylcarbinol was produced from glucose-citrate-broth.
Hydrogen ion concentration;	growth took place at a pH of 6.0
Growth in milk;	casein was slowly digested with slight curd formation.

action on sugars; arabinose, galactose, glycerol, laevulose, mannose, mannitol, xylose were fermented. Ammonia was produced from arabinose and xylose. An inconsistency was noted with lactose. Maltose, sorbitol, saccharose, glycogen, trehalose, inulin, adonitol, rammose, raffinose, dulcitol and salicin were unfermented.

The organism was identified as Bacillus subtilis.

Demonstration of stimulatory effect on starter bacteria growing on a solid medium

The effect of growing the contaminant culture in close proximity to a test culture of starter bacteria. A quantity of yeast-dextrose-agar was added to a series of petri plates. On solidification, a loopful of an eighteen-hour-old milk culture of the test starter was stroked over the surface of the agar. A loopful of a yeast-dextrose-broth culture of the contaminant culture was then stroked in lines near those of the starter culture. The inoculated plates were then incubated at 30°C for 48 hours.

Preparation of cell-free material. The organism was inoculated into yeast-dextrose-broth and incubated

at 30°C for forty-eight hours. After this time the broth was passed through a sterile Seitz filter fitted with an EK grade filter pad. The sterile seitz filtrate prepared in this manner was then dispensed in sterile containers.

Demonstration of stimulatory effect on a solid medium using a surface-streke method. A quantity of yeast-dextrose-agar was prepared. One ml of the seitz filtrate was added to each of two sterile petri plates. Ten ml of molten agar were then poured into each of four petri plates - two containing sterile broth filtrate. The agar and filtrate were thoroughly mixed before the agar became firm and when dry a loopful of an eighteen-hour-old milk culture of the test starter was stroked over the surface of the agar. Incubation was then carried out at 30°C for forty-eight hours.

Demonstration of stimulatory effect on a solid medium using the pour-plate technique. Serial dilutions of the test culture were made in 9 ml quantities of sterile quarter strength Ringer's solution. One ml of the 10^{-5} dilution was added to each of four sterile petri plates by pipette. To two of the plates was added 1 ml of the seitz filtrate. A quantity of yeast-dextrose-agar was prepared and conditioned at 45°C. Ten ml quantities

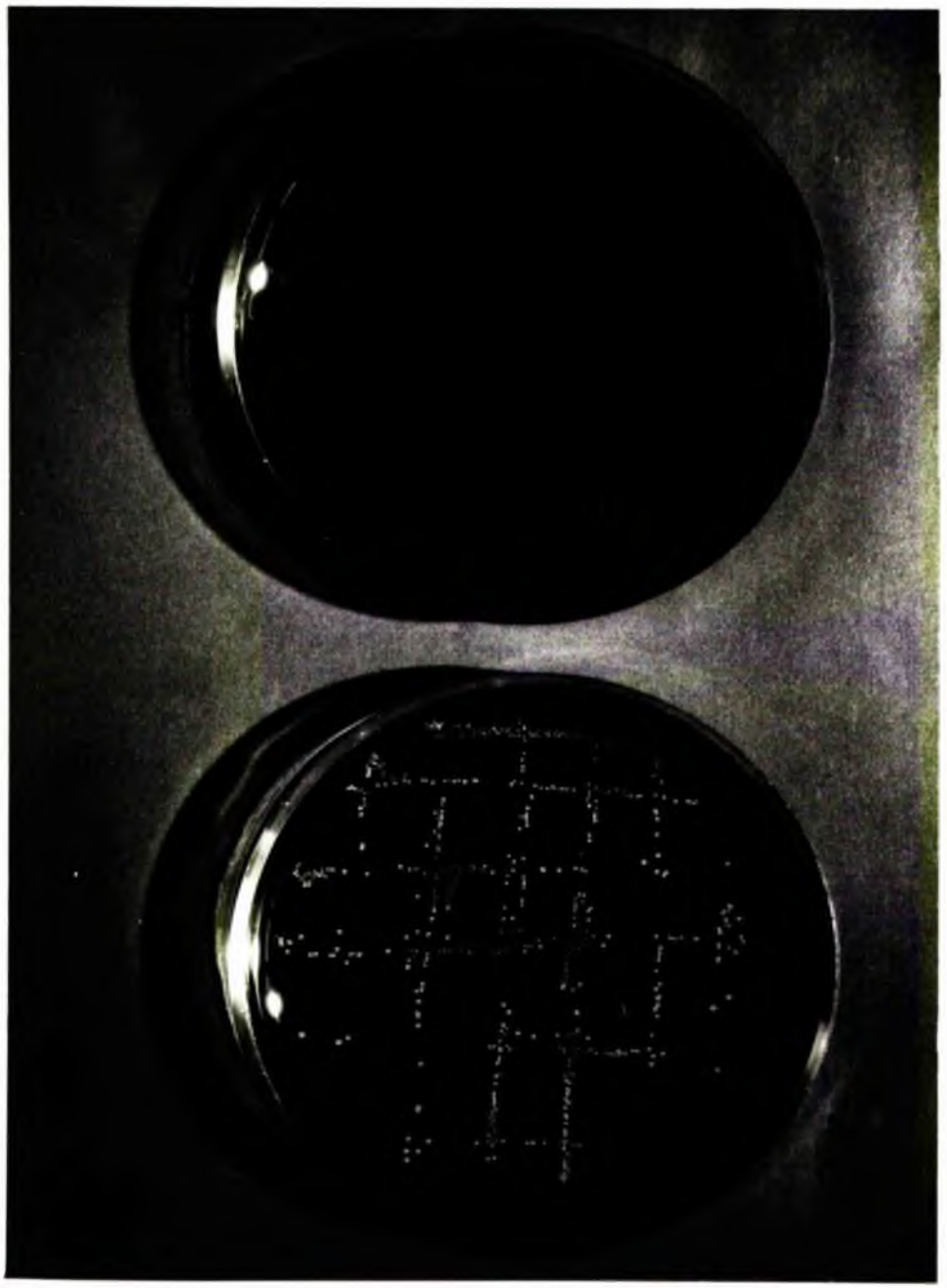
of the agar medium were then added to each plate and following mixing of the materials the plates were incubated at 30°C for forty-eight hours.

The two methods described above do not ensure a complete comparison which would lead to the conclusion that the air organism was causing the stimulation of the growth of the test culture. A further experiment was therefore made. The pour-plate method described above was used with the addition of duplicate plates containing 10 per cent added yeast-dextrose-broth. These conditions offered a full comparison between the growth of the test culture on (a) an undiluted agar medium (b) an agar medium diluted with yeast-dextrose-broth and (c) an agar medium diluted with yeast-dextrose-broth following growth of the contaminant culture - B. subtilis.

Heat stability of the stimulatory material. A quantity of the sterile seitz filtrate prepared in the manner described above was steamed at 100°C for 30 minutes. A further portion was autoclaved at 20 lb/ sq. in. pressure for 15 minutes. Following heat treatment the filtrate was tested by means of the pour-plate technique described above.

RESULTS

An examination of plates 2 - 4 indicates the

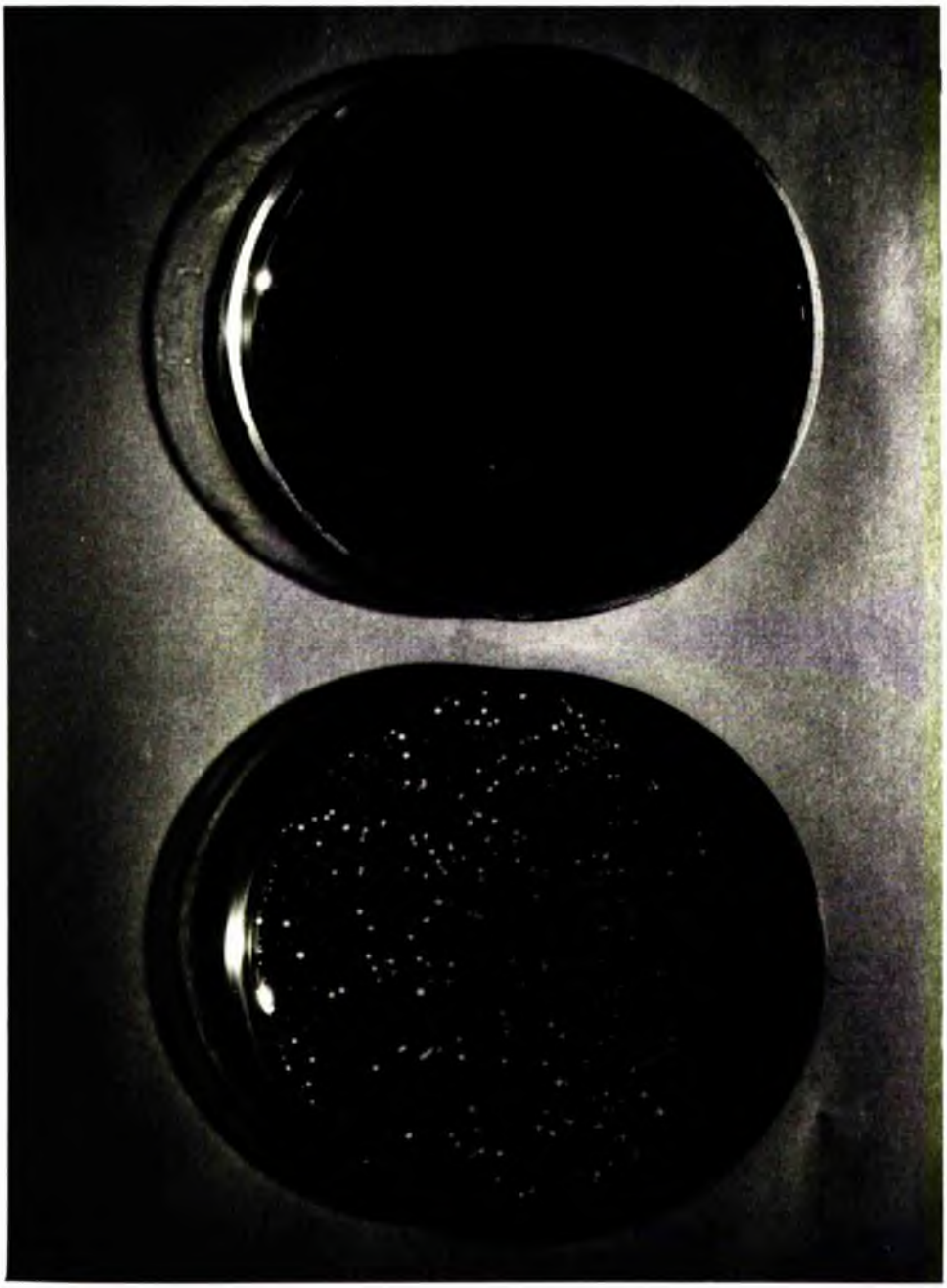


A

B

PLATE 2

The effect of the addition of a cell-free filtrate of yeast-dextrose-broth in which Bacillus subtilis had been grown to yeast-dextrose-agar inoculated with a starter culture by the surface-stroke method. A ..... no added material, B ..... added filtrate



A

B

PLATE 3

The effect of the addition of a cell-free filtrate of yeast-dextrose-broth in which Bacillus subtilis had been grown to yeast-dextrose-agar inoculated with a starter culture by the pour-plate method. A ..... no added material , B ..... added filtrate

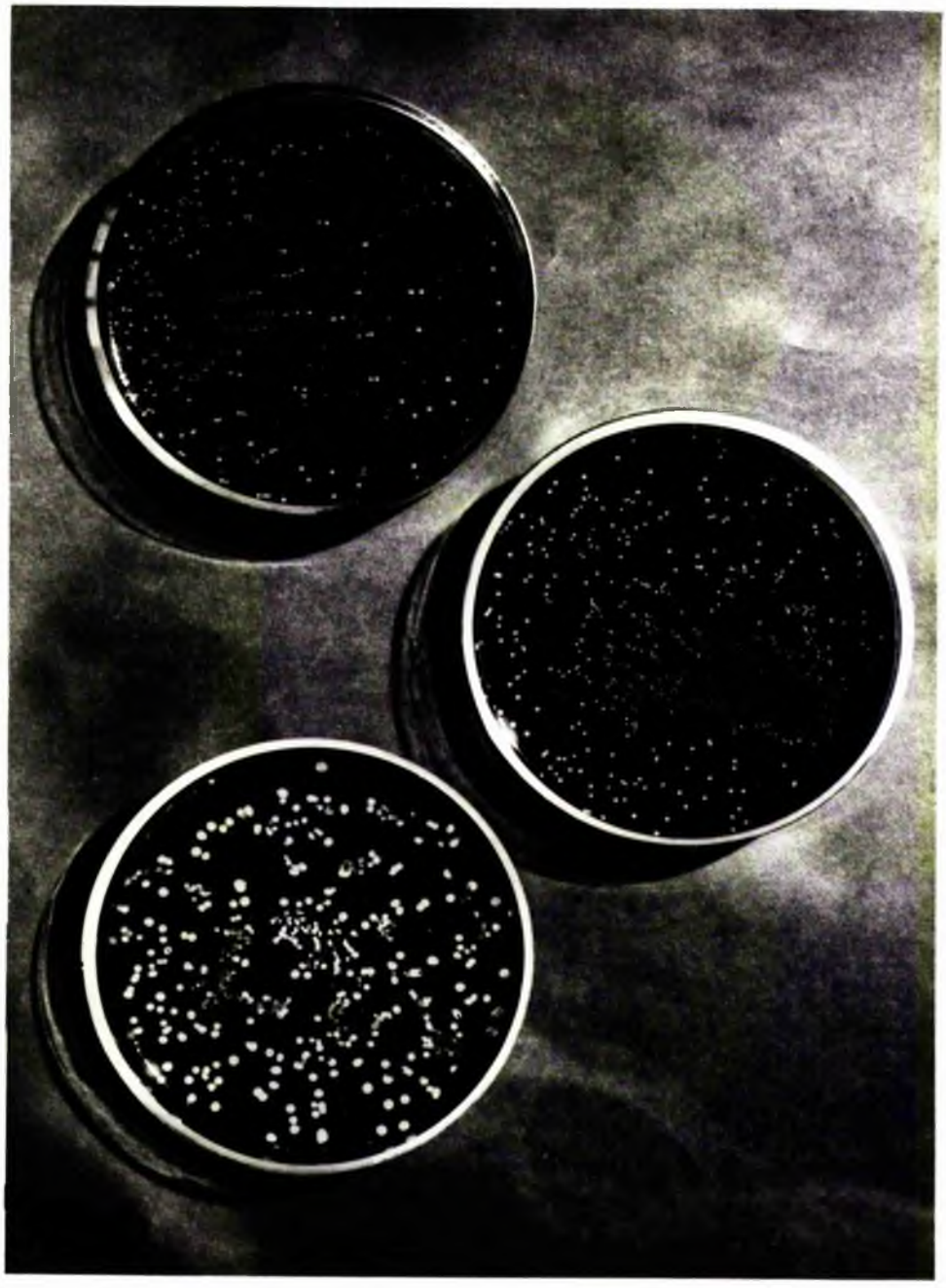


PLATE 4

The effect of the addition of a cell-free filtrate of yeast-dextrose-broth in which Bacillus subtilis had been grown to yeast-dextrose-agar inoculated with a starter culture by the pour-plate method. A ..... no added material, B...., added broth, C ..... added broth filtrate

marked stimulatory action of the filtrate on the growth of a starter culture on an agar medium by both the surface-stroke and pour-plate methods of preparation. An attempt to demonstrate stimulation by growing the test culture and contaminant on an agar medium in alternate lines was vitiated by overgrowing of the test culture by the contaminant. The stimulatory effect could be reproduced at will when a cell-free fluid was used.

A mixed-strain starter culture was used in the experiments and it will be seen from an examination of plates 2 and 3 that not all colonies were stimulated to the same degree although the size of all the colonies is greater in the presence of the broth filtrate. In the case of the surface-stroke plates the difference between the size of the large and small colonies is greater than in the pour-plates. Small colonies were formed although there was no physical crowding apparent on the plate.

Demonstration of the stimulatory effect of B. subtilis on starter bacteria growing in liquid culture

Materials and methods

Test cultures. The following cultures were used:

Streptococcus cremoris: FC/54 J/5

Multi-strain starter: 33

Mixed-strain starters: 90, 87

Broth filtrate. The broth filtrate was prepared by the method described above.

Milk. 103 ml quantities of freshly separated milk were sterilised by steaming at 100°C for 30 minutes followed by 10 lb/ sq. in. pressure for ten minutes.

Methods. The effect of added cell-free filtrate on acid production in milk by a starter culture. Following inoculation of the required number of flasks of milk to provide duplicate tests for each treatment, sterile separated milk, yeast-dextrose-broth and yeast-dextrose-broth filtrate were added in 0.5 to 10 per cent quantities to the inoculated milk. After incubation at 30°C for 5½ hours the titratable acidities were determined by titration with N/9 sodium hydroxide (NaOH) using a 0.5 per cent solution of phenolphthalein as indicator.

The effect of added cell-free filtrate on acid production in milk by a starter culture using a test simulating conditions in cheesemaking. A vitality test of the type described by Whitehead and Cox (196) was made on the three test cultures in milk to which 10 per cent of the cell-free broth filtrate prepared in the manner described

above had been added.

The influence of added broth filtrate on growth of starter bacteria. A determination of the effect of the addition of a broth filtrate of B. subtilis on the viable count of starter bacteria in milk and broth was made. Ten per cent of the broth filtrate was added to (a) sterile separated milk and (b) yeast-dextrose-broth inoculated with 1.0 and 0.1 per cent respectively of an eighteen-hour-old milk culture of the test organism. Control flasks of milk and broth were inoculated with the same amount of culture. After incubation for eighteen hours at 30°C, duplicate serial dilutions of the cultures were made in quarter strength Ringer's solution. The solutions were plated using yeast-dextrose-agar and after forty-eight hours incubation at 30°C the viable bacterial count was determined.

Cheesemaking experiments. The effect of the addition of the cell-free yeast-dextrose-broth filtrate to milk on the rate of acid production during cheese making was determined. The cheesemaking process was carried out by the basic method for Scottish Cheddar cheese where the main feature is the use of a long ripening period before

the addition of rennet. After the addition of 2 per cent of the broth filtrate to the milk in the cheese vat one per cent of an eighteen-hour-old starter was added. Starter was added to the control milk in the same amount. The milks in the control and experimental vats were ripened at 30°C until the required increase in titratable acidity was observed. Later stages of the process are shown in table 55. It was found during the process that the rate of acid production in the milk containing broth filtrate was very much more rapid than that of the control milk. This being so the whey was drained off the experimental vat when the titratable acidity had reached the required level. When the titratable acidity of the whey in the control vat reached the same level the whey was then removed. Variations in rate of acid development are therefore shown as differences in time between certain stages of the process. The curd was milled after the cheddaring period when the correct amount of acid formation had taken place as judged by the hot iron test (96).

RESULTS

An examination of table 52 shows that there is a marked increase in the amount of acid produced by starter cultures on the addition of yeast-dextrose-broth to the milk medium. The addition of cell-free

TABLE 52

Titratable acidity (per cent lactic acid) of (1) milk, (2) milk with added yeast-dextrose-broth and (3) milk with added B. subtilis broth filtrate after 5½ hours incubation at 30°C following inoculation with 1 per cent of starter

Starter	Concentration of added material (%)	Medium		
		Milk	Milk+broth	Milk+filtrate
FC/54/J5	10	0.43	0.595	0.635
33	0.5	0.35	0.40	0.38
	1.0	0.35	0.37	0.40
	2.5	0.37	0.42	0.43
	5.0	0.36	0.43	0.45
	10.0	0.35	0.44	0.47
90	0.5	0.38	0.42	0.43
	1.0	0.37	0.41	0.45
	2.5	0.38	0.48	0.48
	5.0	0.39	0.51	0.52
	10.0	0.38	0.48	0.57
87	1.0		0.50	0.56
	2.5		0.53	0.63
	5.0		0.60	0.65
	10.0		0.57	0.66

filtrate further stimulated acid production only when added in high concentration. One exception was culture 87, a commercial mixed-strain starter, which showed a marked stimulus on the addition of one per cent of the cell-free filtrate. The stimulatory action of broth and broth filtrate was demonstrated for a single-strain culture of Str. cremoris, a multi-strain and a mixed-strain starter.

When the 'vitality' test (196) was used, cultures 87 and 90 gave a marked response to the addition of broth filtrate - table 53. Culture 33 did not, however, react in the same way in the two types of test. In milk culture tests there was a marked stimulus to acid production with added filtrate whereas in the 'vitality' test which closely simulates conditions in cheese manufacture there was little response to the addition of the material.

Table 54 shows that the addition of cell-free broth filtrate of B. subtilis had no effect on the bacterial count of a culture in milk after twenty-four hours incubation at 30°C. A considerable difference in the cell count was found when broth filtrate was added to yeast-dextrose-broth inoculated with a starter culture.

The rate of acid production during cheese manufacture of a culture in milk supplemented with

TABLE 53

Titratable acidity (per cent lactic acid) at various stages of a 'vitality' test (196) made on three commercial cultures in [1] milk and [2] milk with added broth filtrate of B. subtilis

Operation	Time (h-min)	Starter 87		Starter 33		Starter 90	
		Milk	Milk + filtrate	Milk	Milk + filtrate	Milk	Milk + filtrate
Starter added	0.00	0.185	0.185	0.18	0.18	0.175	0.175
Rennet added	0.30	-	-	-	-	-	-
Curd cut	1.30	-	-	-	-	-	-
Maximum scald	2.30	0.12	0.12	0.13	0.125	0.12	0.12
Test and drain	3.30	0.15	0.15	0.135	0.155	0.155	0.13
Test and drain	4.30	0.27	0.29	0.18	0.18	0.20	0.24
Final Test	5.30	0.48	0.53	0.26	0.29	0.33	0.42

TABLE 54

Viable bacterial count after 18 hours incubation at 30°C of (1) milk, (2) milk with added broth filtrate, (3) yeast-dextrose-broth and (4) yeast-dextrose-broth with added broth filtrate of B. subtilis inoculated with a starter culture

Medium	Bacterial Count (per ml)
(1) Milk	112×10^6
(2) Milk + filtrate	111×10^6
(3) Yeast-dextrose-broth	68×10^6
(4) Yeast-dextrose-broth + filtrate	94×10^6

TABLE 55

The effect of the addition of broth filtrate of B. subtilis on the rate of acid production in cheese manufacture

Cheese process record

Milk	Milk (raw) 20 gallons
Type of cheese	Cheddar
Starter	Mixed-strain No. 87
Amount of inoculum	1 per cent
Amount of rennet	1 oz
Weight of curd	Vat 1, 23 lb, Vat 2, 23 lb

Operation		Time (h-min)	Temp. (°F) x	Titratable acidity (%lactic acid)	Hot Iron Test
Starter added	Vat 1.	0.00	85	0.175	
	Vat 2.	0.00	84	0.175	
Rennet added	Vat 1.	1.55	86	0.20	
	Vat 2.	1.55	87	0.20	
Curd cut	Vat 1.	2.35	85	0.14	
	Vat 2.	2.35	86	0.145	
Maximum scald	Vat 1.	3.35	102	0.16	
	Vat 2.	3.35	102	0.16	
Curd settled	Vat 1.	3.45		0.17	
	Vat 2.	4.45		0.185	
Whey run	Vat 1.	4.50		0.25	
	Vat 2.	4.00		0.24	
Curd milled	Vat 1.	6.50			1¼"
	Vat 2.	5.40			1¼"
Curd salted	Vat 1.	7.00			
	Vat 2.	5.50			
Curd hooped	Vat 1.	7.17			
	Vat 2.	6.15			

x The Fahrenheit scale is normally used in cheese manufacture

added broth filtrate was very rapid. The time taken for the titratable acidity of the whey in the control vat to reach the amount required at whey draining was 2 hours 55 minutes; the titratable acidity of the whey in the experimental vat reached the same level in 2 hours - see table 55.

II. STIMULATION OF STARTER BACTERIA BY VARIOUS MATERIALS

In view of the demonstration in the foregoing sub-section of the stimulatory action of added yeast-dextrose-broth when starter cultures were grown in milk it was considered desirable to establish to which of the broth ingredients the stimulus was due. In addition, the author had frequently observed that a large proportion of the colonies formed on an agar medium during the course of culture re-isolation had difficulty in growing in milk. The addition of one per cent of yeast-dextrose-broth to the milk used as the first liquid medium in such cases caused the isolate colonies to coagulate the milk in a very short time.

A. BACTERIA REMOVED FROM A SOLID MEDIUM

Stimulation by yeast-dextrose-broth and various ingredients of the broth

EXPERIMENTAL

Materials and methods

Test cultures. The following starter cultures were used:

Str. cremoris: single-strain cultures WM_3C_{11}, FC/52/14, FD/53 D/1, BT1.

Streptococcous lactis: N.C.T.C. 662.

Multi-strain starter: RM.

Mixed-strain starter: FBA.

Milk media. The yeast-dextrose-broth used in the experiments described in the preceeding sub-section had the following composition; peptone 20 g, lab-lemco (a proprietary form of meat extract) 10 g, sodium chloride 5 g, yeastrel (a proprietary form of yeast extract) 3 g and distilled water 1000 ml.

A series of milk media were prepared with the following composition:

(1) Milk (reconstituted separated)

(2) Milk (fresh separated)

(3) Milk* (r.s.) + 10 per cent yeast-dextrose-broth

(4) Milk (r.s.) + 10 per cent peptone solution (20 g / 1000 ml)

(5) Milk (r.s.) + 10 per cent lab-lemco solution (10 g / 1000 ml)

(6) Milk (r.s.) + 10 per cent sodium chloride solution (5 g / 1000 ml)

(7) Milk (r.s.) + 10 per cent dextrose solution (5 g / 1000 ml)

(8) Milk (r.s.) + 10 per cent yeastrel solution (3 g / 1000 ml)

The milk used in the preparation of medium (1)

* r.s. reconstituted separated

and media (3 - 8) was made from a large batch of spray-dried skim milk powder which had been shown by test to support normal starter growth.

It is well known that milk is not a uniform material either in composition or in its suitability for the growth of starter bacteria. In order to eliminate the possibility that a variation in the basal medium was causing any difference in the reaction of the test starter it was considered advisable to prepare the milk from a large quantity of milk powder. The milk base was prepared by dissolving 100 g of powder in 900 ml of distilled water and adding 1 per cent of litmus indicator. The materials used in the media 3 - 8 were prepared in distilled water and added to the milk base before the complete medium was dispensed in 10.5 ml quantities in test-tubes plugged with cotton wool. Sterilisation was carried out by steaming at 100°C for 30 minutes followed by 10 lb/ sq. in. pressure for 10 minutes. The concentration of solids in the plain milk medium (1) was corrected by the addition of distilled water.

Medium 2 - a control milk medium - was prepared from freshly separated milk in a similar manner.

When ready for use the hydrogen ion concentration of all media was 6.6 ($\pm$ 0.1).

Methods

Changes in physical condition of medium and colour of indicator. Twenty-five ml quantities of molten yeast-lactose-phosphate agar (35) were poured into large petri plates and allowed to solidify. A loopful of an eighteen-hour-old culture of the test organism was then spread over the surface of the medium. After inoculation, the plates were incubated at 30°C for forty-eight hours. Twelve tubes of each milk medium were each inoculated with one single bacterial colony removed in its entirety from the agar surface. A platinum wire sterilised in the bunsen flame was used to cut round the colony, the agar plug on which the bacterial colony was growing being transferred to the tube of milk medium. This procedure ensured that a similar size of inoculum was used for each test. Large plates were used for the growth of each test organism and a large number of isolated colonies were available of similar size and growing in conditions where there was no physical crowding of the bacterial colonies or shortage of essential growth requirements.

On addition of the agar block and bacterial colony to the milk medium the tube was shaken for 1 minute and then incubated at 30°C for one hour. The tube was again shaken for 1 minute and

incubation renewed at 30°C. After twelve and twenty-four hours incubation the tubes were examined for evidence of coagulation of the milk and changes in the litmus indicator. Coagulation was complete when the culture tube of milk medium could be held horizontally without showing any flow of material. Incubation was continued at 30°C for a total of ninety-six hours during which time an examination of the tubes was made at twenty-four hour intervals.

Rate of acid production of the test cultures in various milk media. Two tubes of each milk medium described above but without indicator were inoculated with single colonies of a forty-eight-hour-old culture of the test starter. After various periods of incubation at 30°C the titratable acidity of the contents of the tubes was determined by titration with N/9 sodium hydroxide (NaOH) using a 0.5 per cent solution of phenolphthalein as indicator.

Number of viable organisms present in surface colonies of a test starter growing on an agar medium. The number of viable organisms present in individual colonies of a test starter growing on an agar medium was determined by the pour-plate method (200). Duplicate individual colonies were cut from a forty-

eight-hour-old agar culture of the test organism by the method described above and after the colonies had been added to 100 ml quantities of sterile quarter strength Ringer's solution in stoppered bottles the containers were shaken one hundred times through the distance of one foot. Serial dilutions using 9 and 99 ml blanks of sterile diluent were made. Individual sterile pipettes were used for each transfer. One ml quantities of the dilutions were added to each of three plates followed by 10 ml of yeast-lactose-phosphate agar (83) previously tempered to 45°C. The plates were then incubated at 30°C for forty-eight hours before the colony count was made.

RESULTS

Rate of indicator and physical condition change in various milk media. The results obtained with three representative cultures are shown in tables 56 - 58. In all cases the growth of the test cultures as demonstrated by change in physical condition of the milk media and change in litmus indicator was shown to be much more rapid in the media containing yeast-dextrose-broth or peptone. Peptone was found to be the ingredient mainly responsible for the stimulatory effect of the broth.

TABLE 56

Evidence of bacterial growth as demonstrated by (a) coagulation and (b) change in litmus indicator of 10 ml amounts of (1) milk (reconstituted separated), (2) milk (fresh separated), (3) milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 8) milk (r.s.) plus 10 per cent of the various ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture FC/52/14: single-strain starter of Str. cremoris

Medium	Incubation period (h)											
	24			48			72			96		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Ac
(1) Milk (reconst.separated)	-	-	-	-	-	-	-	-	-	-	-	-
(2) Milk (fresh separated)	-	-	-	-	-	-	-	-	-	S	-	-
(3) Milk (r.s.)+ Y.D.B.	+	+	+									
(4) Milk (r.s.)+ peptone	+	+	+									
(5) Milk (r.s.)+ NaCl	-	-	-	-	-	-	-	-	-	-	-	-
(6) Milk (r.s.)+ dextrose	-	-	-	-	-	-	-	-	-	-	-	-
(7) Milk (r.s.)+yeastrel	-	S	S	S	S	S	+	+	+			
(8) Milk (r.s.)+lemco	-	-	-	-	-	-	-	-	-	-	-	-

\+ reaction complete
\- no change in medium
S slight reaction
r.s. reconstituted separated

TABLE 57

Evidence of bacterial growth as demonstrated by (a) coagulation and (b) changes in litmus indicator of 10 ml amounts of (1) milk (reconstituted separated), (2) milk (fresh separated), (3) milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 8) milk (r.s.) plus 10 per cent of the various ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture WM3C 11: single-strain starter of Str. cremoris

Medium	Incubation period (h)											
	24			48			72			96		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid
(1) Milk (reconst. separated)	-	-	-	-	-	-	M	-	M	+	M	M
(2) Milk (fresh separated)	-	-	-	-	-	-	M	-	-	M	-	-
(3) Milk (r.s.)+ Y.D.B.	+	+	+									
(4) Milk (r.s.)+ peptone	+	+	+									
(5) Milk (r.s.)+ NaCl	-	-	-	-	-	-	M	-	M	+	-	M
(6) Milk (r.s.)+ dextrose	-	-	-	-	-	-	-	-	-	M	-	M
(7) Milk (r.s.)+ yeastrel	-	-	-	+	M	M	+	M	M	+	+	+
(8) Milk (r.s.)+ lemco	-	-	-	-	M	-	+	M	M	-	+	+

\+ reaction complete
\- no change in medium
M marked change in medium
r.s. reconstituted separated

TABLE 58

Evidence of bacterial growth as demonstrated by (a) coagulation and (b) changes in litmus indicator of 10 ml amounts of (1) milk (reconstituted separated), (2) milk (fresh separated), (3) milk (r.s.) plus 10 per cent of yeast-dextrose-broth and (4 - 8) milk (r.s plus 10 per cent of the various ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture RM: multi-strain starter

Medium	Incubation period (h)											
	24			48			72			96		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.
(1) Milk (reconst.separated)	-	-	-	-	-	-	+	S	S	+	S	S
(2) Milk (fresh separted)	-	-	-	-	-	-	+	S	S	+	S	S
(3) Milk (r.s.)+ Y.D.B.	+	+	+									
(4) Milk (r.s.)+ peptone	+	+	+									
(5) Milk (r.s.)+ NaCl	-	-	-	-	-	-	-	-	-	+	S	S
(6) Milk (r.s.)+ dextrose	-	-	-	-	-	-	-	-	-	+	S	S
(7) Milk (r.s.)+ yeastrel	-	S	S	+	S	+	+	+	+			
(8) Milk (r.s.)+ lemco	-	-	-	-	-	-	-	-	-	+	S	S

\+ reaction complete
\- no change in medium
S slight reaction
r.s. reconstituted separated

Yeastrel - a proprietary form of yeast extract - was stimulatory but to a lesser extent, while lemco - a proprietary form of beef extract, dextrose and sodium chloride had no visible effect on the rate of growth of the test cultures. Cultures FC/52/14 and WM3C /11 failed to bring about complete coagulation and indicator change in milk in a ninety-six hour period of inoubation at 30°C. Addition of yeast-dextrose-broth or peptone enabled the single colony inocula to clot the milk media within a twenty-four hour period of incubation and bring about acidification and reduction of the litmus in the same time. The addition of yeastrel brought about an increased rate of growth and cellular activity of the cultures but to a lesser extent than the addition of peptone.

Culture RM coagulated milk in a shorter time than either of the other two test cultures but again the addition of broth, peptone or yeastrel accelerated the growth and acid production of the culture. The addition of broth, peptone or other broth ingredient did not result in any increase in growth or acid production of test culture N.C.T.C. 662 - a strain of Str. lactis. This culture was a slow-growing strain which was not used in cheese manufacture because of the slow rate of acid production. Tests made with this culture are shown in table 59.

TABLE 59

Evidence of bacterial growth as demonstrated by coagulation of 10 ml amounts of (1)milk (reconstituted separated), (2)milk (fresh separated), (3)milk (r.s.) plus 10 per cent of yeast-dextrose-broth and (4 - 7) milk (r.s.) plus certain ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture N.C.T.C. 662: single-strain Str. lactis

Medium	Incubation period (h) 24	48	72	96
(1)Milk(reconst.separated)	-	-	-	+
(2)Milk(fresh separated)	-	-	-	-
(3)Milk (r.s.)+Y.D.B.	-	-	-	+ 7 of 12
(4)Milk (r.s.)+peptone	-	-	-	+ 8 of 12
(5)Milk (r.s.)+dextrose	-	-	-	+ 8 of 12
(6)Milk (r.s.)+yeastrel	-	-	-	+ 8 of 12
(7)Milk (r.s.)+lemco	-	-	-	+ 7 of 12

\+ reaction complete
\- no change in medium
r.s. reconstituted separated

Rate of acid production of test cultures in various milk media. The effect of the addition of yeast-dextrose-broth or its ingredients to milk on the acid production by starter bacteria is shown in table 60. Whereas the titratable acidity of the plain milk medium inoculated with one single surface colony of the test organism growing on an agar medium increased by 0.22 per cent, a similar inoculum changed the titratable acidity of milk containing added broth or peptone by 0.64 and 0.58 per cent respectively. Acid production in milk containing added yeastrel was only slightly higher than in untreated milk. Addition of beef extract had no effect on the increase in titratable acidity of the milk medium after inoculation within the twenty-four hour period of incubation at 30°C and under the same conditions the addition of dextrose or sodium chloride depressed the rate of acid development. When a fast-growing strain of test organism was used - table 61 - the titratable acidity of the plain milk medium after inoculation and incubation for twenty-four hours at 30°C was lower than similarly inoculated and incubated milk media containing yeast-dextrose-broth, peptone or yeastrel. The presence of added dextrose, sodium chloride or lemco had no effect on the rate of acid production of the test cultures.

Bacterial population of surface colonies. The

TABLE 60

Titratable acidity (per cent lactic acid) of 10 ml amounts of $^{(1)}$milk (reconstituted separated), $^{(2)}$milk (fresh separated), $^{(3)}$milk (r.s.) plus 10 per cent yeast-dextrose-broth and $^{(4-8)}$milk (r.s.) plus 10 per cent of the various ingredients of the broth on incubation at 30°C for 24 hours after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture R M : multi-strain starter. Initial titratable acidity of milk 0.18 (per cent lactic acid).

Medium	Coagulation	x Titratable acidity (% lactic acid)
$^{(1)}$Milk (reconst.separated)	-	0.40
$^{(2)}$Milk (fresh separated)	-	0.39
$^{(3)}$Milk (r.s.)+Y.D.B.	+	0.82
$^{(4)}$Milk (r.s.)+peptone	+	0.76
$^{(5)}$Milk (r.s.)+NaCl	-	0.37
$^{(6)}$Milk (r.s.)+dextrose	-	0.35
$^{(7)}$Milk (r.s.)+yeastrel	-	0.44
$^{(8)}$Milk (r.s.)+lemco	-	0.41

x mean of two values
\+ reaction complete
\- no change in medium
r.s. reconstituted separated

TABLE 61

Evidence of bacterial growth as demonstrated by (a) coagulation, (b) change in litmus indicator and (c) change in titratable acidity of 10 ml amounts of (1) milk (reconstituted separated), (2) milk (fresh separated), (3) milk (r.s.) plus 10 per cent of yeast-dextrose-broth and (4 - 8) milk (r.s.) plus 10 per cent of the various ingredients of the broth on incubation at 30°C for 24 hours after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture FD/53 D/1: single-strain starter of Str. cremoris

Medium	Coagulation Reduction Acidification	Titratable acidity (% lactic acid)
(1) Milk (reconst.separated)	Complete	0.73
(2) Milk (fresh separated)	Complete	0.93
(3) Milk (r.s.)+Y.D.B.	Complete	0.88
(4) Milk (r.s.)+peptone	Complete	0.87
(5) Milk (r.s.)+NaCl	Complete	0.71
(6) Milk (r.s.)+dextrose	Complete	0.74
(7) Milk (r.s.)+yeastrel	Complete	0.81
(8) Milk (r.s.)+lemco	Complete	0.76

x mean of two values

r.s. reconstituted separated

TABLE 62

Viable bacterial count of individual colonies from a forty-eight hour old culture of starter bacteria on yeast-lactose-phosphate agar (83)

Culture WM30 11: single-strain starter of *Str. cremoris*

Colony	Bacterial Count (per ml)
A	$^{-}$ 10.6×10^6
B	^{x} 11.4×10^6

$^{-}$ mean value of seven counts

^{x} mean value of nine counts

viable count determined by the pour-plate method (200) showed that the number of organisms in each colony was similar provided the colonies were chosen as being similar in size and were growing in conditions free from physical crowding.

Duration of the stimulatory effect of added yeast-dextrose-broth or peptone. The addition of 10 per cent of either yeast-dextrose-broth or peptone stimulated culture WM3C /11 to coagulate milk within a twenty-four hour incubation period at either 22 or 30°C. Plain milk inoculated with the same amount of culture was coagulated at 30°C after a further forty-eight hours incubation whereas at 22°C coagulation was not complete in a ninety-six hour period. When a further transfer of the coagulated supplemented milk medium was made into plain milk and incubated at 30°C, the time taken for coagulation became prolonged and equal to that of the control. It is evident therefore that the stimulus to acid production only takes place in the presence of the stimulatory material and does not bring about any permanent change in the acid-producing ability of the starter bacteria.

The effect of increasing the yeastrel content of the experimental milk media

EXPERIMENTAL

Having established that peptone was the ingredient

of yeast-dextrose-broth mainly responsible for the stimulus of acid production by starters when this material was added to milk it was of interest to determine whether the amount of stimulation caused by yeastrel could be increased to that of the peptone by a change in the concentration of the material. A series of milk media were prepared as follows:

(1) Milk (reconstituted separated)
(2) Milk (fresh separated)
(3) Milk (r.s.) + 10 per cent yeast-dextrose-broth
(4) Milk (r.s.) + 10 per cent peptone solution (2 per cent)
(5) Milk (r.s.) + 10 per cent yeastrel solution (0.3 per cent)
(6) Milk (r.s.) + 10 per cent yeastrel solution (2 per cent)

The media were prepared by the method described above with and without the addition of litmus indicator.

Methods

The effect of increased yeastrel on the growth of starter bacteria in milk media. The test organism was prepared by the method described above. Twelve tubes of each medium were inoculated with individual surface colonies cut from the agar medium. Changes

* reconstituted separated

in the physical condition of the media were observed during the inoubation period at 30°C. Reduction and acidification of the litmus indicator were noted. The contents of two tubes of each medium without litmus were titrated after inoubation using N/9 sodium hydroxide (NaOH) and a 0.5 per cent solution of phenolphthalein.

The effect of increasing the yeastrel concentration in yeast-dextrose-broth used in milk supplements. Milk media were prepared by the addition to reconstituted skim milk powder of yeast-dextrose-broth, peptone solution, yeast-dextrose-broth from which peptone had been withheld and yeast-dextrose-broth in which the yeastrel content had been increased from 0.3 to 2 per cent. Litmus was added and inoculation of twelve tubes of each medium was made as before, an examination of the tubes being made at intervals during inoubation at 30°C.

RESULTS

The effect of increased yeastrel on the growth of starter cultures in milk media. When the concentration of added yeastrel was raised from 0.3 per cent to 2 per cent the rate of growth and acid production of the test starter cultures was increased. An examination of tables 63 and 64 shows that test cultures RM and WM3C/11 were affected in a similar way. Both cultures were slow to coagulate plain

milk media and bring about change in the indicator. In media containing 0.2 per cent yeastrel the reactions were complete within a forty-eight-hour period at 30°C. A further twenty-four hour period of incubation was required before the reactions were complete in the medium containing 0.3 per cent yeastrel. The addition of yeast-dextrose-broth and/or peptone to milk brought about a completion of the reactions in a shorter time. Test culture FBA - a more active acid-producing strain coagulated the milk medium, reduced and acidified the milk completely within a twenty-four hour period of incubation at 30°C - table 65. When the tubes were examined after twelve hours incubation a third of the tubes containing milk supplemented with peptone were coagulated with complete reduction and strong acidification of the litmus indicator. Growth of the test cultures was slower in the other media.

When media without indicator were used the results of determinations of titratable acidity after various periods of incubation at 30°C confirmed the results of the tests described above. Reference to table 66 shows that the increase in titratable acidity of the control milk media twenty-four hours after inoculation with single colonies of a culture of

TABLE 63

Evidence of bacterial growth as demonstrated by (a) coagulation and (b) changes in litmus indicator of 10 ml amounts of (1) milk (reconstituted separated), (2) milk (fresh separated), (3) milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 6) milk (r.s.) plus certain ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture RM: multi-strain starter

Medium	Incubation period (h)											
	24			48			72			96		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.
(1) Milk (reconst. separated)	-	-	-	-	-	-	+	S	S	+	S	S
(2) Milk (fresh separated)	-	-	-	-	-	-	+	S	S	+	S	S
(3) Milk (r.s.) + Y.D.B.	+	+	+									
(4) Milk (r.s.) + peptone	+	+	+									
(5) Milk (r.s.) + yeastrel (low conc.)	-	S	S	+	S	M	+	+	+			
(6) Milk (r.s.) + yeastrel (high conc.)	M	M	M	+	+	+						

\+ reaction complete
\- no change in medium
S slight reaction
M marked change in medium
r.s. reconstituted separated

TABLE 64

Evidence of bacteria growth as demonstrated by (a) coagulation and (b) changes in litmus indicator of 10 ml amounts of [1] milk (reconstituted separated), [2] milk (fresh separated), [3] milk (r.s.) plus 10 per cent yeast-dextrose-broth and [4-6] milk (r.s.) plus 10 per cent of certain ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour culture of starter bacteria

Culture WM3C 11: single-strain starter of Str. cremoris

Medium	Incubation period (h)											
	24			48			72			96		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.
(1) Milk (reconst. separated)	-	-	-	-	-	-	+	S	S	+	S	S
(2) Milk (fresh separated)	-	-	-	-	-	-	S	S	S	+	S	S
(3) Milk (r.s.)+Y.D.B.	+	+	+									
(4) Milk (r.s.)+peptone	+	+	+									
(5) Milk (r.s.)+yeastrel (low conc.)	-	S	S	+	S	M	+	+	+			
(6) Milk (r.s.)+yeastrel (high conc.)	M	M	M	+	+	+						

\+ reaction complete
\- no change in medium
S slight reaction
M marked change in medium
r.s. reconstituted separated

TABLE 65

Evidence of bacterial growth as demonstrated by (a) coagulation and (b) change in litmus indicator of 10 ml amounts of (1)milk (reconstituted separated), (2)milk fresh separated), (3)milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 6) milk (r.s.) plus certain ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture FBA: mixed-strain starter

Medium	Incubation period (h)					
	12			24		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.
(1)Milk (reconst. separated)	-	M	-	+	+	+
(2)Milk (fresh separated)	-	S	-	+	+	+
(3)Milk (r.s.)+Y.D.B.	-	S	S	+	+	+
(4)Milk (r.s.)+peptone	x+	+	+	+	+	+
(5)Milk (r.s.)+yeastrel (low conc.)	-	S	-	+	+	+
(6)Milk (r.s.)+yeastrel (high conc.)	S	S	S	+	+	+

x two of six tubes
\+ reaction complete
\- no change in medium

M marked reaction
S slight reaction
r.s. reconstituted separated

TABLE 66

Titratable acidity (per cent lactic acid) of duplicate 10 ml amounts of (1)milk (reconstituted separated), (2)milk (fresh separated), (3)milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 6)milk (r.s.) plus 10 per cent of various ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture WM3C 11: single-strain starter of Str. cremoris. Titratable acidity of milk 0.18 (per cent lactic acid)

Medium	Incubation period (h)					
	24			48		
	A	B	Mean	A	B	Mean
(1)Milk (reconst. separated)	0.34	0.33	0.335	0.40	0.46	0.43
(2)Milk (fresh separated)	0.40	0.37	0.385	0.50	0.56	0.53
(3)Milk (r.s.)+Y.D.B.	0.78	0.78	0.78	0.96	0.93	0.945
(4)Milk (r.s.)+peptone	0.78	0.76	0.77	0.83	0.94	0.885
(5)Milk (r.s.)+yeastrel (low conc.)	0.44	0.44	0.44	0.65	0.72	0.685
(6)Milk (r.s.)+yeastrel (high conc.)	0.58	0.58	0.58	0.79	0.77	0.78

r.s. reconstituted separated

WM3C11 was 0.155 per cent whereas that of the milk containing added broth and peptone was 0.60 and 0.59 per cent respectively. The addition of 0.3 per cent yeastrel resulted in an increase in titratable acidity of 0.26 per cent in the test. By increasing the concentration of yeastrel to 2 per cent the titratable acidity of the milk medium rose to 0.40 per cent. After a further twenty-four hour period of incubation the milk supplemented with either broth or peptone again had the highest titratable acidities. The results obtained when culture RM was used were similar - table 67.

In addition to demonstrating the effect of the addition of the various growth stimulants to milk the results obtained show that the method of inoculating the milk media with colonies of similar size cut from an agar medium resulted in good replication of results. Table 66 shows that the greatest difference in titratable acidity between duplicate tubes selected at random after twenty-four hours is 8.1 per cent which is less than the difference which may be expected between duplicate activity tests on the same starter cultures - section 6. After forty-eight hours incubation the greatest difference between duplicate tubes selected at random was 13.3 per cent. The difference between duplicate

TABLE 67

Titratable acidity (per cent lactic acid) of duplicate 10 ml amounts of (1)milk (reconstituted separated), (2)milk (fresh separated), (3)milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 6)milk plus certain ingredients of the broth on incubation at 30°C for 24 hours after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture RM: multi-strain starter

Medium	Titratable acidity (% lactic acid) A	B	Mean
(1)Milk (reconst.separated)	0.35	0.35	0.35
(2)Milk (fresh separated)	0.44	0.45	0.445
(3)Milk (r.s.)+Y.D.B.	0.83	0.86	0.845
(4)Milk (r.s.)+peptone	0.80	0.83	0.815
(5)Milk (r.s.)+yeastrel (low conc.)	0.48	0.47	0.475
(6)Milk (r.s.)+yeastrel (high conc.)	0.57	0.69	0.68

r.s. reconstituted separated

TABLE 68

Titratable acidity (per cent lactic acid) of replicate 10 ml quantities of (1) milk (reconstituted separated), (2) milk (fresh separated), (3) milk (r.s.) plus 10 per cent yeast-dextrose-broth and (4 - 6) milk (r.s.) plus 10 per cent of certain ingredients of the broth, on incubation at 30°C for 18 hours after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Medium	Replicate 1	2	3	4	5	mean
(1) Milk (reconst. separated)	0.74	0.66	0.76	0.74	0.76	0.73
(2) Milk (fresh separated)	0.94	0.93	0.92	0.96	0.92	0.93
(3) Milk (r.s.)+Y.D.B.	0.91	0.98	0.91	0.97	0.92	0.88
(4) Milk (r.s.)+peptone	0.91	0.90	0.89	0.79	0.87	0.87
(5) Milk (r.s.)+yeastrel (low conc.)	0.84	0.76	0.87	0.80	0.81	0.82
(6) Milk (r.s.)+yeastrel (high conc.)	0.94	0.93	0.87	0.85	0.90	0.90

r.s. reconstituted separated

TABLE 69

Evidence of bacterial growth as demonstrated by (a) coagulation and (b) change in litmus indicator of 10 ml amounts of [1]milk (reconstituted separated), [2]milk (fresh separated), milk (r.s.) plus 10 per cent yeast-dextrose-broth and [4 - 6]milk (r.s.) plus certain ingredients of the broth on incubation at 30°C after inoculation with individual colonies of a forty-eight hour old culture of starter bacteria

Culture WM3C 11: single-strain starter of Str. cremoris

Medium	Incubation period (h) 24			48			72			96		
	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.	Coag.	Red.	Acid.
[1]Milk (reconst. separated)	-	-	-	-	-	-	+	S	S	+	M	M
[2]Milk (fresh separated)	-	-	-	-	-	-	+	S	S	+	M	M
[3]Milk (r.s.)+Y.D.B.	M	M	M	+	+	+						
[4]Milk (r.s.)+peptone	M	M	M	+	+	+						
[5]Milk (r.s.)+Y.D.B.-peptone	-	S	S	+	+	+						
[6]Milk (r.s.)+Y.D.B. (increased yeastrel)	M	+	M	+	+	+						

\+ reaction complete
\- no change in medium
M marked change in medium
S slight reaction
r.s. reconstituted separated

tubes was smaller in the tests made on culture RM - table 67. The titratable acidity of five tubes selected at random in a test with a fast-growing strain of starter is shown in table 68.

The effect of increasing the concentration of yeastrel in yeast-dextrose-broth used as a growth supplement. The results of a test using culture WM3C11 are shown in table 69. Coagulation of the milk media was complete in all cases within a forty-eight hour period of incubation at 30°C. The increase in yeastrel content of the broth did not alter the rate of coagulation of the medium.

B. THE ADDITION OF PEPTONE TO MILK INOCULATED WITH ACTIVE LIQUID CULTURES

The work described above has shown that the growth of and acid production by starter bacteria removed from a solid medium could be stimulated by the addition to the medium of yeast-dextrose-broth, peptone and to a lesser extent yeastrel.

It was possible, however, that the stimulus only took place when the bacteria were being changed from a solid medium to a liquid medium. In order to determine the effect of adding peptone and other nutrients to milk inoculated with actively-growing starter bacteria the following experiments were undertaken.

EXPERIMENTAL

Materials and methods

Test cultures. The following cultures were used;

Str. lactis; strains C10, ML3.

Str. cremoris; strains FC/54J/1, FBA/1, B8, C7, C11, C13, HP.

Mixed-strain starters; FD/53S, 71, 91/87, FC/52, 87.

When used, the test strains were eighteen-hour-old cultures in milk prepared at 22°C and had been transferred daily for a period before the tests were made.

Peptone solution. A two per cent solution of peptone in distilled water was prepared by autoclaving the dissolved material at 15 lb/ sq. in. pressure for 20 minutes.

Milk. Freshly separated milk was dispensed in 103 ml quantities in conical flasks plugged with non-absorbent cotton wool and sterilised by steaming at 100°C for 30 minutes followed by autoclaving at 10 lb/ sq. in. pressure for 10 minutes. When required for use the temperature of the milk was adjusted to 30°C.

Peptonised milk solution. A 1.5 per cent solution of a commercial preparation of peptonised

milk was prepared - by dissolving the material in distilled water and autoclaving at 15 lb/ sq. in. pressure for 15 minutes.

Methods

Acid production by starter cultures in milk containing added peptone. Three pairs of flasks of milk were inoculated with 1 per cent of an eighteen-hour-old culture of the test strain prepared at 22°C. To one pair of flasks was added 10 per cent of the peptone solution. The remaining flasks were diluted to the same degree with sterile distilled water or sterile separated milk. After inoculation with 1 per cent of the test culture the flasks were incubated at 30°C for 5½ hours at which point the titratable acidity was determined.

Acid production by starter bacteria in milk containing added peptone in test conditions simulating conditions found in cheese manufacture. The amount of acid produced by starter cultures in milk pasteurised at 60°C for 30 minutes containing 10 per cent of added peptone solution was determined by the 'vitality' test of Whitehead and Cox (196). Two control reactions in which 10 per cent amounts of the pasteurised milk and sterile distilled water were added to the milk were prepared. The various stages of the 'vitality' test were carried out during the 5½ hour incubation period.

Cell multiplication and acid production by starter cultures in milk containing added peptone. Method one described above was used. Flasks of sterile separated milk to which the various solutions had been added were prepared. The viable bacterial count was determined by the pour-plate (209) method, on inoculation and at the end of the incubation period. Serial dilutions of the materials were made in sterile quarter strength Ringer's solution. Five 1 ml portions of the 10^{-5} dilution were plated out on inoculation and a similar number of portions of the 10^{-7} dilution were plated out at the end of the incubation period. It had been previously established that these dilutions would provide plate counts of between 25 and 100 colonies. At the end of the incubation period the titratable acidity of the milks was determined.

The effect of the addition of peptone to milk used for starter propagation. Sterile separated milk in conical flasks was inoculated with 1 per cent of the test culture. Additions of peptone solution, sterile distilled water and sterile separated milk were made and the flasks were incubated at 22°C for 18 hours. Hydrogen ion concentration and titratable acidity of the milks were determined at the end of the propagation period. Duplicate flasks of sterile separated milk were inoculated with one per cent of

the cultures. After 5½ hours at 30°C the titratable acidity and pH of the milks were determined.

The effect of the addition of peptone to milk on the acid produced by a starter culture during cheese manufacture. Milk and milk to which 2 per cent of peptone solution had been added were compared in cheesemaking experiments. Peptone solution was added to the pasteurised milk to produce a concentration of peptone of 0.04 per cent. After the addition of 1 per cent of an eighteen-hour-old milk culture of the starter the milks were ripened at 30°C until the titratable acidity of the milk had increased by 0.02 per cent when the rennet was added. The later stages of the process are shown in full in the cheesemaking record - table 77. Since it was found that the rate of acid production was greatly increased in the milk containing peptone it was decided to drain off the whey at what was considered to be a suitable level of acidity. The whey was drained off the control vat at the same titratable acidity. Variations in rate of acid production show as differences in time between certain stages of the process.

A comparison between the effect of adding peptone and peptonised milk on the rate of acid development by starter bacteria. Ten per cent of peptone solution, peptonised milk and sterile distilled water were

added to a series of flasks of sterile separated milk prepared by the method described above and inoculated with 1 per cent of the test culture. After 5½ hours at 30°C the titratable acidity of the milks was determined.

RESULTS

Acid production by starter cultures in milk containing added peptone. Addition of peptone solution to produce a peptone concentration of 0.2 per cent in sterile separated milk resulted in an increase in the rate of acid production by starter cultures. The results obtained in tests using a single-strain culture of Str. cremoris are shown in table 70. On the first test the culture produced acid at a rapid rate in the control milk whereas the rate of acid production was very much lower in the second test. On both occasions, however, the addition of peptone to the milk in which the culture was growing resulted in a marked stimulus to acid production. When two mixed-strain starters were tested - table 71 - the addition of peptone encouraged acid production by the starter culture. An examination of table 72 shows that with two exceptions the addition of 0.2 per cent peptone resulted in a marked increase in the amount of acid produced by the test cultures in milk during the 5½ hour

TABLE 70

Titratable acidity (per cent lactic acid) of [1] milk and [2] milk containing 10 per cent peptone solution after 5½ hours incubation at 30°C following inoculation with 1 per cent starter

Starter FBA 1: single-strain Str. cremoris

Test	Medium	x Titratable acidity (% lactic acid)
A	Milk	0.465
	Milk + peptone	0.635
B	Milk	0.33
	Milk + peptone	0.55

x mean of two values

TABLE 71

Titratable acidity (per cent lactic acid) of (1) milk, (2) milk plus 10 per cent sterile distilled water and (3) milk plus 10 per cent peptone solution after 5½ hours incubation at 30°C following inoculation with 1 per cent starter

Starter	Medium	x Titratable acidity (% lactic acid)
FD/53 S	(1) Milk	0.44
	(2) Milk + sterile distilled water	0.39
	(3) Milk + peptone solution	0.65
71	(1) Milk	0.32
	(2) Milk + sterile distilled water	0.28
	(3) Milk + peptone solution	0.40

x mean of two values

TABLE 72

Titratable acidity (per cent lactic acid) of (1)milk, (2)milk plus 10 per cent distilled water, (3)milk plus 10 per cent peptonised milk and (4)milk plus 10 per cent peptone solution after 5½ hours incubation at 30°C following inoculation with 1 per cent starter

Starter	Medium			
	Milk	Milk + distilled water	Milk + peptonised milk	Milk + peptone
E8	0.43	0.37	0.50	0.47
C7	0.38	0.42	0.49	0.46
C11	0.47	0.46	0.56	0.56
C13	0.36	0.30	0.445	0.425
HP	0.50	0.46	0.50	0.505
ML3	0.545	0.545	0.595	0.61
C10	0.45	0.37	0.47	0.505
91	0.495	0.47	0.535	0.51
129	0.525	0.50	0.555	0.55

incubation period of the test. The exceptions were HP, a single-strain starter of Str. cremoris which when tested gave no indication of stimulation and 129, a mixed-strain starter which was stimulated to a very limited extent.

Acid production by starter bacteria in milk containing added peptone in test conditions simulating those found in cheese manufacture. The results suggesting a marked stimulus to acid production as judged by the 5½ hour test in milk were confirmed when the Whitehead and Cox (196) 'vitality' test was used. Individual results obtained in duplicate tests with one test culture are presented in table 73 while table 74 shows the final titratable acidities with four test cultures. In each case the titratable acidity at the end of the test period is much higher in milk to which peptone was added than in untreated milk. A close agreement between duplicate tests was found.

Cell multiplication and acid production by starter cultures in milk containing added peptone in relation to increase in bacterial numbers. In this trial a single-strain culture of Str. cremoris was used. The results obtained from three determinations are presented in table 75. One interesting feature was the low hydrogen ion concentration found in the milk containing

TABLE 73

Titratable acidity (per cent lactic acid) readings at certain stages of a 5½ hour 'vitality' test (196) of a starter in (1) milk and (2) milk containing added peptone

Starter FC/52: mixed-strain culture

Operation	Time (h-min)	Medium: Milk			Medium: Milk + distilled			Medium: Milk + peptone		
Test and drain	3.30	0.15	0.15	0.15	0.14	0.14	0.14	0.16	0.16	0.16
Test and drain	4.30	0.24	0.24	0.24	0.235	0.23	0.235	0.295	0.30	0.30
Test and drain	5.30	0.45	0.45	0.45	0.425	0.435	0.43	0.67	0.66	0.665
Final test	6.30	0.65			0.60			0.83		

TABLE 74

Titratable acidity (per cent lactic acid) readings at the end of a 5½ hour 'vitality' test (196) of starters in [1] milk and [2] milk containing added peptone

Starter	Medium	
	Milk	Milk + peptone
91/87	0.40	0.46
FC/52	0.45	0.665
87	0.49	0.56
FD/53 S	0.31	0.52

TABLE 75

Titratable acidity (per cent lactic acid), hydrogen ion concentration and bacterial count of (1) milk, (2) milk plus 10 per cent distilled water and (3) milk plus 10 per cent of a peptone solution on inoculation with 1 per cent of a starter and after an incubation period of 5½ hours at 30°C

Starter FC/54 J/1: single-strain Str. cremoris

	Milk		Milk + water		Milk + peptone	
	On inoculation	After incubation	On inoculation	After incubation	On inoculation	After incubation
*						
Titratable acidity (% lactic acid)	0.225	0.49	0.225	0.44	0.225	0.50
pH	6.4	5.2	6.4	5.25	6.4	5.0 x
Bacterial count (per ml)	3.7×10^6	337×10^6	4.4×10^6	266×10^6	3.8×10^6	330×10^6

x milk coagulated
* mean of three values

added peptone after the test period compared with that of the untreated milk despite the titratable acidities being similar. This would suggest that a stronger acid had been formed in the presence of peptone. Knudsen (92) has suggested that if in the souring of milk by a pure culture and by a mixed-strain starter the same titratable acidity but a different hydrogen ion concentration is found then it may be concluded that it is not the same acid which has been formed in both instances. Czulak (33) has expressed the same opinion in a study of strains of Streptococcus diacetilactis.

The flasks of milk to which peptone had been added were coagulated at the end of the test period. There was no significant difference in the bacterial numbers at the end of the test period in milk and milk with added peptone. It must therefore be assumed that the lower hydrogen ion concentration of the milk containing peptone was due to an increase in the cell activity of the test culture.

The effect of the addition of peptone to milk used for starter propagation. Reference to table 76 shows that the addition of peptone to the milk used for the propagation of starter cultures had no stimulatory action on the acid production by the culture when re-transferred into a plain milk medium. In one test where a mixed-strain culture was used - table 76, the

TABLE 76

Titratable acidity and hydrogen ion concentration during propagation of a culture in (1)milk, (2)milk plus 10 per cent distilled water and (3)milk plus 10 per cent peptone solution and in milk on inoculation with 1 per cent of the culture prepared in the above media and after a 5½ hour incubation period at 30°C

Culture FC/54 J: mixed-strain starter

Test	Milk		Milk + distilled water		Milk + peptone	
	Acidity (% lactic acid)	pH	Acidity (% lactic acid)	pH	Acidity (% lactic acid)	pH
Culture media on inoculation	0.225	6.4	0.225	6.4	0.225	6.4
x After 18 h at 22°C	0.705	4.7	0.635	4.6	0.68	4.55
Test milk on inoculation	0.225	6.4	0.225	6.4	0.225	6.4
- After 5½ h at 30°C	0.46	5.35	0.45	5.4	0.41	5.5

x mean of two values

- mean of four values

addition of peptone to the milk used for propagation resulted in a slight impairment in the activity of the culture in plain milk on the subsequent transfer.

The effect of added peptone in cheese manufacture. Reference to table 77 in which a comparison is shown between acid production in cheese manufacture in milk with and without added peptone indicates that the rate of acid development is greatly increased in the presence of added peptone. In the experiments the concentration of added peptone was 0.04 per cent. The time taken for the starter to produce the required increase in titratable acidity between curd cutting and whey draining in the plain milk was 2 hours which is the normal time between these stages. The same titratable acidity was reached in a shorter time - 1 hour 25 minutes - in the milk containing added peptone. Detailed titratable acidity determinations made during this period of increasing acid production in a second cheesemaking trial are shown in table 78. The effect of the addition of peptone on acid production became more marked before the whey running stage. The required acidity was reached in the milk containing added peptone 80 minutes earlier than in plain milk.

TABLE 77

The effect of the addition of 0.04 per cent peptone to the milk used for cheese manufacture on the rate of acid production by a starter

Cheese process record

Milk	Whole (raw) 10 gallons
Type of cheese	Cheddar
Starter	Mixed-strain No. 71
Amount of inoculum	1 per cent
Amount of rennet	½ oz
Weight of curd	Vat 1 (contol), 10½ lb
	Vat 2 (added peptone), 10½ lb

Operation		Time (h-min)	Temp. (°F) x	Titratable acidity (% lactic acid)	Hot iron test
Starter added	Vat 1	0.00	86	0.18	
	Vat 2	0.00	86	0.18	
Rennet added	Vat 1	1.50	86	0.20	
	Vat 2	1.50	86	0.20	
Curd cut	Vat 1	2.35	85	0.145	
	Vat 2	2.35	86	0.14	
Maximum scald	Vat 1	3.35	102	0.175	
	Vat 2	3.35	102	0.205	
Curd settled	Vat 1	4.06		0.19	
	Vat 2	3.55		0.22	
Whey run	Vat 1	4.35		0.25	
	Vat 2	4.00		0.25	
Curd milled	Vat 1	6.25			1¼"
	Vat 2	5.50			1¼"
Curd salted	Vat 1	6.40			
	Vat 2	5.55			
Curd hooped	Vat 1	6.45			
	Vat 2	6.00			

[x]The Fahrenheit scale of temperature is used in cheesemaking

TABLE 78

The rate of acid production by a starter in cheese manufacture using (1) milk and (2) milk containing 0.04 per cent added peptone. Titratable acidity (per cent lactic acid) readings at intervals in the process between curd cutting and whey draining

Operation	Time (h-min)	Temp. (°F)	Titratable acidity (% lactic acid) (1) Milk	(2) Milk + peptone
Curd cut	0.00	86	0.12	0.12
Scalding	0.30	92	0.14	0.145
	0.45	97	0.145	0.165
	1.00	102	0.155	0.18
Maximum scald	1.15	103	0.16	0.19
Curd settled	1.30	102	0.18	0.21
Whey run	1.45			0.24 Free whey
				0.265 From curd
	1.50			0.29
	3.05		0.26 Free whey	
			0.29 From curd	
	3.10		0.31	

The effect of the addition of peptonised milk on the rate of acid production by a starter culture in milk. The addition of a commercial preparation of peptonised milk to sterile separated milk stimulated acid production by starter bacteria. Results obtained with several test cultures are shown in table 72. Test strain HP was not stimulated by either peptone or peptonised milk.

DISCUSSION

It has been demonstrated that a strain of *B. subtilis* was able to produce substances which stimulated the growth of starter bacteria on solid media. The stimulus was demonstrated when a sterile cell-free solution prepared by filtration of the yeast-dextrose-broth medium in which the organism was growing was added to yeast-dextrose-agar inoculated with starter bacteria. Two common methods of inoculation were used, viz. (a) the pour-plate and (b) the surface-streak techniques. Similar results were obtained by both methods. When cell-free preparations were added to milk inoculated with test cultures the rate of acid production was increased. The stimulus due to the 'growth factor' produced by the culture of

B. subtilis was observed only when large amounts of cell-free material were used. The yeast-dextrose-broth used in the preparation of the stimulatory material was found to stimulate the acid production by the starter bacteria. The additional response of the culture to the broth after growth of B. subtilis varied with the test culture. When culture 87, a mixed-strain starter, was used there was a marked increase in stimulation at all levels of added material. With culture 90, added stimulus only took place when a very large addition of material was made. The addition of the cell-free material to milk did not alter the bacterial count produced by a starter at the end of a twenty-four hour incubation period. Addition of cell-free filtrate did, however, produce a higher cell count in yeast-dextrose-broth. Experiments proved that the stimulatory principle was stable to heat treatment at 100°C and autoclaving at 15 lb/ sq. in. pressure.

The associative action of bacteria has been reported previously and Rice (135) has considered the stimulus to be due to the production of a readily available source of nitrogen when associative growth was in milk. While yeast-dextrose-broth provides a readily available source of nitrogen it is also obvious that the prior growth of B. subtilis in yeast-dextrose-broth increases the stimulatory action.

In an investigation to determine which of the ingredients of yeast-dextrose-broth were stimulatory to starter bacteria two techniques were used. The first of these was used with test cultures growing on solid medium. When starter cultures are plated and single colonies picked off into milk it is frequently found that a large proportion of the cultures do not grow readily. Peptone was shown to have a very marked influence on the growth and cell activity of cultures freshly isolated from solid medium. The addition of a proprietary form of meat extract of high nitrogen content did not exert any stimulatory influence on growth or cell activity. This is surprising since an analysis of the material shows that it contains 9 per cent total nitrogen which consists of considerable amounts of the proteose, peptone and amino forms. The main constituents of this material are similar to those of peptone. When yeastrel - a proprietary form of yeast extract - was added to the milk medium, growth of the starter bacteria was stimulated but to a lesser extent than peptone. The addition of the monosaccharide, dextrose, did not bring about any more rapid growth of the starter bacteria. Determinations of titratable acidity of the various milk media after various periods of incubation confirmed the results obtained by making observations of change in

physical condition of the media and litmus indicator. In all tests there was a close agreement between replicate tubes.

While the stimulatory effect of broth, peptone or yeastrel was most marked with the slow-growing strains of starter bacteria the addition of these materials to milk encouraged the growth and acid production of fast-growing organisms in a similar manner. One culture of Str. lactis was not stimulated by any of the ingredients of yeast-dextrose-broth. This culture normally produced acid at a very low rate. The consistency in the results obtained between replicate tubes both in visual examination tests and when determinations of acid production were made confirmed the accuracy of the method of inoculation. Bacterial counts of the individual colonies showed that the number of cells was similar in different colonies provided they were of equal size and growing in conditions free from physical crowding.

The effect of the addition of peptone to milk inoculated with active milk cultures of starter bacteria was determined in the second series of tests. Stimulation of acid production by starter cultures was confirmed in a test at 30°C, by a 'vitality' test (196) which simulated closely the conditions

found in cheese manufacture and in actual cheesemaking. A variety of cultures was tested. Single-strain cultures of Str. cremoris and Str. lactis behaved in the same way as multi- and mixed-strain cultures. In cheese manufacture the rate of acid production from the stage at which the curd was cut became rapid by comparison with that of the same culture in plain milk. Stimulation of bacteria was only for one generation. In experiments in which peptone was added to the milk used for starter propagation it was found that the activity of the subsequent transfer in milk was not increased. The activity of one culture propagated in milk containing added peptone was lower than the control propagated in plain milk. In this test a mixed-strain starter was used. The hydrogen ion concentration of the culture prepared in the supplemented milk medium was significantly lower than the control.

It was shown that the stimulus to acid production by the addition of peptone to milk was due to an increase in the cell activity of the culture and not to an increase in cell numbers.

Since the addition of peptonised milk to the milk medium stimulated the bacterial cells it is evident that milk provides essential growth factors for starter bacteria. Peptonised milk contains

the degradation products of the proteins, albumins and globulins of milk. The nitrogen of milk is therefore more readily available for bacterial assililation than the primary proteins of milk.

Changes in the fermentation reactions of various organisms have been reported from time to time. A reference to a member of the coli-aerogenes group failing to ferment lactose was made by Neisser (118) in 1906. When this culture was plated out secondary strains were formed which were able to ferment this sugar. Dealing with the lactic streptococci, Yawger and Sherman (203) reported the isolation from milk of strains of Str. lactis which failed to curdle milk. Only a small amount of acid was formed by the cultures in milk. One strain gradually became able to ferment lactose and clot milk.

The question of the nutrition and growth requirements of the lactic streptococci is by no means of recent origin. In 1929 Anderegg and Hammer (4) reported on the proteolytic powers of certain strains of Str. lactis. These workers raised the question of the relationship between protein decomposition and rate of coagulation of milk inoculated with Str. lactis. Did certain organisms grow slowly because they were unable to attack the protein or did they not attack the protein because

they grew so slowly? These workers referred to data not included in the paper which indicated that when peptone or certain amino-acids were added to milk inoculated with slow-coagulating organisms an increased rate of coagulation took place. It was suggested that the organisms were limited in their growth in normal milk through their inability to satisfy their nitrogen requirements. Protein decomposition occurred with cultures which coagulated milk rapidly while no breakdown of protein took place with cultures which produced acid slowly.

In milk inoculated with slow-coagulating strains the addition of acid did not bring about any increase in proteolytic action. This result would suggest that acid production by slow-coagulating strains is related to loss of proteolytic activity. Protein decomposition by strains of Str. lactis was due to enzyme action. Results indicated that while the enzyme did not show the rapid and extended activity of some proteolytic enzymes produced by bacteria, decomposition did proceed to the amino nitrogen stage.

Collins and Nelson (27) have shown that when Str. lactis was grown in milk there was an increase in the soluble nitrogen content of the milk. The greatest increase in soluble nitrogen took place

during the period of rapid growth and acid production. The speed at which liberation of nitrogenous degradation products took place decreased during the stage when the bacterial population was declining. This was due either to a reduction in metabolic activity of the cells or to the reaction of the medium becoming available for the continued action of the enzyme system concerned.

Harriman and Hammer (69) have confirmed the opinion of others that fast acid-producing starters were more actively proteolytic than cultures which formed acid at a slower rate.

There have been many reports on stimulation of lactic acid bacteria by various materials. Chu and Williams (26) have presented evidence that the effect of peptone in stimulating the growth of Lactobacillus casei was probably due to a combination of growth factors rather than to the presence of a single 'peptone factor'. Smith (159) has reported on an unidentified growth factor present in yeast extract which stimulated strains of Str. lactis which failed to grow in a medium consisting of various vitamins, purine bases, a pryimidine and a salt mixture. Other materials have been shown to exert a stimulatory influence on lactic acid bacteria.

The presence in whey of an unidentified factor which stimulated L. casei was made by Virtanen, Karstom, Jorma and Kahra (185). The work of these authors substantiates the opinions of others that where stimulation occurs it is due to an increase in individual cell activity and not to an increase in the number of cells. Hansen (67) has stated that little or no stimulation was observed when Str. lactis and Str. cremoris were grown in media enriched by their own cell content. Rogers and Greenbank (139) reported that cell-free broth filtrates of Str. lactis stimulated the initial growth of Str. lactis but limited the final population.

Since the experimental work described in this section was carried out, Garvie and Mabbitt (53) have reported on the effect of peptone on a slow acid-forming variant strain of an active starter culture. They have shown that acid production could be temporarily stimulated by the addition of peptides and / or amino-acids obtainable from casein by acid or enzymatic hydrolysis. Their conclusion was that change from a fast to a slow culture which frequently occurs on continued transfer was due to the loss of ability to utilise the nitrogen compounds

of milk. In addition to considering the loss of activity by a culture on continued transfer they suggested a relationship between the availability of nitrogen in milk and the inhibition of starter bacteria due to the condition known as 'winter slowness' previously reported by Czulak and Meanwell (38) and Jago (85). Jago (85) found that the addition to 'winter slow' milk of extracts of fowl liver, separator slime and yeast extract overcame the inhibitory principle inherent in the milk which was shown to be closely associated with the fat globules. Jago's work supported the theory of Czulak and Meanwell (38) that inhibition of susceptible starter bacteria in 'winter milk' was due to the inability of the organisms to synthesise some growth-stimulating factor normally formed by non-susceptible cultures. They also suggested that inhibited cultures had a reduced activity in winter milk because of an unknown growth factor which they require and which is not available because of the presence in the milk of a heat-labile inhibitory substance.

The work described in this section has shown that fast-acid producing strains of starter bacteria were stimulated in the same way as slow strains. Some exceptions were, however, found. Not all slow acid-

forming cultures were stimulated by the addition of yeast extract, yeast-dextrose-broth or peptone. On the other hand some fast-growing strains were not stimulated by the provision of readily available nitrogen.

The importance of the form of available nitrogen is shown by the experiments which demonstrated that the addition to milk of lemco, a proprietary form of beef extract rich in nitrogen - did not result in any stimulation of the starter bacteria.

Provision of sugar in the monosaccharide form did not influence the amount of acid formed by the test strains. Cultures which fermented lactose slowly were unable to ferment dextrose. That milk is a source of growth factors for the lactic acid streptococci was shown by the marked stimulation of acid production of starter bacteria following the addition to milk of a proprietary form of peptonised milk.

SUMMARY

A strain of *B. subtilis* found to stimulate the growth of starter bacteria on an agar medium during the routine re-isolation of a starter culture was shown to produce a heat-stable stimulatory substance during growth in yeast-dextrose-broth. A sterile seitz-filtrate of a yeast-dextrose-broth culture of

B. subtilis stimulated colony formation by starter bacteria on an agar medium and acid production by starter cultures in milk.

This investigation confirmed the work of others on associative action between various bacteria including B. subtilis and the organisms used in starter cultures for cheesemaking.

During the course of the investigation it was observed that yeast-dextrose-broth stimulated acid production by starter bacteria. A full investigation was undertaken to determine which of the ingredients of the broth was responsible for this effect. Surface colonies of starter cultures growing on an agar medium and added to milk media prepared from a constant base material - milk powder - and the various ingredients of the yeast-dextrose-broth were stimulated to produce acid and thereby bring about change in the physical condition of the media which contained yeast-dextrose-broth, peptone or yeastrel (a proprietary form of yeast extract).

Lemco (a proprietary form of meat extract) rich in the breakdown products of proteins did not stimulate the starter bacteria. Dextrose and sodium chloride were also without effect.

The stimulatory effect of yeast-dextrose-broth, peptone and yeastrel was demonstrated with slow- and

fast-growing cultures. The results obtained by observing the rate of coagulation of the milk media and acidification and reduction of the litmus indicator were confirmed by determinations of titratable acidity of indicator-free media after various periods of incubation.

The stimulatory effect of peptone was shown to be of a temporary nature, increased acid production resulting from a stimulus to cell activity and not cell multiplication.

Addition of peptone to the milk used for propagating starters resulted in an increase in acid production by the organisms but impaired the acid-producing ability of the subsequent transfer in plain milk.

The addition to milk of 0.04 per cent of peptone (bacteriological) resulted in a rapid increase in acid production during cheese manufacture.

A commercial preparation of peptonised milk containing the breakdown products of milk proteins stimulated acid production by seven of nine test cultures. The stimulus to acid production was similar to that produced by peptone.

The dependence of acid production by starter bacteria on a readily available source of nitrogen was demonstrated.

SECTION EIGHT

CITRATE-UTILISING ACTIVITY OF CERTAIN STARTER BACTERIA

CITRATE-UTILISING ACTIVITY OF CERTAIN STARTER BACTERIA

In the two previous sections the acid-producing ability of organisms used as starters in cheese manufacture has been considered. A second form of activity is associated with certain of these organisms and it is pertinent to consider this characteristic.

Citric acid which exists in milk - as one of the minor constituents - to some 0.15 - 0.20 per cent (43) - is utilised by the decarboxylase systems of the homofermentative organism Streptococcus diacetilactis and the hetero-fermentative bacteria belonging to genus Leuconostoc.

A considerable amount of work has been carried out on the biological activity of starter bacteria since Storch (167) described the organisms responsible for the formation of flavour compounds in butter cultures. The group of bacteria which he described in 1919 - some twenty-nine years after he first introduced pure cultures for cream souring (166) - have been known by various names including betacocci,

associated organisms and leuconostocs. Bergey (16) classified these organisms in the genus Leuconostoc of the tribe Streptococcoceae.

The presence of leuconostocs in starters used in cheese manufacture has been demonstrated (33) and since the majority of the cheese manufactured in Great Britain is produced with the aid of mixed-strain starters which frequently contain these organisms an examination of their bacteriological reactions is of interest.

In 1939 Matuszewski et al. (113) described an organism, homo-fermentative in action, possessing the property of utilising citric acid in the formation of diacetyl and various other products including carbon dioxide. Swartling (172) carried out a detailed study of a number of citric acid-fermenting streptococci some of which had been designated Streptococcus citrophilus, obtained from various sources. This worker reached the conclusion that all the homo-fermentative citric acid-fermenting bacteria which he examined belonged to a distinct group for which he suggested the specific epithet diacetilactis. Czulak (33) has commented on the presence of Streptococcus diacetilactis in a number of commercial starters in use in Australia while Järvik and Kjell (86) have demonstrated the presence

of this organism in starters used in Swedish cheese manufacture. Zeilinska and Hiscox (204) examined Cheddar cheese curd and found considerable numbers of group N streptococci which resembled Str. diacetilactis and the organism Str. citrophilus described by van Beynum and Pette (176). The presence of such organisms in cheese means that they gained entry to the cheese either as contaminants from the milk or equipment or were added in the starter culture. Starter cultures are frequently open to contamination in cheesemaking factories and the author has found that contamination with citrate-fermenting organisms is common. In one particular instance starters were found to have become contaminated in a creamery within a period of fifteen days of the supply of the pure culture. In this case the original cultures supplied to the factory were non-aroma, non-gas-producing starters of Streptococcus cremoris. The bacteriological control of the starters in this creamery was very poor, despite which the starters produced acid at a satisfactory rate in the cheesemaking process. On examination after a period of time the cultures were found to produce a positive reaction in the creatine test (61), indicating the presence of contaminants able to produce flavour compounds.

Isolation and purification of strains present in the starter confirmed that there was present a considerable number of organisms giving positive creatine tests and producing gas in the test of Gibson and Abd-el-Malek (55).

The presence of citric acid-fermenting organisms in cheese starters whether by intention or by accidental contamination warrants an examination of certain bacteriological properties of these organisms and factors affecting them.

Utilisation of citric acid

Van Beynum and Pette (176) have shown that the leuconostocs convert one molecule of citric acid into two molecules of carbon dioxide, one and a half molecules of acetic acid and measurable amounts of ethyl alcohol. Slade and Workman (158) have described the breakdown of citric acid through oxaloacetic or acetic acids to succinic or pyruvic acids with the formation of carbon dioxide. The pyruvic acid is then reduced to lactic acid or hydrolysed to acetic and formic acids, carbon dioxide and hydrogen being formed.

Slade (157) agrees with this scheme for the breakdown of citric acid. Homo-fermentative lactic acid bacteria have been shown (24) to attack citric acid with the formation of carbon dioxide. This

utilisation of citric acid can take place in the absence of carbohydrates. Campbell and Gunsalus (25) have reported that homo-fermentative lactic acid cocci which utilise citric acid produced similar fermentation products to the hetero-fermentative lactic organisms.

Demonstration of gas production

Several methods are available for demonstrating the formation of gas by starter bacteria.

Hassouna and Allen (72) reported that the starter organisms - Streptococcus lactis, Str. cremoris, Streptococcus paracitrovorus in pure culture or in combination gave no evidence of gas formation in either separated milk, separated milk with yeast extract, or in evaporated milk when a capillary tube method (3) of gas detection was employed. When absorption titration methods were used a culture of Str. diacetilactis was shown to produce considerable amounts of gas from separated milk. These workers demonstrated the difficulties associated with the detection of gas produced by certain starter bacteria when they reported that cultures of Str. paracitrovorus which did not produce gas in capillary-tube or absorption-titration methods produced sufficient gas to cause the deformation of tins of evaporated milk into which they had been inoculated.

Gibson and Abd-el-Malek (55) described a method in which they used a fortified nutrient gelatin medium in conjunction with an agar-agar seal in a test tube. They recommended this method especially for the detection of small amounts of gas which would have been undetected in liquid media due to gas passing into solution.

At a later date Sandine, Elliker and Anderson (142) described a simple apparatus for measuring the gas produced by starter bacteria. More recently, Sandine, Elliker, Wilster, Stein and Anderson (143) have used a method dependent on the formation of gas in a rennet curd prepared in a suitable container such as a beaker.

Factors affecting the utilisation of citric acid

Hydrogen ion concentration. One of the most important of these is the hydrogen ion concentration of the medium. Under alkaline conditions 1 molecule of citric acid yielded two molecules of acetic acid, 1 molecule of formic acid and carbon dioxide. In the same experiments it was found that a change of pH from an alkaline to an acid condition resulted in the production of considerable amounts of acetylmethylcarbinol.

Gibson and Abd-el-Malek (55) considered the most suitable pH for the production of carbon dioxide by citrate-fermenting organisms to be 5.5 to 6.0.

Hammer and Werkman (66) reported that the fermentation of citric acid in a broth medium by Streptococcus citrovorus and Str. paracitrovorus varied with the initial pH of the medium. At relatively high pH smaller amounts of carbon dioxide, volatile acids, acetylmethylcarbinol and diacetyl were formed than at lower pH values. The greater part of the decomposition took place when the pH of the medium was below 5.0. In addition to affecting the fermenting capacity of the bacterial cells, pH plays a part in the rate of growth of the citrate-fermenting bacteria. The optimum pH for the growth of the leuconostocs has been reported to be 5.5 in milk and 6.5 in broth (129). A pH of 5.5 was shown to promote a higher growth rate of leuconostocs than 4.4 (29). The maximum pH for growth of the leuconostocs was shown to be 7.5 in broth media.

Temperature. Temperature is a very important factor in the growth and cellular activity of most bacteria. It is no less so with the leuconostocs or other citrate-fermenting organisms. A temperature of 23°C has been described (129) as being the optimum for the leuconostocs. Gibson and Abd-el-Malek (55) studied the biochemical characteristics of citrate-fermentating homo-fermentative streptococci at the optimum temperature. In cheesemaking the temperature

varies from 30°C to 40°C that is, from the optimum to the near maximum for growth of many starter bacteria.

Pressure. Oliver (126) has reported that the amount of carbon dioxide produced in a culture of Str. diacetilactis was dependent on the proportion of carbon dioxide in the atmosphere. When cultures were grown in sealed flasks the accumulating CO_2 raised the pressure and the total CO_2 produced was much less than in those cultures in which the gas was removed as it was formed. These results suggested that synthesis of the decarboxylase was inhibited by high carbon dioxide pressures.

Variations in gas production

Gas production has been reported (312) to be a very variable property of citric acid-fermenting organisms. Sandine, et al. (142) have classified starter cultures into low, medium and high gas-producers and have used this classification as a means of selecting starter cultures for cottage cheese manufacture. It was shown by these workers that the production of excessive carbon dioxide by starter cultures rated as high in gas production resulted in a defect condition known as 'floating curd'.

Cultures of the hetero-fermentative streptococci

and Str. diacetilactis were found to give slight seasonal variations in biochemical properties (54). These variations were explained by the possibility of a seasonal change in the composition of milk during the months of December and January. The variant forms were partly or wholly unable to form volatile acids and were unable to ferment citric acid. The possibility of variation in gas production is greater with mixed-strain starters where the proportions of the component strains of the mixture may vary considerably.

In view of the possible relationship between gas production by component strains of starter cultures used in cheesemaking and the texture of cheese it was considered to be valuable to examine the effect of added citric acid on gas production in a milk medium.

I PRELIMINARY INVESTIGATION

EXPERIMENTAL

Materials and methods

Test culture. A mixed-strain starter BU 13 was selected for the trial. This culture was normal in growth and had a high acid-producing activity when examined by the Anderson and Meanwell test (5). The characteristics of the component strains of starter BU 13 are shown in table 79.

Sterile separated milk. Fresh separated milk was dispensed in 10.5 ml portions in test tubes. The test tubes were plugged with cotton wool and the milk was sterilised by steaming at 100°C for 30 minutes followed by 10 lb / sq. in. pressure for 10 minutes.

Agar-agar. Two per cent agar-agar was prepared by dissolving agar-agar powder in distilled water, dispensing in 4 ml portions in test tubes closed with rubber bacteriological seals and sterilising at 15 lb / sq. in. pressure for 20 minutes.

Sodium citrate solution. A 10 per cent (w/v) solution of tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) was prepared in distilled water. This solution was dispensed in screw-capped bottles and sterilised at 15 lb / sq. in. pressure for 20 minutes. Supples and Bellis (170) have shown that citric acid is not destroyed by such heating.

Method. Gibson and Abd-el-Malek (55) devised a medium for the demonstration of carbon dioxide formation from citrate by streptococci which consisted of nutrient gelatin supplemented with 3 per cent of a citrate-citric acid mixture, 0.25 per cent of yeast extract, 10 per cent tomato juice and 0.5 per cent glucose. In the examination of homo-fermentative bacteria the glucose was omitted.

In the investigation reported below a simple medium prepared from sterile separated milk and agar-agar was preferred since it corresponded more closely to the material used in cheesemaking. An agar curd was preferred to a rennet curd because of the tendency of the latter to shrink as acid is produced.

To six tubes of sterile separated milk were added varying amounts of tri-sodium citrate solution to increase the concentration of citric acid by 0.048, 0.240, and 0.480 per cent. After addition of the citrate the tubes were shaken to mix the contents and were then allowed to stand for 30 minutes to allow complete interaction between the citrate and milk. Each tube was then inoculated with 1 per cent of an eighteen-hour-old milk culture of starter BU 13. Two tubes of the same batch of milk were inoculated and acted as controls - the citric acid content being that naturally occurring in the milk.

A similar number of 4 ml quantities of 2 per cent agar-agar were dissolved and cooled to 48°C.

Each tube of inoculated milk was given a final mixing before being poured into the tube of molten agar-agar. After inverting once the tubes were adjusted to 22°C and incubated at this temperature for 72 hours.

After 24 hours incubation the tubes were

examined for visible signs of gas formation as shown by openings in the agar-milk curd. Table 80 below details the results of the examination.

RESULTS

After 24 hours incubation at 22°C there was a considerable formation of gas holes in the medium where the citric acid content had been increased by 0.24 per cent. Since there was no further increase in gas hole formation in the low citrate tubes following a further period of incubation it appeared that all the citrate had been utilised in the first twenty-four hour incubation period.

The preliminary investigation was repeated with culture BU 13 and with a further two mixed-strain cultures - the results being shown in table 81.

The biochemical reactions of the component strains of BU 11 and BU 12 are given below in table 82.

Incubation beyond the twenty-four period did not result in a formation of gas holes in agar-milk tubes free from gas holes at the end of the initial incubation period.

As will be seen from table 81 culture BU 12 produced gas holes at lower citric acid concentrations than either BU 11 or BU 13. This suggested a possible variation between cultures in the amounts

TABLE 79

Cultural and biochemical reactions of the organisms combined in the mixed-strain starter BU 13

Component strain	Creatine test (61)	Gas Production (55)	Precipitin ring test Group N serum (148)	Growth at 40°C (119)	Growth in a medium containing 4% NaCl (119)
HP	-	-	+	-	-
FC/55D12	-	-	+	-	-
FC/55D4	+	+	+	-	-
FR	+	+	-	+	+
AB	+	+	-	+	+

TABLE 80

Gas production by a mixed-strain starter in an agar–milk curd containing various amounts of citric acid

Tube numbers	Citric acid content of medium (% (w/w) anhydrous)	Gas hole formation Incubation period (h) 24	72
1 & 2	Base	-	-
3 & 4	Base + 0.048	-	-
5 & 6	Base + 0.240	+	+
7 & 8	Base + 0.480	+	+

TABLE 81

Gas production by mixed-strain starters in an agar-milk curd containing various amounts of citric acid

Starter	Tube number	Citric acid content of medium (% (w/w) anhydrous)	Gas hole formation Incubation period (h) 24	72
BU 11	1 and 2	Base	-	-
	3 and 4	Base + 0.048	-	-
	5 and 6	Base + 0.240	+	+
	7 and 8	Base + 0.480	+	+
BU 12	1 and 2	Base	-	-
	3 and 4	Base + 0.048	+	+
	5 and 6	Base + 0.240	+	+
	7 and 8	Base + 0.480	+	+
BU 13	1 and 2	Base	-	-
	3 and 4	Base + 0.048	-	-
	5 and 6	Base + 0.240	+	+
	7 and 8	Base + 0.480	+	+

TABLE 82

Cultural and biochemical reactions of the individual strains of two mixed-strain starter cultures

Starter	Component Strain	Creatine test (61)	Gas Production (55)	Precipitin test (Group N) (148)	Growth at 40°C in Y.D.B. (120)	Growth in Y.D.B. + 4 % NaCl (120)
BU 11	R6	-	-	+	-	-
	FD/56M/1	+	+	+	-	+
	HDV 4 A/1	+	+	+	+	+
	F F 1	+	+	+	+	+
	AB	+	+	+	+	+
BU 12	K	-	-	+	-	-
	91 4	+	+	+	+	+
	DRC 1	+	+	+	-	+
	FF 2	+	+	-	-	+
	FC/55D 4	+	+	+	+	+

of the various products of citric acid breakdown.

Following this preliminary trial a full investigation was carried out and is detailed below.

II. FULL INVESTIGATION

A. LABORATORY TESTS

EXPERIMENTAL

Materials and methods

Test cultures. The following cultures were used:

Mixed-strain starters; BU 13, R1, R2, R 33, 87, H 30 and 91.

Single-strain starters of Str. diacetilactis; FD 104, 176, 129/3, DRC 1, DRC 2, 129/5 and FD/55N/2.

Single-strain starter of Str. cremoris; HP.

Sterile separated milk. Fresh whole milk free from antibiotics was separated by passage through a separator in order to remove the fat. The skim milk so prepared was dispensed in 10.5 ml quantities in test tubes which were then plugged with non-absorbent cotton wool. The milk was sterilised in an autoclave by heating to 100°C for 30 minutes followed by 10 lb / sq. in. pressure for 10 minutes.

Sodium citrate solution. A 10 per cent (w/v) solution of tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in distilled water was prepared and after dispensing in screw-capped bottles was sterilised at 15 lb. / sq. in. pressure for 20 minutes.

Agar-agar. Two per cent (w/w) agar-agar was prepared by dissolving agar-agar powder in distilled water, dispensing the material when dissolved in 4 ml amounts in test tubes and sterilising at 15 lb / sq. in. pressure for 20 minutes.

Part 1. The effect of added sodium citrate on gas production by starter bacteria in an agar-milk curd. In order to estimate the minimum amount of added citrate required to result in visible gas production in a milk agar curd six levels of added citrate were selected.

The method was essentially the same as that described in the preliminary investigation. In order to ensure that the agar-milk curd consistency was similar in all cases, distilled water was added to the tubes to compensate for any change in volume brought about by the addition of sodium citrate solution.

The ingredients of the various tubes are shown in table 83. Incubation was carried out at 22°C for 24 and 72 hour periods. At the end of this time the tubes were examined for evidence of gas production.

After incubation, an examination of the tubes indicated a very noticeable increase in the gas hole formation with increase in citric acid content of

TABLE 83

Contents of agar-milk tubes used in gas production tests of starter bacteria

Tube No.	Milk (sterile separated) (ml)	Sodium citrate (ml)	Added citric acid (% (w/w) anhydrous)	Water (sterile distilled)	Culture (ml)
1 and 2	10	-	-	1.0	0.1
3 and 4	10	0.1	0.036	0.9	0.1
5 and 6	10	0.2	0.072	0.8	0.1
7 and 8	10	0.3	0.108	0.7	0.1
9 and 10	10	0.4	0.144	0.6	0.1
11 and 12	10	0.5	0.180	0.5	0.1
13 and 14	10	1.0	0.360	-	0.1

TABLE 84

Gas production by a mixed-strain starter in an agar-milk curd containing various amounts of citric acid

Starter	Tube number	Citric acid content of medium (% (w/w) anhydrous)	Gas hole formation Incubation period (h) 24	72
BU 13	1 and 2	Base	-	-
	3 and 4	Base + 0.036	+	+
	5 and 6	Base + 0.072	+	+
	7 and 8	Base + 0.108	+	+
	9 and 10	Base + 0.144	+	+
	11 and 12	Base + 0.180	+	+
	13 and 14	Base + 0.360	+	+

TABLE 85

Gas production by a mixed-strain starter in an agar-milk curd made with (1) sterile separated and (2) sterile whole milk and containing various amounts of citric acid

Tube number	Type of milk	Amount of added sodium citrate (% (w/w) anhydrous citric acid)	Gas production after incubation
1 and 2	Whole	Base	-
3 and 4	Separated	Base	-
5 and 6	Whole	0.036	-
7 and 8	Separated	0.036	+
9 and 10	Whole	0.180	+
11 and 12	Separated	0.180	+
13 and 14	Whole	0.360	+
15 and 16	Separated	0.360	+

the milk. At low citric acid levels the gas holes, if present, were small and independent. In the tubes in which the citric acid was increased by 0.18 and 0.36 per cent the gas formation was so intense that large fissures were formed. An increase in the citric acid content of the medium of 0.036 per cent was sufficient to cause gas hole formation in the agar-milk curd - table 84.

While sterile separated milk was used almost exclusively the effect of added sodium citrate on gas production in whole milk was examined.

Part of a portion of fresh milk was separated by passage through a separator. The skim milk produced was dispensed in 10.5 ml amounts in test tubes and sterilised for use in the method described above. The whole milk portion was similarly treated. A gas production test was carried out with additions of citric acid ranging from 0.036 to 0.360 per cent in each type of milk by the method detailed above using culture BU 13.

Examination after the 24 and 72 hour incubation periods indicated that the whole milk medium had fewer gas holes present - table 85.

Part 2. The effect of added sodium citrate on the hydrogen ion concentration of milk. Since it was expected that the addition of sodium citrate

to milk would influence the hydrogen ion concentration of the medium, a series of determinations was made to establish the amount of change in hydrogen ion concentration.

To 100 ml of sterile separated milk was added varying amounts of sterile 10 per cent (w/v) tri-sodium citrate solution described above. Distilled water was added to equalise the amount of dilution in each flask. After 30 minutes the hydrogen ion concentrations were determined potentiometrically by means of a Cambridge pH meter. A glass electrode was used in conjunction with a Calomel reference electrode. The results are given below - table 86.

Since the addition of agar-agar does not affect the pH of a medium (107) the figures given in the table 86 would represent the hydrogen ion concentration of the complete agar-milk curd described above.

The effect of added sodium citrate on hydrogen ion concentration was shown to be equal for raw whole milk and sterilised skim milk. Sodium citrate was added to split samples of raw whole milk and sterile separated milk in order to raise the citric acid content by 0.55 per cent. The results of pH determinations are given in table 87.

In order to show that the copious gas production

with added sodium citrate was due to increased citric acid content and not to the effect of pH, a portion of separated milk was split into four aliquots. To two aliquots was added sufficient sterile sodium citrate to raise the citric acid content by:

(a) 0.036 and (b) 0.180 per cent.

One aliquot of each type of milk was allowed to remain unstandardised. The hydrogen ion concentration of the second group was standardised to the pH of the untreated milk by the addition of sterile 25 per cent lactic acid.

After inoculation, duplicate 10 ml portions of each of the control and the four treated milks were pipetted into sterile test tubes containing 4 ml of molten 2 per cent agar-agar at a temperature of 48°C. After mixing, the temperatures were adjusted to 22-23°C and incubation was carried out for 24 and 72 hour periods. An examination made at this time indicated little difference in gas hole formation between the media having the pH of the original milk and the higher value resulting from addition of sodium citrate - table 87(a).

An increase of 0.036 per cent in the citric acid content of the medium was sufficient to cause a noticeable change in hydrogen ion concentration. It was shown therefore, that while differences in

TABLE 86

Hydrogen ion concentration values of sterile separated milk following the addition of various amounts of sodium citrate

Sample	Amount of added sodium citrate (% (w/w) anhydrous citric acid)	* pH
1 and 2	-	6.63
3 and 4	0.055	6.69
5 and 6	0.110	6.74
7 and 8	0.165	6.81
9 and 10	0.220	6.93
11 and 12	0.275	7.00
13 and 14	0.550	7.21

* mean of two values

TABLE 87

Changes in hydrogen ion concentration of (1) raw whole and (2) sterile separated milk following the addition of sodium citrate

Milk sample	Amount of added sodium citrate (% (w/w) anhydrous citric acid)	pH On addition	pH After 30 minutes
Raw whole milk	-	6.65	6.65
Raw whole milk	0.55	7.00	7.25
Sterile separated milk	-	6.65	6.65
Sterile separated milk	0.55	7.01	7.24

TABLE 87(a)

Gas production by a mixed-strain starter in an agar-milk curd of varying hydrogen ion concentration

Tube numbers	Citrate content of medium (% (w/w) anhydrous citric acid)	pH	Gas formation Incubation period (h) 24	72
1 and 2	Base	6.57	-	-
3 and 4	Base + 0.036	6.57	+	+
5 and 6	Base + 0.036	6.57	+	+
7 and 8	Base + 0.180	6.58	+	+
9 and 10	Base + 0.180	6.79	+	+

actual amount of carbon dioxide produced may vary with changes in pH in the order of 0.3 pH units, the dramatic effect of increased gas production resulting in the formation of numerous gas holes and fissures in the agar-milk curd was caused by the increased citric acid content and was not due to a change in the hydrogen ion concentration of the medium.

Part 3. The effect of temperature on gas production by starter bacteria in an agar-milk curd. The optimum temperature for the growth of betacocci has been given (129) as 23°C. Bergey (16) gives the optimum temperature for Leuconostoc citrovorum as 21-25°C and Leuconostoc dextranicum as 20-25°C. The optimum temperature for homo-fermentative species is 30-33°C (16). In Cheddar cheesemaking the temperature varies from 30°C at the beginning to 40°C by the middle of the manufacturing technique, and falling to 25°C at the end of manufacture. From 25°C the temperature of the cheese falls slowly until it reaches that of the air in the curing room which is maintained at 13-16°C.

Duplicate tubes were prepared for five levels of citric acid in addition to the control for three temperatures, 22, 30 and 37°C by the method described

above.

After incubation for 24 hours an examination was carried out. The results are detailed in table 88 below.

At an incubation temperature of 37°C there was a very marked increase in the gas formation compared with the lower temperatures. An increase in the citric acid content of the medium of 0.072 per cent resulted in a reaction at 37°C similar to that brought about by an increase of 0.180 per cent citric acid at incubation temperatures of 22 and 30°C. In addition the control milk tubes gave an increased amount of gas formation at 37°C compared with the lower temperatures.

A single-strain culture of Str. diacetilactis FC/55D/4 was subjected to the same test conditions and was found to behave in a similar manner. The effect of increase in temperature appeared to have the same effect as an increase in the citrate level. In the experiments with the single-strain culture of Str. diacetilactis there was no visible gas formation in the control milk at the level of 0.23 per cent citric acid and at 0.266 it was very slight, the gas holes being small and discrete when the incubation temperature was 22°C. At incubation temperatures of 30 and 37°C gas holes were formed in the agar-milk curd in considerable numbers at citric acid

TABLE 88

The effect of temperature on the production of gas holes by a mixed-strain starter in an agar-milk medium at various concentrations of citric acid

Group	Citric acid content of medium (% (w/w) anhydrous)	Temperature (°C)	Examination after 24 hours incubation
A	0.230	22	Slight gas hole formation
	0.266		Slight gas hole formation
	0.302		More numerous gas holes
	0.338		Gas holes becoming more
	0.374		numerous and larger with
	0.410		increase in citric acid content of the medium
B	0.230	30	
	0.266		
	0.302		Gas hole formation at all
	0.338		levels of citric acid was
	0.374		more intense than at 22°C
	0.410		
C	0.230	37	Gas holes
	0.266		Gas holes
	0.302)		
	0.338)		Numerous gas holes and large
	0.374)		fissures
	0.410)		

levels of 0.23 and 0.266 per cent. As was found with the mixed-strain culture BU 13, the most marked gassing occurred at an incubation temperature of 37°C.

There are three possible reasons for the apparent increase in visible gas production at higher temperatures.

Firstly, an increase in incubation temperature may cause the gas locked inside the medium to expand and result in the formation of cracks and fissures. Secondly it may be due to a more complete and rapid utilisation of the citric acid with a more rapid formation of carbon dioxide. Thirdly, higher temperatures may alter the yield of carbon dioxide produced from citric acid. Since further incubation of the low temperature tubes did not result in any increase in gas hole formation, it would appear that the breakdown of citric acid was complete after twenty-four hours. In order to prove this theory sodium citrate solution was added to sterile separated milk to give four concentrations of citric acid. One per cent of an eighteen-hour-old milk culture of the single-strain culture of Str. diacetilactis FC/55D/4, was added to each portion of milk after samples had been taken for

citric acid determination. After the inoculated milk had been mixed, incubation was carried out at 22°C for 24 hours. At the end of this time, samples of milk which was now coagulated were taken for a further determination of the citric acid content.

From table 89 it will be seen that in all cases the breakdown of citric acid was complete in twenty-four hours.

The rate of breakdown of citric acid by strains of Str. diacetilactis was determined in a further experiment. Sterile tri-sodium citrate solution was added to sterile separated milk to raise the citric acid content to approximately 0.25 per cent (w/w) anhydrous. Six tubes of the citrate-fortified milk were each inoculated with 1 per cent of the test culture. Duplicate tubes were then incubated at each of three test temperatures, 22, 30 and 37°C. Citric acid determinations by a method based on that of Babad and Shtrikman (7) were made on the original milk and on the various milks after a six-hour period of incubation. The acid-producing ability of the test cultures was determined by 'activity' tests (5) carried out at the three incubation temperatures. The detailed results of the tests are given in table 89 (a) It will be seen that while the rate of

TABLE 89

Citric acid content of various milk media on inoculation with a single-strain starter of Str. diacetilactis and after twenty-four hours incubation at 22°C

Milk Sample	Citric acid (% (w/w) anhydrous)	
	On inoculation	After incubation
1	0.163	0.002
2	0.199	0.000
3	0.271	0.002
4	0.343	0.000

TABLE 89(a)

Destruction of citric acid in a milk medium by single-strain cultures of Str. diacetilactis at various temperatures

Test culture	Incubation period (h)	Citric acid content (% (w/w) anhydrous) of milk media Incubation temp. (°C) 22	30	37
DRC 1	0	0.248	0.248	0.248
	6	0.215	0.086	0.067
Amount of citric acid destroyed (% (w/w))		0.033	0.162	0.181
* 'Activity' of culture		0.24	0.47	0.48
DRC 2	0	0.248	0.248	0.248
	6	0.228	0.219	0.217
Amount of citric acid destroyed (% (w/w))		0.020	0.029	0.031
* 'Activity' of culture		0.23	0.42	0.43
176	0	0.248	0.248	0.248
	6	0.210	0.120	0.138
Amount of citric acid destroyed (% (w/w))		0.038	0.128	0.110
* 'Activity' of culture		0.27	0.51	0.56

* Titratable acidity (per cent lactic acid) of milk inoculated with 1 per cent of culture and incubated for 6 hours

breakdown of citric acid was similar for the three cultures at 22°C there was a marked variation in the destruction of citric acid by the different test cultures at 30 and 37°C. The rate of destruction of citric acid was not related to the acid-producing activity of the culture.

The rate of breakdown of citric acid by Str. diacetilactis was much more rapid at 30 and 37°C than at 22°C.

Part 4. Variations in gas formation by different cultures at 22°C. Six mixed-strain starters were compared for ability to produce visible signs of gas formation in the agar-milk curd test described above at an incubation temperature of 22°C. One batch of milk was used in tests in order to ensure that the base material was similar for all the cultures. The materials were prepared in the manner detailed above and the method was carried out as described above. Detailed results are given in table 90.

A further period of incubation at 22°C over a twelve-day period did not markedly alter the results. Slight gas formation became noticeable with cultures, R 1 and 91 at 0.297 and 0.333 per cent citric acid. Culture R 2 gave a very slight visible gas production at 0.189 per cent citric acid.

TABLE 90

A comparison of the gas production by several mixed-strain starters in agar-milk media containing various amounts of citric acid

Citric acid content of medium (% (w/w) anhydrous)	Gas hole formation after 24 hours at 22°C					
	Starters					
	R 1	R 2	R 33	87	H30	91
0.153	-	-	-	-	-	-
0.189	-	-	S	S	-	-
0.225	-	S	M	M	-	-
0.261	-	M	M	M	-	-
0.297	-	M	M	M	-	-
0.333	-	M	M	M	-	-

S - slight gas formation
M - marked gas formation resulting in gas holes and fissures

TABLE 91

Cultural and biochemical reactions of strains of Str. diacetilcatis used in gas-production tests

Strain	Precipitin test Reaction to Group N serum (148)	Growth at 40°C (119)	Growth in presence of 4% NaCl (119)	Production of NH_3 from Arginine (124)	Creatine test (61)
FD 104	+	+	-	+	+
176	+	+	+	+	+
129/8	+	-	+	-	+
DRC 1	+	-	+	+	+
DRC 2	+	+	+	+	+
129/5	+	-	+	+	+
FD/55N/2	+	+	+	+	+

TABLE 92

A comparison of the amount of gas produced by strains of Str. diacetilactis in an agar-milk curd containing various amounts of citric acid

Citric acid content of medium (% (w/w) anhydrous)	Gas hole formation after 24 hours at 22°C						
	FD104	FD/85N/2	176	129/8	DRC 1	DRC 2	129/5
0.195	-	-	-	-	-	-	-
0.231	S	S	S	S	S	-	M
0.267	M	S	M	M	S	-	M
0.303	M	M	M	M	M	S	M
0.339	M	M	M	M	M	S	M
0.375	M	M	M	M	M	S	M
0.750	M	M	M	M	M	M	M

S - slight gas hole formation, i.e. holes few and discrete

M - very marked formation of gas holes and fissures

A similar experiment was carried out with seven single-strain cultures of Str. diacetilactis. The biochemical reactions of the cultures are given in table 91.

The detailed results of the examination following incubation are presented in table 92.

Culture DRC 2 which had biochemical reactions identical with culture 176 gave a very much smaller gas formation than any of the other six cultures. Culture 129/5 produced the most marked formation of gas holes.

Part 5. Rate of breakdown of citric acid at temperatures used in cheese manufacture. In order to examine the extent and speed of breakdown of citric acid as a possible reason for the variation in the gas-producing activity of the cultures experiments with temperature conditions similar to those occurring in cheesemaking procedure was carried out.

Sterile tri-sodium citrate solution was added to 100 ml amounts of sterile separated milk to raise the citric acid content by 0.108 per cent (w/w). After 30 minutes, 1 per cent of an eighteen-hour-old culture was added and the materials mixed. The inoculated flask was then placed in a water bath, thermostatically controlled at 30°C by means of a

contact thermometer used in conjunction with a circulating pump and heater and maintained at this temperature for two hours. The temperature was then slowly raised to 38.8°C in 30 minutes and maintained at 38.8°C for 1½ hours. At this stage the temperature was reduced, the flasks of milk being incubated at 30°C for a further two hours. Samples were then withdrawn by means of a sterile pipette and immediately submitted to citric acid determination by a method based on that of Babad and Shtrikman (7). The temperature of the milks was then lowered to 22°C and incubation was continued for 18 hours. At the end of the twenty-four hour period a further series of citric acid determinations was carried out. The results are shown in tables 93 and 94. It will be seen from the results that the speed at which citric acid is broken down is a very variable factor.

Of the five strains of Str. diacetilactis which were tested, strain DRC 1 destroyed citric acid at the most rapid rate. This culture destroyed 0.235 per cent (w/w) of citric acid in six hours. Three of the five test strains of Str. diacetilactis destroyed all the citric acid within the twenty-four hour period at temperatures equivalent to those during cheese manufacture and the initial

TABLE 93

Destruction of citric acid in a milk medium by one strain of Str. cremoris and five strains of Str. diacetilactis in 6 and 24 hours at temperatures ranging from 22 to 40°C

Culture	Strain of organism	Incubation period (h)	Citric acid content (% (w/w) anhydrous citric acid)	Reduction in citric acid content (%)
HP	Str. cremoris	-	0.380	-
		6	0.370	2.6
		24	0.338	11.1
DRC 1	Str. diacetilactis	-	0.380	-
		6	0.145	61.8
		24	0.041	86.6
DRC 2	Str. diacetilactis	-	0.380	-
		6	0.336	11.6
		24	0.157	41.3
176	Str. diacetilactis	-	0.380	-
		6	0.243	36.1
		24	0	100
129/5	Str. diacetilcatis	-	0.380	-
		6	0.296	22.1
		24	0	100
104	Str. diacetilactis	-	0.380	-
		6	0.275	27.6
		24	0	100

TABLE 94

Destruction of citric acid in a milk medium by three mixed-strain starters in 6 and 24 hours at temperatures ranging from 22 to 40°C

Culture	Incubation period (h)	Citric acid content of medium (% (w/w) anhydrous)	Reduction in citric acid content (%)
91	0	0.373	-
	6	0.351	5.9
	24	0.015	96.0
R 1	0	0.373	-
	6	0.347	6.96
	24	0.032	91.1
R 33	0	0.373	-
	6	0.310	16.9
	24	0.013	97

pressing of the cheese. Test culture HP, a single-strain starter of Str. cremoris included for comparison purposes reduced the citric acid content of the milk by eleven per cent.

The three mixed-strain test cultures were less active than the strains of Str. diacetilactis during the primary six-hour incubation period but there was a variation between the three cultures in citric acid destruction.

B. CHEESEMAKING EXPERIMENTS

EXPERIMENTAL

In view of the demonstration of increased gas production in agar-milk media, it was considered desirable to carry out cheesemaking experiments with milk to which sodium citrate had been added.

The addition of sodium citrate to milk sequesters the calcium which is present in milk to the extent of 0.15 - 0.20 per cent (43) with the result that the coagulating action of rennet is impaired or completely destroyed.

Three levels of citrate addition were selected for the cheesemaking experiments on the basis of variation in the citrate content of milk previously reported in the literature. The levels selected were 0.036, 0.072 and 0.108 expressed as per cent (w/w) anhydrous citric acid.

A preliminary experiment was carried out to establish the degree of loss of rennet action following addition of citrate and the means whereby it could be restored. A full description follows.

PRELIMINARY TO CHEESE MANUFACTURE

Procedure

Three two litre portions of whole milk, previously pasteurised to 69.4°C in an H.T.S.T. plate heat exchanger were placed in large beakers. The first portion acted as a control milk and to the second and third portions was added sodium citrate in sufficient quantity to raise the citric acid content by 0.036 and 0.108 per cent which covered the range selected. The volume of material in each beaker was standardised by the addition of distilled water.

After 30 minutes the hydrogen ion concentration of each portion was determined electrometrically and the pH of the citrated milks was standardised to that of the control untreated milk by the addition of the necessary amount of 25 per cent (v/v) lactic acid.

One and a half per cent of an eighteen-hour-old milk culture of starter BU 13 was then added to each portion of milk and after mixing, the milks were incubated at 30°C for 1 hour. This step was

similar to the 'ripening' period common to the cheesemaking process practised in Great Britain.

0.7 ml of a commercial preparation of rennet was then added to each portion of milk at a temperature of 30°C. The times taken for the first signs of coagulation were noted and are shown in table 95 below.

It was apparent at this stage that in order to make cheese successfully the normal rennet action had to be restored by the addition of calcium ions. Any addition of calcium should, however, be the minimum requirement. The addition of calcium chloride to milk in order to bring about a firm body in cheese has been practised (183) for some time where the amount of naturally occurring calcium in milk is low.

The preliminary experimental technique was repeated. After 30 minutes of the hour incubation period at 30°C had elapsed varying amounts of a solution of calcium chloride were added at the rate given below and the time taken for coagulation noted for each milk. Coagulation times are shown in tables 96 and 97.

The effect of the addition of sodium citrate was not directly proportional to the amount of

TABLE 95

The effect of the addition of sodium citrate to milk on the time taken for coagulation of the milk by a commercial preparation of rennet

Amount of added sodium citrate (%(w/w) anhydrous citric acid)	pH after addition of citrate	pH standardised	Coagulation time (min)
-	6.60	6.60	12
0.036	6.65	6.61	26
0.108	6.72	6.62	60

TABLE 96

The effect of the addition of calcium chloride to milk in restoring the rennet action impaired by the prior addition of sodium citrate

Amount of added sodium citrate (%(w/w) anhydrous citric acid)	pH after addition of citrate	pH after standardisation	Amount of added $CaCl_3$ (%(w/v))	Coagulation time (min)
-	6.72	6.72	-	12
0.108	6.90	6.76	0.05	22
0.108	6.90	6.76	0.1	5

TABLE 97

The effect of the addition of calcium chloride to milk in restoring rennet action impaired by the prior addition of sodium citrate

Amount of added citrate (% (w/w) anhydrous citric acid)	pH after standardisation	Amount of added $CaCl_3$ (% (w/v))	Coagulation time (min)
-	6.62	-	13.5
0.036	6.67	0.025	19.0
0.072	6.7	0.05	10.5
0.108	6.67	0.075	8.5

added material since proportionally increasing amounts of calcium chloride gave very divergent times for coagulation. By trial and error the amounts of calcium chloride required to produce similar coagula with varying amounts of added sodium citrate were obtained.

CHEESE MANUFACTURE

Materials and Equipment

Milk. Mixed milk which had been flash pasteurised by heating to 69.4°C in a plate heat exchanger was used in the cheesemaking experiments. The milk was tested for freedom from coliform bacteria by the method of Wilson (200).

Starters. Several mixed-strain cultures containing citrate-fermenting bacteria were used. The description of these cultures has been given elsewhere in this section and the actual culture used in each experiment detailed will be given.

Rennet. A commercial preparation of rennet was used in the cheese manufacture.

Equipment. An assembly of four stainless steel vats fitted with water jackets and mechanical paddle-type agitators was used in the cheesemaking trials - plate 5. The assembly of equipment ensured that every vat received exactly the same

mechanical treatment. The curd-whey mixture was stirred mechanically at a uniform speed. The ancillary equipment used was common to all cheese manufacture.

Procedure

Having established that the addition of sodium citrate to milk affected (a) the hydrogen ion concentration and (b) the calcium system several preliminaries were carried out on the milk before cheese manufacture was commenced. The amount of pH variation, the method of restoring the pH and the method of restoring rennet action have been described in the preliminary investigation. The procedure for making Cheddar cheese has been described elsewhere in this thesis, and it is sufficient here to detail the main points in the procedure in an actual trial.

Following a thorough mixing the pasteurised milk was distributed in four ten-gallon portions in the cheese vats which had previously been sterilised with a commercial preparation of sodium hypochlorite. The temperature of the milk was adjusted to $86^{\circ}F$.

Sodium citrate solution was added to three experimental milks in sufficient quantity to raise the citric acid content by 0.036, 0.072 and 0.108

per cent. An equivalent dilution of the control vat was made with distilled water. After thirty minutes pH determinations were carried out electrometrically and the hydrogen ion concentration of milks in the experimental vats was standardised to that of the control milk by the addition of the required amount of 25 per cent (v/v) lactic acid.

Table 98 below details the pH change on citrate addition.

The pH of the milk of vat 4 was restored to that of the control vat by the addition of 25 per cent (v/v) lactic acid at the rate of 0.01 ml per 10 ml of milk. The addition of proportionately less lactic acid restored the pH of the milks containing smaller amounts of added citrate to an equal degree. The required amount of acid was diluted in 10 ounces of water and stirred into the milk. Care was taken to avoid a sudden lowering in pH which would have resulted in local clotting if the pH fell to the iso-electric point at pH 4.6.

Following standardisation of hydrogen ion concentration, 1½ per cent of an eighteen-hour-old milk culture of starter R 2 was added, the temperature of the milk being 86°F.

Ripening of the milk at 86°F continued for thirty minutes before the required amount of the

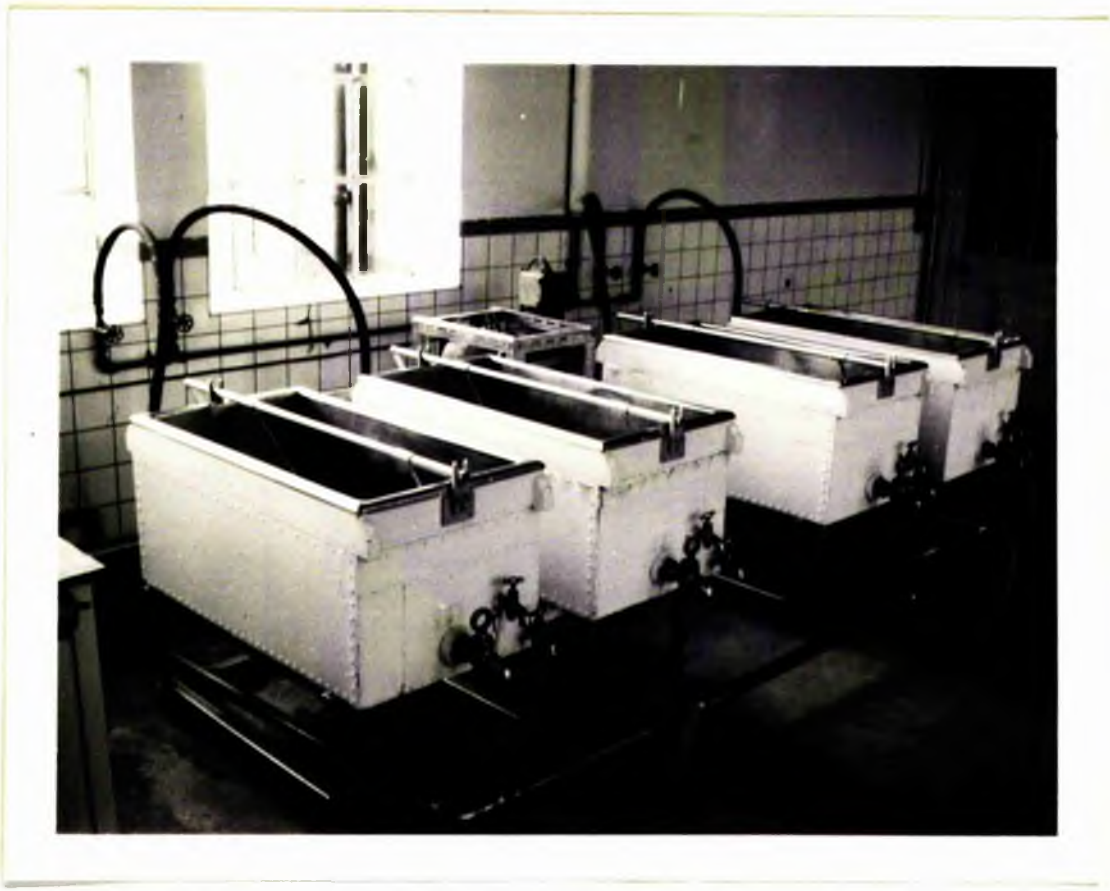


PLATE 5

The assembly of equipment used in cheesemaking experiments

calcium chloride solution was added to each experimental vat with a suitable dilution of the control milk with distilled water. Following the preliminary experiments it had been established that the required additions of calcium chloride were 0.032, 0.047 and 0.064 per cent for the vats to which sodium citrate had been added at levels of 0.036, 0.072 and 0.108 per cent (expressed as per cent (w/w) anhydrous citric acid).

The milk was ripened, following starter addition, for a total of 1 hour 52 minutes. At this stage it was decided that the acid development was satisfactory and rennet was then added at the normal rate of use - 1 ounce to 20 gallons of milk.

The first sign of coagulation was noted. The details are given below - table 99.

The various stages in the cheesemaking procedure following rennet addition are given in detail in the cheesemaking process record - table 100 and for the other test cultures in tables 101 - 104.

Particular attention was paid to ensuring that the amount of salted curd put into each cheese hoop was exactly the same. In order to ensure that each cheese received the same pressure, one cheese from each vat was placed in the same press and all four

TABLE 98

Change in hydrogen ion concentration of whole milk used for cheese manufacture on the addition of sodium citrate

Vat number	Amount of added sodium citrate (% (w/w) anhydrous citric acid)	pH
1	-	6.51
2	0.036	6.65
3	0.072	6.68
4	0.108	6.72

TABLE 99

Time taken for the first signs of coagulation to appear following the addition of a commercial rennet preparation to the milks (used for cheese manufacture) to which sodium citrate and calcium chloride had been added

Vat number	Citric acid level (% (w/w) anhydrous)	Amount of added calcium chloride (% (w/v)	Coagulation time (min)
1	Base	-	10
2	Base + 0.036	0.032	9
3	Base + 0.072	0.047	9
4	Base + 0.108	0.064	12

TABLE 100

Process record of Cheddar cheese manufacture using milks containing various amounts of citric acid

Series 1.	Milk	Whole (pasteurised to 69.4°C) - 10 gallons	Coliform bacteria - ve in 1 ml		
	Starter	Mixed-strain R 2	Amount of inoculum		1¼ per cent
	Amount of rennet	⅓ oz	Amount of salt		1½ oz
	Weight of individual cheese on hooping	4½ lb			

		Vat 1	Vat 2	Vat 3	Vat 4
Amount of added sodium citrate (%(w/w) anhydrous citric acid)		-	0.036	0.072	0.108
Starter added	x Time (h-min)	0.00	0.00	0.00	0.00
	✓ Temp. (°F)	86	86	86	86
	- Acidity (% lactic acid)	0.155	0.16	0.16	0.16
Rennet added	Time	1.52	1.52	1.52	1.52
	Temp.	86	86	86	86
	Acidity	0.175	0.18	0.185	0.19
Coagulation	Time	2.02	2.01	2.01	2.04
Curd cut	Time	2.37	2.37	2.37	2.37
	Temp.	86	86	86	86
	Acidity	0.125	0.125	0.13	0.135
Maximum scald	Time	3.42	3.42	3.42	3.42
	Temp.	103	103.5	103	102.5
	Acidity	0.14	0.14	0.15	0.15
Curd settled	Time	4.17	4.18	4.22	4.20
	Acidity	0.14	0.145	0.16	0.175
Whey run	Time	5.48	5.49	5.49	5.37
	Acidity	0.23	0.24	0.24	0.24
Curd milled	Time	7.33	7.40	7.46	7.27
Curd hooped	Temp.	82	81.5	81.5	83

x Time from the start of cheesemaking
✓ °Fahrenheit which is the temperature scale used in cheesemaking
- Titratable acidity (per cent lactic acid)

TABLE 101

Process record of Cheddar cheese manufacture using milks containing various amounts of citric acid

Series 2. Milk Whole (pasteurised to 69.4°C) - 10 gallons Coliform bacteria - ve in 1 ml
Starter Mixed-strain 129 Amount of inoculum 1¼ per cent
Amount of rennet ⅓ oz Amount of salt 1½ oz
Weight of individual cheese on hooping 4½ lb

		Vat 1	Vat 2	Vat 3	Vat 4
Amount of added sodium citrate (%(w/w) anhydrous citric acid)		-	0.036	0.072	0.108
Starter added	Time (h-min)	0.00	0.00	0.00	0.00
	Temp. (°F)	86	86	86	86
	Acidity (% lactic acid)	0.155	0.155	0.155	0.155
Rennet added	Time	1.45	1.45	1.45	1.45
	Temp.	86	86	86	86
	Acidity	0.175	0.175	0.18	0.19
Coagulation	Time	1.54	1.55	1.56	1.55
Curd cut	Time	2.30	2.30	2.30	2.30
	Temp.	85	85	85	85
	Acidity	0.125	0.13	0.14	0.14
Maximum scald	Time	3.30	3.30	3.30	3.30
	Temp.	103	103	103.5	103
	Acidity	0.14	0.145	0.15	0.155
Curd settled	Time	3.55	3.55	3.55	3.55
	Acidity	0.15	0.15	0.17	0.17
Whey run	Time	4.47	4.49	4.49	4.49
	Acidity	0.25	0.245	0.245	0.245
Curd milled	Time	6.29	6.26	6.39	6.39
Curd hooped	Temp.	81	79	78	79

x Time from the start of cheesemaking
/ °Fahrenheit which is the temperature scale used in cheesemaking
- Titratable acidity (per cent lactic acid)

TABLE 102

Process record of Cheddar cheese manufacture using milks containing various amounts of citric acid

Series 3.	Milk	Whole (pasteurised to 69.4°C) - 10 gallons	Coliform bacteria - ve in 1 ml		
	Starter	Mixed-strain R 33	Amount of inoculum		1½ per cent
	Amount of rennet	½ oz	Amount of salt		1½ oz
	Weight of individual cheese on hooping	4½ lb			

		Vat 1	Vat 2	Vat 3	Vat 4
Amount of added sodium citrate (% (w/w) anhydrous citric acid)		-	0.036	0.072	0.108
Starter added	×Time (h-min)	0.00	0.00	0.00	0.00
	≠Temp. (°F)	86	86	86	86
	-Acidity (% lactic acid)	0.155	0.155	0.155	0.155
Rennet added	Time	1.34	1.36	1.39	1.41
	Temp.	86	86	86	86
	Acidity	0.18	0.175	0.185	0.19
Coagulation	Time	1.44	1.46	1.49	1.52
Curd cut	Time	2.24	2.26	2.29	2.33
	Temp.	85.5	85	85	85.5
	Acidity	0.125	0.13	0.13	0.14
Maximum scald	Time	3.30	3.31	3.31	3.33
	Temp.	102.5	102.5	102.5	103
	Acidity	0.15	0.155	0.16	0.175
Curd settled	Time	3.55	3.56	4.02	3.53
	Acidity	0.165	0.175	0.18	0.20
Whey run	Time	4.27	4.28	4.28	4.03
	Acidity	0.275	0.27	0.29	0.225
Curd milled	Time	6.07	6.08	6.10	5.38
Curd hooped	Temp.	79	78	79	79

× Time from start of cheesemaking
≠ °Fahrenheit which is the temperature scale used in cheesemaking
- Titratable acidity (per cent lactic acid)

TABLE 103

Process record of Cheddar cheese manufacture using milks containing various amounts of citric acid

Series 4.	Milk	Whole (pasteurised to 69.4°C) - 10 gallons	Coliform bacteria - ve in 1 ml	
	Starter	Mixed-strain R 33	Amount of inoculum	1½ per cent
	Amount of rennet	½ oz	Amount of salt	1½ oz
	Weight of individual cheese on hooping	4½ lb		

		Vat 1	Vat 2	Vat 3	Vat 4
Amount of added sodium citrate (% (w/w) anhydrous citric acid)		-	0.036	0.072	0.108
Starter added	x Time (h-min)	0.00	0.00	0.00	0.00
	≠ Temp. (°F)	86	86	87	86
	- Acidity (% lactic acid)	0.14	0.14	0.14	0.14
Rennet added	Time	1.35	1.32	1.36	1.39
	Temp.	85.5	86	85.5	87
	Acidity	0.17	0.175	0.18	0.20
Coagulation	Time	1.45	1.42	1.49	1.52
Curd cut	Time	2.15	2.15	2.17	2.22
	Temp.	-	-	-	-
	Acidity	0.12	0.13	0.14	0.145
Maximum scald	Time	3.20	3.20	3.24	3.27
	Temp.	103	103	103	103
	Acidity	0.145	0.15	0.165	0.17
Curd settled	Time	3.45	3.45	3.49	3.47
	Acidity	0.155	0.165	0.18	0.20
Whey run	Time	4.25	4.18	4.21	3.57
	Acidity	0.24	0.26	0.29	0.245
Curd milled	Time	6.10	6.01	6.07	5.42
Curd hooped	Temp.	76	78	78	79

x Time from start of cheesemaking

≠ °Fahrenheit which is the temperature scale used in cheesemaking

- Titratable acidity (per cent lactic acid)

TABLE 104

Process record of Cheddar cheese manufacture using milks containing various amounts of citric acid

Series 5. Milk Whole (pasteurised to 69.4°C) - 10 gallons Coliform bacteria - ve in 1 ml
Starter Vats 1 and 2 Mixed-strain No. 129
3 and 4 Single-strain HP
Amount of rennet ½ oz Amount of Inoculum 1½ per cent
Weight of individual cheese on hooping 4½ lb Amount of salt 1½ oz

		Vat 1	Vat 2	Vat 3	Vat 4
Amount of added sodium citrate (% (w/w) anhydrous citric acid)		-	0.108	-	0.108
Starter added	x Time (h-min)	0.00	0.00	0.00	0.00
	≠ Temp. (°F)	83	85	86	85
	- Acidity (% lactic acid)	0.16	0.16	0.16	0.16
Rennet added	Time	1.40	1.40	1.40	1.42
	Temp.	86	86	86	86
	Acidity	0.18	0.19	0.17	0.19
Coagulation	Time	1.52	1.51	1.53	1.54
Curd cut	Time	2.21	2.25	2.21	2.31
Maximum scald	Time	3.23	3.31	3.26	3.41
	Temp.	103	103.5	100	100
	Acidity	0.14	0.15	0.14	0.17
Curd settled	Time	3.51	3.55	3.51	4.06
	Acidity	0.15	0.165	0.155	0.19
Whey run	Time	4.34	4.33	4.21	4.21
	Acidity	0.24	0.24	0.22	0.22
Curd milled	Time	6.19	6.19	6.16	6.18
Curd hooped	Temp.	76	77	77	75

x Time from start of cheesemaking
≠ °Fahrenheit which is the temperature scale used in cheesemaking
- Titratable acidity (per cent lactic acid)

were then pressed equally for the required time.

The cheese were cured in an air-conditioned curing room at a temperature of 12-15°C with a humidity of not less than 85°R.H.

RESULTS

Visual and organoleptic tests made on cheese three days after manufacture

Series 1. When three days old one cheese each from vats 1 and 4 were cut vertically through the centre and examined for closeness of texture and aroma by a panel of four persons where were unaware of the previous treatment. The observers were experienced in examining cheese critically for the characteristics mentioned above. Opinions made by the observers are given below - table 105.

Opinion expressed by the various observers was strongly in support of the view that the cheese made from milk with added sodium citrate was more open in texture and more full in aroma than the control cheese made from milk with normal citric acid content. The observers described the aroma as being similar to that of butter made from cream which had been 'ripened' with a butter starter.

Series 2. An examination of the type described above was carried out on a cheese from vats 1, 2, 3

TABLE 105

Visual and organoleptic tests made on cheese manufactured from milk containing various amounts of citric acid

Observer	Texture	Aroma
1	No. 7 more open than No. 2	No. 7 - higher in aroma No. 2 - more attractive
2	No. 7 greater number of small gas holes than No. 2	Flavour higher in No. 7
3	No. 7 more evidence of bacterial open texture	Flavour higher in No. 7
4	Little difference between cheese but more small openings evident in No. 7	Aroma fuller in No. 7

Cheese No. 2 from Vat No. 1 - control

Cheese No. 7 from Vat No. 4 - 0.108 per cent added citric acid

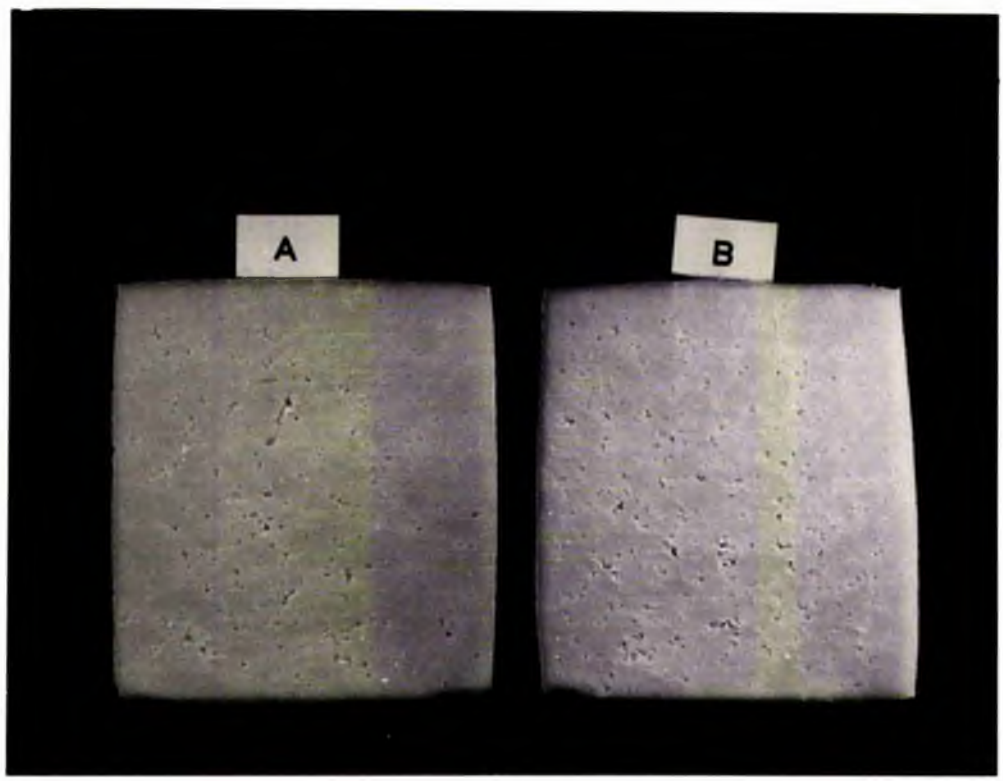


PLATE 6

The effect of increasing the citric acid content of milk used for cheesemaking. The vertical sections of cheese examined three days after manufacture are shown. Cheese A made from milk containing the natural amount of citric acid. Cheese B made from milk to which sodium citrate was added to raise the citric acid content of the milk by 0.108 per cent ((w/w) anhydrous)

and 4 in the manner described for Series 1. The general opinion of the group of observers was similar to that for series 1, i.e. a more open texture and stronger aroma was found with the cheese made from high citric acid content milk.

Series 3. A cheese from vats 1, 2 and 4 was prepared for examination and submitted to the group of observers. A progressive degree of open texture was found with increasing citric acid content. The high citrate cheese again had a more marked aroma.

Series 4. This series of cheese were similar to those of series 4. The tendency for a greater degree of open texture and higher aroma were found in the cheese made from milk with increased citric acid.

Series 5. The cheese from vats 1 and 2, made with a mixed-strain starter, again showed the tendency to more open texture in the citrated milk than the control. There was no similar tendency in the cheese made from the same milk using a single-strain culture. The control cheese from vat 3 was slightly more open in texture than that from vat 4.

Formation of aromatic compounds in cheese examined three days after manufacture. The creatine test described by Hammer (61) has been used

as a qualitative and semi-quantitative test for the detection of the formation of diacetyl and its immediate precursors in the selection of cultures suitable for use in buttermaking. The test is based on the formation of a red compound in the presence of the strong alkaline solution and a guanadine base.

A modified test was used as a means of comparing the amounts of the aromatic compounds formed in the cheese.

Method

Sampling. One core was withdrawn with a cheese sampling iron from each end of the cheese. The outer inch of the core was discarded and the remainder passed through a fine grater.

Procedure. 10 g of finely grated cheese were macerated with 25 ml of distilled water. When the consistency of the mixture was smooth and free from lumps, the paste was placed in a centrifuge tube and spun at 6,000 rev/min for 10 minutes.

10 ml of the supernatent liquid were then placed in two clean test tubes with a small quantity of creatine and 10 ml of a 40 per cent (w/w) aqueous solution of sodium hydroxide added. Rubber stoppers were placed in the test tubes which were then inverted twice in order to mix the contents.

TABLE 106

A comparison by a modified creatine test (61) of the amount of diacetyl plus acetylmethylcarbinol produced in cheese made from milk containing various amounts of citric acid

Series	Starter	Vat No.	Citric acid content of milk (% (w/w) anhydrous)	Colour formation
1	R 2 (mixed-strain)	1	Base	++
		4	Base + 0.108	+++
2	129 (mixed-strain)	1	Base	+
		2	Base + 0.036	++
		3	Base + 0.072	+++
3	R 33 (mixed-strain)	1	Base	++
		2	Base + 0.036	+++
		4	Base + 0.108	++++
4	R 33 (mixed-strain)	1	Base	++
		4	Base + 0.108	++++
5	129 (mixed-strain)	1	Base	++
		2	Base + 0.108	++++
	HP (single-strain of Str.cremoris)	3	Base	-
		4	Base + 0.108	-

After mixing, the stoppers were removed and the contents of the tubes exposed to the atmosphere for forty-five minutes at which time the depth of the red bands of colour formed near the surface were compared. Comparisons between samples tested on the same day can be made easily and accurately but tests carried out on different days cannot be compared accurately without reference to colour standards.

Results

Table 106 gives the comparisons made between the cheese. In every case the cheese made from the citrate-fortified milk had a higher flavour content than the cheese made from milk of natural citric acid content.

The test was found to provide a rapid means of distinguishing between small and large amounts of flavour compounds. The cheese made with single-strain non-aromatic starter gave no reaction in the test.

Visual examination of cheese at maturity

The duplicate cheese were maintained at a temperature of 12 - 15°C and humidity of not less than 85° R.H. for a period of 14 weeks before final examination.

At the end of the maturing period the cheese

TABLE 107

Order of desirability of flavour - taste and smell - of cheese made from milk of different citric acid content

Series	Placing	Vat number	Citric acid content of milk (% (w/w) anhydrous)
1	1st	2	Base + 0.036
	2nd	4	Base + 0.108
	3rd	1	Base
	4th	3	Base + 0.072
2	1st	1	Base
	2nd	2	Base + 0.036
	3rd	4	Base + 0.108
	4th	3	Base + 0.072
3	1st	3	Base + 0.072
	2nd	4	Base + 0.108
	3rd	1	Base
	4th	2	Base
4	1st	2	Base + 0.036
	2nd	3	Base + 0.072
	3rd	1	Base
	4th	4	Base + 0.108
5	1st	2	Base + 0.108
	2nd	1	Base
	* 3rd	3	Base
	* 4th	4	Base + 0.108

* Starter HP; Str. cremoris

were cut to give a vertical section through the centre of the cheese.

Series 1. The progressive degree of open texture associated with increase in citric acid found in the examination of young cheese was evident at this time although the open texture was in the form of large fissures and not small holes and fissures.

Series 2, 3, 4, 5. There was no significant difference between the group cheese in the series. The small holes found at an early examination were absent, and while some cheese had a slightly open texture, the occurrence was haphazard and appeared to be unrelated to the experimental details previously described.

Organoleptic tests on cheese at maturity

In four out of the five series, cheese made from milk to which sodium citrate had been added to raise the citric acid content by 0.036 per cent were judged to be superior to the others in flavour and aroma. The cheese made from milk containing only the natural content of citric acid was placed first in this examination in only one series - table 107.

Compositional analyses of cheese at maturity

Methods

Sampling of cheese for fat, moisture and

hydrogen ion concentration. Four cores were removed from each cheese by means of a sampling iron, two being taken from each end. The outer inch of the cores was discarded, the remainder being passed through a cheese grater. The grated cheese was thoroughly mixed and placed in an airtight container until tested.

Sampling of cheese for flavour estimation by distillation. 300 g from the cheese centre were grated and placed in an airtight container until the test was made.

Estimation of fat. The amount of fat present was estimated by the Gerber method described in B.S. 696: Part 2: 1955 (19).

Estimation of moisture. The method of Richmond was used (43).

Hydrogen ion concentration. Method (a) of B.S.770: 1952 (18) was used. A glass electrode was used in conjunction with a calomel reference electrode.

Estimation of diacetyl and acetylmethylcarbinol. A steam distillation method was used (94).

Results

Fat and Moisture. The results obtained indicated that there were no major differences between the cheese in each group in the amount of

fat and moisture present. The detailed values are shown in table 108.

Hydrogen ion concentration. Determinations made on the mature cheese confirmed the estimates of titratable acidity during the cheesemaking process. All cheese developed the normal amount of acidity. An examination of table 108 shows that the pH values of the cheese in series 3 and 4 made with the same starter and different milks were similar.

Flavour. The chemical estimations of diacetyl plus acetylmethycarbinol confirmed the semi-quantitative test used on young cheese. The results presented in table 108 show that there was a marked increase in flavour compounds with increase in the amount of citric-acid present in the milk.

It will also be seen that there was a marked variation between the starters in the amount of flavour compounds produced. Starter 129 produced lower values than either of the other mixed-strain starters. The values obtained from the cheese of series 3 and 4 made on different days show good replication. Starter HP, a single-strain culture of Str. cremoris produced only small amounts of flavour compounds.

TABLE 108

Analyses at maturity of cheese made from milk containing various amounts of citric acid

Series	Starter	Vat number	Citric acid content of milk (% (w/w) anhydrous)	Fat (%)	Moisture (%)	pH	Flavour compounds (mg per 300 g of sample)	Moisture in fat-free substances (%)
1	R 2	1	Base	34.2	33.60	5.11	30.4	51.13
		2	Base + 0.036	34.3	34.13	5.05	34.3	51.95
		3	Base + 0.072	35.0	33.59	5.02	41.4	51.68
		4	Base + 0.108	34.2	33.86	5.10	49.0	51.46
2	129	1	Base	34.0	33.93	5.05	13.6	51.41
		2	Base + 0.036	34.2	33.50	5.02	20.5	50.91
		3	Base + 0.072	33.2	34.87	4.96	23.0	52.20
		4	Base + 0.108	34.1	34.92	4.98	24.0	52.91
3	R 33	1	Base	33.4	32.88	4.84	22.9	49.38
		2	Base + 0.036	32.5	34.02	4.92	28.8	50.43
		3	Base + 0.072	32.5	34.66	4.87	37.9	51.36
		4	Base + 0.108	32.3	33.99	4.83	44.8	50.21
4	R 33	1	Base	31.3	35.42	4.90	21.1	51.55
		2	Base + 0.036	31.4	35.96	4.90	34.7	52.43
		3	Base + 0.072	33.1	34.32	4.90	38.5	51.31
		4	Base + 0.108	31.8	35.26	4.92	46.5	51.69
5	129	1	Base	32.4	34.36	5.02	8.2	50.84
		2	Base + 0.108	31.4	36.01	4.98	33.3	52.49
	HP	3	Base	32.6	33.91	4.93	2.6	50.29
		4	Base + 0.108	32.3	33.88	4.90	4.4	50.03

DISCUSSION

Less is known about *Str. diacetilactis* - the third member of the Group N streptococci - than either of the other two, *Str. lactis* and *Str. cremoris*. This is perhaps not surprising since this organism was first described and named by Matuszewski, *et al.* (113) as recently as 1936. That it belongs to serological group N of Mattick and Shattock (110) has been conclusively proved (172,17). Its position within the group, however, is less well defined. Bergey (16) considers it to be synonomous with *Str. cremoris*. Swartling (172) did not accept this opinion and presented several good reasons why it should be considered a distinct species. Many of its biochemical reactions are intermediate between *Str. cremoris* and *Str. lactis*. By its ability to produce diacetyl from citrate it is distinguished from the other two homo-fermentative streptococci. *Str. diacetilactis* is a common constituent of starter cultures used for cheese manufacture (33). The author has considered the effect of added citrate on the gas-producing activity of *Str. diacetilactis* and has shown that the formation of gas in a semi-solid agar curd is

a characteristic of the strain of the organism. The formation of gas holes in a semi-solid material such as cheese or the agar-milk curd used by the author in the experimental work described above is dependent on the volume of gas produced being in excess of that which may be taken up in solution by moisture present in these materials. The naturally occurring citric acid - present in milk to some 0.15 - 0.20 per cent (43) - seldom appeared to be sufficient to result in the formation of gas holes in an agar-milk curd inoculated with cultures of Str. diacetilactis or mixed-strain starters containing this organism and / or strains of the genus Leuconostoc.

Gibson and Abd-el-Malek (55) suggested a nutrient gelatin medium for use in detecting gas production by streptococci. This method was not specifically designed for the detection of gas formed by starter bacteria. A second method for detecting gas production by starter bacteria was described by Czulak (33) and involved the use of pasteurised milk and rennet. The fact that the basic materials used in this test were not sterile must detract somewhat from its value. The method adopted by the author and described above involving

the use of sterile separated milk as the basic material, a supplement of sterile sodium citrate solution - the material necessary for the formation of carbon dioxide - and solidification by agar-agar appears to offer a simple but effective means of demonstrating gas production by starter bacteria.

Gas production by strains of Str. diacetilactis and mixed-strain starters takes place at 22, 30 and 37°C. At the higher temperatures gas-hole formation was found to be much more intense than at 22°C and resulted in an effect similar to that produced by high concentrations of citric acid.

Variation in the formation of gas holes in an agar-milk curd at different concentrations of citric acid is apparently a characteristic of the strain of culture unrelated to its acid-producing ability. That variations exist in the rate of breakdown of citric acid by strains of Str. diacetilactis is in agreement with the variations in other characteristics of this organism apparent in results reported by Swartling (172).

Practical cheesemakers have expressed the opinion that the incidence of open texture in cheese of the Cheddar variety is more pronounced at some seasons of the year than at others. In particular, the summer months appear to promote open texture to

the extent that cheese may be downgraded because of the undesirable nature of the condition.

Sherwood (154) carried out considerable research on the cause of 'slit-openness' in cheese, and established that certain strains of betacocci were capable of producing the carbon dioxide responsible for gas hole formation. Cheese affected by this condition evolved much more carbon dioxide than normal cheese free from the condition. The production of gas appeared to be favoured by higher than normal hydrogen ion concentration in the cheese. Hansen et al. (68) who prepared cheese with large quantities of betacocci found that the cheese were extremely open in texture. It has thus been established by the work quoted above and by other workers (192), that gas production by organisms able to utilise citrates and present in mixed-strain starters, may result in open texture in cheese. Not all carbon dioxide formed inside cheese forms gas holes. Part of the carbon dioxide is evolved through the rind; logically the greatest transference is from the area of cheese immediately within the rind. Schulz (144), established that the moisture in cheese was able to hold approximately 18 per cent of the total amount of carbon dioxide formed in cheese by fermentation, the remaining 82 per cent

either escaping through the rind, or finding its way into the mechanical openings in the cheese by means of the water phase. An accumulation of carbon dioxide at these positions in the cheese resulted in the formation of gas holes, the distribution of the gas holes being controlled by the number of cavities initially present in the cheese. Gas hole formation in Herrgärd cheese has been shown by Swartling and Lindgren (173), to be due to the breakdown of citric acid by starter cultures containing Str. diacetilactis, Leuconostoc citrovorum and Leuconostoc dextranicum.

The shape of the gas holes formed in cheese has been studied by van Beynum and Pette (178) who reported that the shape of the hole was largely dependent on the hydrogen ion concentration of the cheese. At high pH conditions the gas holes which formed were round in appearance, whereas a low pH condition favoured the formation of holes of slit appearance. An open-textured condition in which the gas formation resulted in large fissures in the cheese, was traced to the presence of Str. citrovorus.

The cheesemaking and cultural experiments described above, have shown that the citric acid content of the milk used in cheese manufacture is a possible source of variation in gas production in

cheese made with mixed-strain starters, or where contamination with citrate-fermenting bacteria takes place.

Richmond (43), states that the citric acid content of milk varies between 0.15 and 0.20 per cent. A review of the literature dealing with citric acid in milk established that there are considerable variations in the amount of this material present.

Supples and Bellis (170), reached the conclusion that there was a marked variation in the citric acid content of the milk from individual animals which was due to the individuality of the animal. The figures given in table 109 illustrate the greatest reported variations in the citric acid content of milk from cows of the same breed, at the same stage of lactation and being fed on similar rations.

Overby (130), attributed the superiority of the milk from Jersey cows in flavour production by butter cultures to the relatively high citric acid content of the milk. Fabris (47), on the other hand, was unable to establish any difference between breeds when comparing the citric acid content of milk of cows of the Ftiesian and Brown Swiss breeds. Allen (2), however, believed that many of the figures purporting to show variations in the citric

TABLE 109

Reported variation in the citric acid content of milk from individual cows

Investigators	Range	Citric acid content (% anhydrous)
Supplee and Bellis (170)	Low	0.121
	High	0.182
Rice and Markley (136)	Low	0.116
	High	0.199
Storgårds (168)	Low	0.122
	High	0.258
Holm, Webb and Deysher (77)	Low	*0.238
	High	*0.265

* average figures over one lactation

acid content of milks were due to the use of unreliable methods.

Variations in the citric acid content of samples of bulked milk have been commented on by various workers.

Sherwood and Hammer (155), concluded that there was no significant seasonal variation although the values of 335 determinations ranged from 0.07 to 0.33 per cent. The same conclusion was reached by Arup (6), who analysed samples of retail milk. On the other hand, Heinemann (74), who examined samples of separated milk powders produced over a season concluded that there was a small but definite seasonal variation in the citric acid content of the samples. A further report at the same time (13), indicated considerable seasonal variation; milk produced in the winter month of November had the lowest amount of citric acid. Eilers and Jense (45), again stressed the possibility of seasonal variations in citric acid content of milk in 1945. Holwerda (78), examined samples of bulked skim milk received at a number of dairy factories in Friesland over a twelve month period, and found the lowest values during the period, November to January, at 0.138 - 0.130 per cent. The values rose until a maximum was reached in

April (0.173 per cent), and thereafter the level remained high from May to October.

That a considerable seasonal variation occurs in milk, has been further established by Nickerson (122,123), working in California.

There appears therefore, to be considerable evidence in support of the existence of a marked variation in citric acid content of bulk milk supplies. This being so the amount of gas production in cheese and secondly, the amount of aromatic compounds formed will therefore vary considerably. It is not claimed by the author that this is the only reason for apparent differences in gas-producing activity of starter cultures in cheese manufacture.

In addition to the considerations of acid production by starter cultures, the breakdown of citric acid must be further considered. It has been shown that the rate of breakdown of citric acid is a variable factor in starter bacteria. The method described above, in which the amounts of residual citric acid - present in milk during growth of strains of Str. diacetilactis and mixed-strain starters - was determined after six and twenty-four hours at temperatures within the range experienced in cheesemaking offers a useful means of comparing the rate of breakdown of citrate by different

strains of starter bacteria. Determinations made by this method indicated that amounts of citric acid greater than those occurring naturally in milk are destroyed within the twenty-four hour test period, one strain being able to destroy 0.213 per cent of citric acid within the primary six-hour period - the normal duration of the cheesemaking process.

In the opinion of the author, therefore, a culture which utilises the citric acid completely within the manufacturing period and before the cheese is pressed will produce a cheese of closer texture than that made with a culture which is unable to utilise the major part of the citric acid until after the cheese has been placed in the mould and sealed by the application of pressure in such a way as to prevent the carbon dioxide being liberated freely into the atmosphere.

Gas hole formation in Dutch cheese has been associated (15) with delayed gas production and a recent report (137) of work carried out in New Zealand concludes that the open texture condition commonly found with mixed-strain starters was most severe when the gas was formed immediately after manufacture.

The addition of citric acid to milk inoculated

with citrate-fermenting starter bacteria did not always bring about the expected increase in gas hole formation in the agar-milk curd. It would appear therefore that attention must be given to the amounts of the various products of citrate breakdown by starter bacteria.

In addition to gas production, flavour-producing activity of citrate-fermenting bacteria must be further considered. van Niel, Kluyver and Derx (179) have reported that diacetyl was the substance responsible for the characteristic flavour of butter made from cream 'ripened' by starter. The role of diacetyl in the flavour of cheese has never been satisfactorily established. When cheese is examined during the curing period in order to determine its quality the 'grader' relies to a large extent on the smell which he finds on the freshly drawn cheese core. There is little doubt that cheese made with mixed-strain starters which produce diacetyl from citric acid have a much more pronounced aroma than cheese made with single-strain starters which do not form diacetyl. It is obvious therefore that the presence or absence of flavour compounds such as diacetyl must influence the grader's assessment of the cheese.

During a trial conducted by the author of non-aroma, non-gas-producing single-strain starters in

a commercial cheese factory, the opportunity was presented of comparing cheese made using the above type of culture, with those made by means of mixed-strain cultures containing citrate-fermenting organisms. The flavour scores of the two types of cheese made on the same day in the factory and examined by five persons is given in table 110. Twelve cheese of each type were examined.

The cheese which were critically examined by five observers unaware of the previous treatment had all been judged to be in grade 1 when examined by the official grader and were free from off-flavours and major defects. It is emphasised here that there is great difficulty in assessing the results of organoleptic tests such as those for aroma and flavour. In the above mentioned examination the values given by the observers, who were experienced judges of cheese differed markedly for the same cheese. In one particular case the score points awarded varied from 50 points - the maximum - awarded by observers A and B, to 45 points by C, 47 points by D and finally to 48 points by E.

It will, however, be seen from table 110 that the flavour score for both groups of cheese is higher for those made with the mixed-strain starters.

TABLE 110

A comparison of the points awarded for flavour - taste and smell - to cheese made with single- and mixed-strain starters at a commercial cheese factory

Age of cheese (weeks)	Type of starter	Flavour score* (possible 50)
12	Single-strain	47.0
	Mixed-strain	47.6
16	Single-strain	47.2
	Mixed-strain	47.8

* mean of five observations

When the cheese made in series 6 of the cheesemaking experiments described above were examined, those made with the single-strain non-aroma- producing starter HP were distinctly lacking in aroma.

Calbert and Price (22) has reported that small amounts of diacetyl appear to be necessary for the formation of the typical flavour and aroma of Cheddar cheese.

It is probable therefore, that the citrate-utilising activity of certain starter bacteria is a very vital one in the formation of the desirable cheese flavour - taste and aroma.

Since it has been shown that a large proportion of the citric acid content of the cheese milk is destroyed during the manufacturing period, the seasonal difference in atmospheric temperature is unlikely to be a major factor in any seasonal increase in the open texture defect in cheese. Most cheese factories ensure that cheese are cured at temperatures between 12 and 15°C. Failure to maintain this low temperature would undoubtedly tend to increase the danger of excess gas hole formation because of the expansion of the gas held in the cheese as the temperature of the cheese and gas increased.

It is of interest to speculate how the citric acid content of milk has been affected by the increase in hygienic quality of milk in recent years.

The presence in a milk supply of large numbers of contaminant bacteria - many of them able to utilise citrate, would result in the citrate being rapidly destroyed, provided temperature conditions were right for growth and cellular activity. Since the beginning of the present century the increased use of refrigeration to cool milk to a temperature at which bacterial multiplication is slow if at all, has combined with a greater emphasis on the methods of clean milk production to produce a milk supply containing few contaminant organisms.

Present day cheese is therefore made from milk, hygienically handled, efficiently cooled on the farm and delivered to the factory within a few hours of its production. Addition of the practice of early pasteurisation on its arrival at the factory ensures that the citric acid content of the milk is largely intact. This being so it is available for conversion into various products including diacetyl and carbon dioxide if the citrate-utilising bacteria gain access to the milk either by intent or accident.

Cultures of Str. diacetilactis are common

components of mixed-strain starters used extensively for cheesemaking in Great Britain. So far little attention has been given to this organism in its effect on cheese. It may well be that the various products of its cellular activity are now of greater importance than formerly due to recent modifications in cheesemaking techniques. Changes in cheesemaking methods introduced in recent years have been concerned with a shortening of the time required for the process. Since the early years of the century the time taken for cheese manufacture has been reduced from 7½ hours to 6¼ hours - the modern Scottish creamery practice - and recently to the 3 hours 20 minutes of the so-called 'short-time' method of Czulak et al. (36). This curtailment of the process has coincided with the introduction of new techniques in treating the cheese during the hooping, pressing and curing stages. These developments, in the opinion of the author, have resulted in the rapid formation of a surface seal thereby creating a barrier against the free escape of gas formed within the cheese. It may well be that the cause of the marked open texture common to much of the cheese made by the 'short-time' method of Czulak et al. (36) is due to the much reduced time available for destruction of citric acid by the starter bacteria before hooping of the curd

takes place.

The bacteria selected for use in cheesemaking are isolated from milk or commercial starters. Following isolation and purification, a long series of tests is undertaken before the cultures are considered suitable for use in the cheese vat. This series of tests begins with the rejection of strains which grow slowly in milk at 22°C and continues with an evaluation of the acid-producing ability of the cultures at the temperatures employed in cheese manufacture. Having established that the rate of acid production of the isolates is suitable, tests for the production of inhibitory substances (80) and compatibility (34) are undertaken to ensure that the isolates may be used with other starter cultures. Selected cultures are fully typed by the recognised cultural and biochemical reactions (16,120). The final stages of culture selection is based upon the establishment of a phage relationship pattern (119,195).

Many of these tests have been used with Str. cremoris and Str. lactis. The importance of Str. diacetilactis as a starter organism used in cheese manufacture in Great Britain cannot be too strongly emphasised and in the opinion of the author tests to determine the citrate-utilising activity

of strains of this organism should be made part of the accepted procedure for the selection of bacteria suitable for use as cheese starters.

SUMMARY

Attention is drawn to the significance of the presence in mixed-strain starters of organisms capable of utilising citric acid.

The effect of increasing the citric acid content of an agar-milk medium was investigated. The intensity of gas-hole formation was greater with increase in the citric acid concentration from the base level natural to milk. Increase in the citric acid content of 0.18 and 0.36 per cent produced similar results.

Gas hole formation was complete within a twenty-four incubation period at 22°C, determinations of residual citric acid made at the end of the incubation indicating that the citric acid had been destroyed in this time.

Variations in the extent of gas-hole formation by different cultures was demonstrated.

Gas-hole formation by a mixed-strain starter and a strain of Str. diacetilactis was more marked at 37°C than at 22 or 30°C. High temperature resulted in gas-hole formation similar to that produced at 22°C by increasing the citric acid

content of the medium.

Determinations of the residual citric acid during the growth of starter bacteria in milk indicated that breakdown of citric acid was greater at 30 and 37°C than at 22°C.

An agar-milk medium fortified with 0.5 per cent sodium citrate is suggested as a useful material for detecting gas-forming organisms in starters.

The rate at which single-strain cultures of Str. diacetilactis and mixed-strain starters utilised citric acid at temperatures experienced during cheese manufacture was determined. A marked variation in the speed of citric acid breakdown was observed. The most rapid citrate-utilising organism was able to destroy the normal citric acid content of milk within the six-hour test period at temperatures ranging from 30 to 38.8°C.

The effect of increasing the citric acid content of milk used for cheese manufacture was investigated. Preliminary investigations concerned the effect of added sodium citrate on the hydrogen ion concentration of milk and on the coagulation by rennet. The addition of sodium citrate increased the hydrogen ion concentration of milk and seriously impaired rennet action. The hydrogen ion concentration of

citrated milk could be satisfactorily restored by the addition of lactic acid while the rennet action was repaired by the addition of calcium chloride.

Cheese made with mixed-strain starters containing citrate-utilising bacteria from milk to which sodium citrate had been added had considerably more gas-hole formation than those made from milk containing only the natural content of citric acid. The aroma of the cheese made from the high citric acid milk was much greater than that of the cheese made from normal milk.

The author discusses the possibility of seasonal variations in the open-texture defect in cheese being linked with variations in the amount of citric acid present in milk and advocates the use of a test to determine the citrate-utilising ability of starter bacteria in the selection of suitable cultures for cheesemaking.

SECTION NINE

THESIS SUMMARY

THESIS SUMMARY

The object of the work described in this thesis was to investigate some of the many factors affecting the activity of the lactic acid-producing streptococci used in cheesemaking. Original work is presented in three sections, two dealing with factors affecting the acid-producing activity and the third, the citrate-utilising activity of bacteria used in cheese manufacture.

Section 1 describes the history of cheesemaking with particular reference to the Cheddar and Dunlop varieties of cheese in relation to the use of preparations of bacteria capable of forming lactic acid from lactose. Changes in the knowledge, scale and technique of cheese manufacture are commented on and emphasis is placed on the very decisive role of the acid-forming bacteria in modern cheese manufacture following the elimination of the natural acid-producing flora of the milk through increased hygienic production of milk and the pasteurisation of milk in the factory. The development of methods for the control of starter cultures is detailed.

Section 2 deals with the several functions performed by starters - preparations of lactic acid bacteria - in cheesemaking.

Section 3 relates to the basic fermentation required in cheese manufacture - that of lactose to lactic acid.

Section 4 reviews the basis of and methods available for determining the acid-producing ability of starter cultures.

Section 5 deals with the bacteriological aspects of the bacteria used in cheese manufacture.

Section 6 deals with the effect of the conditions of propagation of starter cultures on their subsequent acid-producing ability. An investigation into the most suitable temperature for the daily transfer of starters was made. It was clearly demonstrated that of four temperatures tested viz. 20, 22, 30 and 37°C, 22°C was the most suitable for the production of active acid-producing cultures. Work carried out by previous workers dealing with this subject had been with cultures containing more than one strain. The investigation carried out by the author involved the use of single-strain cultures of the bacteria used either singly or in combination for cheese manufacture. Reaction to propagation temperature was shown to be a

characteristic firstly of species and secondly of strain. The possibility of differentiating Streptococcus cremoris and Streptococcus lactis by their reaction to high propagation temperature is discussed. The effect on the acid-producing ability of starters of refrigerating the cultures before use is described. Starter cultures maintained at 4°C for up to forty-eight hours after maturity suffered no damage in acid-producing ability.

The period of logarithmic cellular activity of starter cultures was determined. The acid-producing ability of cultures used in their logarithmic phase of growth was compared with mature stationary phase cultures in laboratory tests and cheese manufacture. Logarithmic phase cultures were found to possess acid-producing abilities at least equal to those of the conventional stationary phase cultures. The value of logarithmic phase cultures to the cheesemaker is discussed with particular reference to the short time required for production of a large amount of starter culture of this type. The significance of the titratable acidity of the logarithmic phase culture as an indication of its potential acid-producing properties are considered.

Section 7 describes the effect of various biological materials on starter bacteria. Bacillus

subtilis was shown to stimulate growth of and acid production by starter bacteria through the formation of a heat-stable growth factor in yeast-dextrose-broth. Addition of yeast-dextrose-broth to milk stimulated acid production by lactic acid streptococci. The stimulatory ingredients of the broth were shown to be peptone and yeastrel - a proprietary form of yeast extract. Addition of peptone to milk reduced the time taken for coagulation of the medium by starter bacteria. The stimulatory effect of peptone, shown to be of a temporary nature, was demonstrated with cultures removed from a solid medium and others regularly transferred in milk. A wide range of test cultures of varying acid-producing ability was tested.

That milk possesses growth factors for the lactic streptococci was shown by the stimulatory effect demonstrated when peptonised milk was added to a growth medium inoculated with starter bacteria. Acid production in cheese manufacture using milk supplemented with peptone was found to be much more rapid than in normal milk.

Section 8 deals with the citrate-utilising activity of certain starter bacteria, with particular reference to the homo-fermentative organism *Streptococcus diacetilactis*. Gas production by this

organism was demonstrated by a simple test involving the use of an agar-milk curd supplemented with sodium citrate. Variations in gas formation by cultures of Str. diacetilactis and mixed-strain starters containing this organism were noted. The citrate concentration normally present in milk was destroyed by a strain of Str. diacetilactis within a twenty-four test period. Strains of Str. diacetilactis and mixed-strain starters were found to vary in the rate of breakdown of citric acid. Variation in the rate of breakdown of citric acid was a strain characteristic distinct from acid-producing activity. Cheese made from milk to which various amounts of sodium citrate were added proved that natural variations in the citric acid content of milk could result in a noticeable increase in the gas-hole formation in cheese when starters containing citrate-fermenting organisms were used. The suggestions is made that citrate-utilising activity should be considered in the selection of starters for use in cheese manufacture.

Section 10 details published and unpublished work referred to by the author during the preparation of this thesis.

SECTION TEN

REFERENCES

REFERENCES

1 Aiton, W. 1811 General view of the Agriculture of the County of Ayr. Glasgow: A. Napier.

2 Allen, L.A. 1931 J. Dairy Res., 3, 1.

3 Allen, L.A. 1939 J. Dairy Res., 10, 1.

4 Anderegg, L.T. and Hammer, B.W. 1929 J. Dairy Sci., 12, 114.

5 Anderson, E.B. and Meanwell, L.J. 1942 J. Dairy Res., 13, 58.

6 Arup, P.S. 1938 Analyst, 63, 635.

7 Babad, J. and Shtrikman, N. 1951 J. Dairy Res., 18, 72.

8 Babel, F.J. 1946 J. Dairy Sci., 29, 589.

9 Babel, F.J. 1957 J. Dairy Sci., 40, 604.

10 Babel, F.J. 1952 Purdue Agr. exp. Sta., Bull. 573.

11 Babel, F.J. 1955 J. Dairy Sci., 38, 705.

12 Baker, J.C., Brew, J.D. and Conn, H.J. 1919 N.Y.(Geneva) Agr. exp. Sta., Tech. Bull. 74.

13 Bataafsche Petroleum Co., Amsterdam. 1942-43 Versl.algem. ned. Zuivelb., 's Gav., 1942-1943. (Dairy Sci. Abstr., 7, 204, (1945-46)).

14 Beijerinck, M.W. 1912 Folia Microbiologica, Delft, 377. (Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology, 6th Edn., London: Bailliere, Tindall and Cox.)

15 Belousov, A. 1957 Mol Prom., 18, 9. (Dairy Sci. Abstr., 19, 626,(1957-58)).

16 Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology, 6th Edn., London: Baillière, Tindall and Cox.

17 Briggs, C.A.E. 1952 J. Dairy Res., 19, 167.

18 British Standards Institution, 1952 B.S. 770: 1952.

19 British Standards Institution, 1955 B.S. 696: Parts 1 and 2: 1955.

20 Buchanan, R.E., St. John-Brooks, R. and Breed, R.S. 1949 J. gen. Microbiol., 3, 444.

21 Burdett, O. 1935 A little book of Cheese. London: Gerald Howe Ltd.

22 Calbert, H.E. and Price, W.V. 1948 J. Dairy Sci., 31, 713.

23 Campbell, J.R. 1898 Trans. Highd. Agric. Soc. Scot., 10.

24 Campbell, J.J.R. and Gunsalus, I.C. 1942 J. Bact., 44, 721, (Dairy Sci. Abstr., 5, 30 (1943-44)).

25 Campbell, J.J.R. and Gunsalus, I.C. 1944 J. Bact., 48, 71.

26 Chu, E.J.-H. and Williams, R.J. 1944 J. biol. Chem., 155, 9.

27 Collins, E.B. and Nelson, F.E. 1949 J. Dairy Sci., 32, 652.

28 Conn, H.W. 1889-1900 Conn. Agr. exp. Sta., Ann. Rpts. (Knudsen, S. 1931 J. Dairy Res., 2, 137).

29 Cox, G.A. 1945 J. Dairy Res., 14, 28.

30 Cox, G.A. and Whitehead, H.R. 1931 J. Dairy Res., 2, 164.

31 Crawford, R.J.M. 1956 Dairy Ind., 21, 534.

32 Crawford, R.J.M., Galloway, J.H., Smillie, D.M., Crawley, J. and Reiter, B. 1959 Dairy Ind., 24, 183.

33 Czulak, J. 1953 Aust. J. appl. Sci., 4, 462.

34 Czulak, J. and Hammond, L.A. 1954 Aust. J. Dairy Tech., 9, 15.

35 Czulak, J. and Hammond, L.A. 1956 Aust. Dairy Rev., 24, 11.

36 Czulak, J., Hammond, L.A. and Meharry, H.J. 1954 Aust. Dairy Rev., 22, 18.

37 Czulak, J. and Keogh, B. 1957 Aust. J. Dairy Tech., 12, 54.

38 Czulak, J. and Meanwell, L.J. 1951 Proc. Soc. appl. Bact., 14, 1.

39 Czulak, J. and Naylor, J. 1956 J. Dairy Res., 23, 120.

40 Dahlberg, A.C. and Ferris, F. 1945 J. Dairy Sci., 28, 771.

41 Dahlberg, A.C. and Kosikowsky, F.V. 1947 J. Dairy Sci., 30, 165.

42 Dahlberg, A.C. and Kosikowsky, F.V. 1948 J. Dairy Sci., 31, 305.

43 Davis, J.G. and Macdonald, F.J. 1952 Richmond's Dairy Chemistry. 5th Edn., London: Charles Griffin & Co. Ltd.

44 Decker, J.W. 1905 Cheese Making. Columbus, Ohio: Berlin Printing Company.

45 Eilers, H. and Jense, W.F. 1945 Versl. landb. Onderzoek., Minist. Landb.'s Grav., 50(15)G, 1081. (Dairy Sci. Abstr., 9, 143.(1947-48)).

46 Evans, A. 1918 J. Agr. Res., 13, 235.

47 Fabris, A. 1951 Mondo Latte 1951, 598. (Dairy Sci. Abstr., 14, 473. (1952)).

48 Feagan, J.T. 1956 Aust. J. Dairy Tech., 11, 149.

49 Fleischman, W. 1896 The Book of the Dairy. London: Blackie and Son.

50 Fream, W. 1892 Elements of Agriculture. 4th Edn., London: Murray.

51 Frobisher, M. Jr. 1947 Fundamentals of Bacteriology. 3rd Edn., Philadelphia and London: W.B. Saunders Company.

52 Fussell, G.E. 1955 Dairy Engng., 72, 53.

53 Garvie, E.I. and Mabbitt, L.A. 1956 J. Dairy Res., 23, 305.

54 Gibshman, M.R. 1948 Mikrobiologiya, 17, 279. (Dairy Sci. Abstr., 10, 120. (1948-49)).

55 Gibson, T. and Abd-el-Malek, Y. 1945 J. Dairy Res., 14, 35.

56 Golding, N.S. 1953 Proc. 22nd. Ann. State Coll. of Wash. Inst. of Dairying. p.53.

57 Golding, N.S., Amundson, H. and Wagenaar, R.O. 1943 J. Dairy Sci., 26, 909.

58 Golding, N.S. and McCorkle, L. 1953 Proc. XIIIth Int. Dairy Congr., 3, 1209.

59 Golding, N.S., McCorkle, L. and Milich, F. 1951 Proc. 32nd. Ann. Meet. Western Div. A.D.S.A.

60 Hammer, B.W. 1920 Iowa Agr. exp. Sta., Res. Bull. 63.

61 Hammer, B.W. 1935 J. Dairy Sci., 18, 579.

62 Hammer, B.W. and Babel, F.J. 1943 J. Dairy Sci., 26, 83.

63 Hammer, B.W. and Baker, M.P. 1926 Iowa Agr. exp. Sta., Res. Bull. 99.

64 Hammer, B.W. and Baker, M.P. 1928 Iowa Agr. exp. Sta., Res. Bull. 106.

65 Hammer, B.W. and Sherwood, F.F. 1923 Iowa Agr. exp. Sta., Res. Bull. 80.

66 Hammer, B.W. and Workman, C.H. 1940-42 Rpts. Iowa Agr. exp. Sta., 1940-41, 145, 1941-42, 165 (Dairy Sci. Abstr., 6, 32. (1944-45)).

67 Hansen, P.A. 1941 J. Dairy Sci., 24, 969.

68 Hansen, H.C., Bendixen, H.F. and Theophilus, D.R. 1933 J. Dairy Sci., 16, 121.

69 Harriman, L.A. and Hammer, B.W. 1931 J. Dairy Sci., 14, 40.

70 Harrison, F.C. 1905 Ontario Agr. Coll., Bull. 141.

71 Hartlib, S. 1655 Legacy of Husbandry. London. (Simon, A.L. 1956 Cheeses of the World. 1st. Edn., London: Faber and Faber.)

72 Hassouna, M.M. and Allen, L.A. 1939 J. Dairy Res., 10, 7.

73 Hastings, E.G., Evans, A.C. and Hart, E.B. 1912 Wis. Agr. exp. Sta., Res. Bull. 25.

74 Heinemann, B. 1944 J. Dairy Sci., 27, 773.

75 Hiscox, E.R. 1941 Chem. & Ind., Lond., 60, 31:563.

76 Hlynka, I. and Hood, E.G. 1947 J. Dairy Sci., 30, 827.

77 Holm, G.E., Webb, R.H. and Deysher, E.F. 1932 J. Dairy Sci., 15, 331.

78 Holwerda, K. 1945 Neth. Milk Dairy J., 8, 115.

79 Horrall, B.E. and Elliker, P.R. 1950 J. Dairy Sci., 33, 245.

80 Hoyle, M. and Nichols, A.A. 1948 J. Dairy Res., 15, 398.

81 Hucker, G.J. and Marquardt, J.C. 1926 N.Y. (Geneva) Agr. exp. Sta., Tech. Bull. 117.

82 Hucker, G.J. and Pederson, C.S. 1930 N.Y. (Geneva) Agr. exp. Sta., Tech. Bull. 167. (Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th Edn., London: Bailliêre, Tindall and Cox.)

83 Hunter, G.J.E. 1946 J. Dairy Res., 14, 283.

84 Hunter, G.J.E. and Whitehead, H.R. 1949 J. Dairy Res., 16, 368.

85 Jago, G.R. 1954 J. Dairy Res., 21, 111.

86 Jarvik, M. and Kjell, S. 1956 Mejeritek. Medd., 17, 59. (Dairy Sci. Abstr., 18, 106 (1946-47)).

87 Johns, C.K. 1953 Canad. J. agric. Sci., 33, 586.

88 Johns, C.K. and Berard, H.L. 1943 J. Dairy Res., 13, 127.

89 Jones, L.J. 1955 Pubn. of the National Dairy Association of New Zealand Ltd.

90 Joshi, N.V. and Ram Ayyar, C.S. 1936 Indian J. vet. Sci., 6, 141. (Swartling, P.F. 1951 J. Dairy Res., 18, 256.).

91 Karnad, R. 1939 Indian J. vet. Sci., 9, 349. (Dairy Sci. Abstr., 2, 54. (1940-41)).

92 Knudsen, S. 1931 J. Dairy Res., 2, 137.

93 Krishnaswamy, M.A. and Babel, F.J. 1951 J. Dairy Sci., 34, 374.

94 Laboratory Manual 1949 Methods of Analysis of Milk and its Products. 2nd Edn. Milk Industry Foundation: Washington, U.S.A.

95 Leber, H. 1959 Milk Pl. Mon., 39, 40.

96 Leitch, R.H. 1932 Cheddar Cheese-making, Glasgow. Scottish Agric. Publishing Co.

97 Lewis, J.E. 1956 J. Soc. Dairy Tech., 9, 123.

98 Lister, J. 1873 Quart. J. Micro. Sci., 13, 380. (Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th. Edn., London: Bailliere, Tindall and Cox.).

99 Lister, J. 1878 Quart. J. Micro. Sci., 18, 177. (Breed R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th. Edn., London: Bailliere, Tindall and Cox.).

100 Lloyd, F.J. 1899 Report on Cheese Making. London; H.M. Stationery Office.

101 Lloyd, F.J. 1891 J. Bath and W. Eng. Soc., 2. (Moir, G.M. 1942 N.Z. Dept. Agric. Bull. 162.).

102 Loftus Hills, G. 1956 Private communication.

103 Macdonald, J. 1908 Stephen's Book on the Farm. 5th. Edn., Edinburgh and London: Blackwood.

104 McDowall, F.H. 1953 The Buttermaker's Manual. 1, 730, Wellington: New Zealand University Press.

105 McDowall, F.H. and Dolby, R.M. 1936 J. Dairy Res., 7, 156.

106 McInerney, T.J. 1920 J. Dairy Sci., 3, 227.

107 Mackie, T.J. and McCartney, J.E. 1953 Handbook of Practical Bacteriology. 9th Edn., Edinburgh and London: E. & S. Livingstone Ltd.

108 Marshall, C.E. 1900 Mich Agr. Coll., Bull. 183.

109 Marshall, M.S. 1920 J. Dairy Sci., 3, 406.

110 Mattick, A.T.R. and Shattock, P.M.F. 1943 Nature, Lond., 151, 278.

111 Matuszewski, T. 1935 Roczniki Nauk Rolniczych i Lesnych, 34, 403. (Biol. Abstr., 10, (1936)).

112 Matuszewski, T. and Supinska, J. 1935 Roczniki Nauk Rolniczych i Lesnych, 35, 249. (Biol. Abstr., 10. (1936)).

113 Matuszewski, T., Pijanowski, E. and Supinska, J. 1936 Trav. Soc. Sci. Varsovie, 21. (Swartling, P.F. 1951 J. Dairy Res., 18, 256.).

114 Meanwell, L.J. 1956 Private communication.

115 Meanwell, L.J. and Simons, J.M. 1953 Proc. XIIIth. Int. Dairy Congr., 3, 1109.

116 Morgan, C.F.V. and Curle, J. 1928 N.Z.J. Agric., 37, 305.

117 Morris, T.A. 1955 Aust. J. Dairy Tech., 10, 65.

118 Neisser, M. 1906 Cent. Bakt., I Abt., 36, 98. (Yawger, E.S. and Sherman, J.M. 1937 J. Dairy Sci., 20, 83.).

119 Nichols, A.A. and Hoyle, M. 1948 J. Dairy Res., 15, 409.

120 Nichols, A.A. and Hoyle, M. 1949 J. Dairy Res., 16, 167.

121 Nichols, A.A. and Ineson, P.J. 1947 J. Dairy Res., 15, 99.

122 Nickerson, T.A. 1957 J. Dairy Sci., 40, 600.

123 Nickerson, T.A. 1957 Conv. Proc. Milk Ind. Found. (Lab. Sect.), 1957, 21. (Dairy Sci. Abstr., 20, 333, (1958)).

124 Niven, C.F. Jr., Smiley, K.L. and Sherman, J.M. 1942 J. Bact., 43, 651.

125 Oliver, J. 1894 Milk, Cheese and Butter. London: Crosby Lockwood and Son.

126 Oliver, W.H. 1952 J. gen. Microbiol., 7, 329.

127 Orla-Jensen, S. 1916 Maelkeri-Bacteriologi, 36. (Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th. Edn. London: Bailliere, Tindall and Cox.).

128 Orla-Jensen, S. 1919 The Lactic Acid Bacteria. Copenhagen: Ejner Munksgaard.

129 Orla-Jensen, S. and Faulenborg, G. 1940 Sbl. Bakt.II, 102, 289. (Dairy Sci. Abstr., 8, 29. (1946-47)).

130 Overby, A.J. 1946 K.VetHojsk. Aarsskr., 1946, 33. (Dairy Sci. Abstr., 8, 106. (1946-47)).

131 Pasteur, L. 1857 Compt. Rend. Acad. Sci., 45, 915. (Hammer, B.W. Dairy Bacteriology. 3rd Edn. New York: John Wiley and Sons Ltd.).

132 Petts, J.W. and Lolkema, H. 1950 Neth. Milk Dairy J., 4, 197.

133 Reiter, B. 1956 Dairy Ind., 21, 877.

134 Rice, E.B. 1942 Dairy Ind., 7, 229.

135 Rice, E.B. 1949 Dairy Ind., 14, 498.

136 Rice, F.E. and Markley, A.L. 1924 J. Dairy Sci., 7, 468.

137 Robertson, P.S. 1957 J. Dairy Res., 24, 235.

138 Robinson, A.D. and Samson, H.S. 1946 Canad. J. Res., 24, 5. (Dairy Sci. Abstr., 8, 119. (1946-47)).

139 Rogers, L.A. and Greenbank, G.R. J. Bact., 19, 181.

140 Rosenbach, F.J. 1884 Mikroorganismen Bei /wun Dinfektion Syrankheiten Des Menschen, 22. (Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th. Edn., London: Bailliere, Tindall and Cox.

141 Sanders, G.P. 1953 United States Department of Agriculture, Agr. Handbook 54.

142 Sandine, W.E., Elliker, P.R. and Anderson, A.W. 1957 Milk Prod. J., 48, 12.

143 Sandine, W.E., Elliker, P.R., Wilster, G.H., Stein, R.L. and Anderson, A.W. 1957 J. Dairy Sci., 40, 1646.

144 Schulz, M.E. 1953 Kieler Milchwirtschaft. Forsch-Berichte. 5, 379. (Dairy Sci. Abstr., 15, 999 (1953).).

145 Scott, R. 1954 Dairyman, 71, 233.

146 Shattock, P.M.F. 1945 Abstr. Proc. Soc. appl. Bact., 1944, 69.

147 Shattock, P.M.F. 1945 Chem. & Ind., Lond., 1945, 53.

148 Shattock, P.M.F. 1949 J. gen. Microbiol., 3, 80.

149 Sheldon, J.P. 1911 The Standard Cyclopedia of Modern Agriculture and Rural Economy. London: The Gresham Publishing Company.

150 Sherman, J.M. 1955 J. Dairy Sci., 38, 1184.

151 Sherman, J.M. and Hodge, H.M. 1934 J. Dairy Sci., 17, 497.

152 Sherman, J.M. and Wing, H.U. 1935 J. Dairy Sci., 18, 657.

153 Sherman, J.M. and Wing, H.U. 1937 J. Dairy Sci., 10, 165.

154 Sherwood, I.R. 1939 J. Dairy Res., 10, 326.

155 Sherwood, F.F. and Hammer, B.W. 1926 Iowa Agr. exp. Sta., Res. Bull. 90.

156 Singleton, W.M. 1906 N.Z. Dept. Agric., Bull. 7.

157 Slade, H.D. 1942 Iowa Sta. Coll. J. Sci., 17, 123. (Dairy Sci. Abstr., 4, 179 (1942-43)).

158 Slade, H.D. and Werkman, C.H. 1941 J. Bact., 41, 675. (Dairy Sci. Abstr., 3, 192, (1941-42)).

159 Smith, F.R. 1943 J. Bact., 46, 369.

160 Smith, S. 1810 General View of the Agriculture of Galloway. London: Richard Phillips.

161 Smith, W. 1900 Practical Guide to Dairying. Edinburgh: Banks & Co.

162 Steele, J. 1794 An essay on Manufacturing Milk into Butter and Cheese and on Calf-feeding etc. Intended for the Perusal and Benefit of Farmers. Glasgow: W. Miller.

163 Stevenson, W. 1909 West of Scotland Agric. Coll., Bull. 50.

164 Stevenson, W. 1911 The Standard Cyclopedia of Modern Agriculture and Rural Economy. London: The Gresham Publishing Company.

165 Stevenson, W. 1911 The Standard Cyclopedia of Modern Agriculture and Rural Economy. London: The Gresham Publishing Company.

166 Storch, V. 1890 Nogle Undersøgelser over Flødens Syrning. 18 Beretn. fra Forsøglab. (Knudsen, S. 1931 J. Dairy Res., 2, 137.).

167 Storch, V. 1919 Fortsatte Undersøgelser over Fremstillingen af Syrevarekære. 102 Beretn. fra Forsøgslab. (Knudsen, S. J. Dairy Res., 2, 137.)

168 Storgårds, T. 1948 Meijevitiet. Aikakausk., 10, 168. (Dairy Sci. Abstr., 10, 138. (1948-49)).

169 Storrs, F.C. and Anderson, E.B. 1949 Proc. XIIth. Int. Dairy Congr., 2, 605.

170 Supplee, G.C. and Bellis, B. 1921 J. biol. Chem., 48, 453.

171 Suzuki, S.K., Hastings, E.G. and Hart, E.B. 1910 J. biol. Chem., 7, 431. (van Slyke, L.L. and Price, W.V. 1949 Cheese. 3rd. Edn., New York: Orange Judd Publishing Co. Inc.).

172 Swartling, P.F. 1951 J. Dairy Res. 18, 256.

173 Swartling, P.F. and Lindgren, B. 1952 Medd. Mejeriförs Malmo No.36 (Dairy Sci. Abstr., 16, 63. (1954)).

174 Thomé, K.E. and Pollack, E. 1948 Acta Agric. Suecana, 3, 75. (Dairy Sci. Abstr., 11, 192. (1949-50)).

175 Toens, P. and Hammer, B.W. 1925 Iowa Agr. exp. Sta., Res. Bull. 133 (Rice, E.B. 1942 Dairy Ind., 7, 229.)

176 van Beynum, J. and Pette, J.W. 1936 Versl. Landbouwk. Ond. 42, 361. (Hammer, B.W. and Babel, F.J. 1943 J. Dairy Sci., 26, 83.

177 van Beynum, J. and Pette, J.W. 1939 J. Dairy Res., 10, 250.

178 van Beynum, J. and Pette, J.W., 1943 Milch. Zbl. 72, 81. (Dairy Sci. Abstr., 7, 54. (1945-46)).

179 van Niel, C.B., Kluyver, A.J. and Derx, H.G. 1929 Biochem. Z., 210, 234. (Hammer, B.W., and Babel, F.J. 1943 J. Dairy Sci., 26, 83.).

180 van Slyke, L.L. and Baker, J.C. 1919 N.Y. (Geneva) Agr. exp. Sta., Tech. Bull., 70.

181 van Slyke, L.L. and Bosworth, A.W. 1907 N.Y. (Geneva) Agr. exp. Sta., Tech. Bull., 4.

182 van Slyke, L.L. and Bosworth, A.W. 1916 N.Y. (Geneva) Agr. exp. Sta., Tech. Bull., 48.

183 van Slyke, L.L. and Price, W.V. 1949 Cheese. 3rd Edn., New York: Orange Judd Publishing Co. Inc.

184 van Tieghem, P.E.L. 1878 Ann. Sci. Nat., 6, Ser., 7, 170. (Breed, R.S., Murray, E.G.D. and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th.Edn., London: Bailliere, Tindall and Cox.).

185 Virtanaen, A.I., Karstrom, H., Jorma, J. and Kahra, L. 1941 Zeitschr. physiol. Chem. 269, 259. (Biol. Abstr., 19. (1945)).

186 Walker-Tisdale, C.W. and Jones, J. 1920 Butter and Cheese. London: Sir Isaac Pitman & Sons Ltd.

187 Walker-Tisdale, C.W. and Woodnutt, W.E. 1919 Practical Cheesemaking. London: Headley Bros.(Publishers) Ltd.

188 Walter, H.E., Sadler, A.M., Malkames, J.P.Jr. and Mitchell, C.D. 1953 United States Department of Agriculture, BDI-Inf-158.

189 Weigmann, H. 1890 Milch-Ztg., 19, 593, 944. (Knudsen, S. 1931 J. Dairy Res., 2, 137).

190 Whitehead, H.R. 1931 J. Dairy Res., 2, 76.

191 Whitehead, H.R. 1943 Dairy Ind., 8, 120.

192 Whitehead, H.R. 1947 Aust. J. Dairy Tech., 2, 149.

193 Whitehead, H.R. 1955 Private communication.

194 Whitehead, H.R. 1956 Dairy Engng., 73, 159.

195 Whitehead, H.R. and Bush, E.J. 1957 J. Dairy Res., 24, 381.

196 Whitehead, H.R. and Cox, G.A. 1932 N.Z.J. Sci. Tech., 13, 304.

197 Whitehead, H.R. and Harkness, W.L. 1954 Aust. J. Dairy Tech., 9, 103.

198 Whitehead, H.R. and Hunter, G.J.E. 1942 J. Dairy Res., 13, 64.

199 Whitehead, H.R. and Sargent, J.D. Starters for Cheese Manufacture. Dairy Research Inst. Pubn. 174. Wellington: E.V. Paul.

200 Wilson, G.S. 1935 The Bacteriological Grading of Milk. London: H.M. Stationery Office.

201 Wilson, G.S. and Miles, A.A. 1946 Principles of Bacteriology and Immunity, 3rd. Edn., London: Edward Arnold & Co.

202 Wright, R.C. and Tramer, J. 1957 J. Dairy Res., 24, 174.

203 Yawger, E.S. Jr. and Sherman, J.M. 1937 J. Dairy Sci., 20, 83.

204 Zielinska, M. and Hiscox, E.R. 1954 J. Dairy Res., 21, 238.