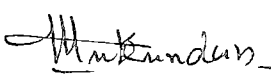
DECLARATION

I hereby declare that this thesis entitled "STUDIES ON MICROFLORA IN BOILED MILK" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.



M. MUKUNDAN.

Mannuthy,
29-7-1978.

CERTIFICATE

Certified that this thesis entitled "STUDIES ON MICROFLORA IN BOILED MILK" is a record of research work done independently by Sri. M. Mukundan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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I dedicate this thesis to my beloved mother.

M. MUKUNDAN.

C O N T E N T S

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INTRODUCTION

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The bovine population of Kerala State during 1972 was 3.3 million comprising of 2.86 million cattle and 0.47 million buffaloes. The total milk production in the State during 1973-74 was estimated as 0.44 million tonnes (Nagarcenkar, 1977). The dairy industry in India at present is spread over the whole country in innumerable small units in a very disorganised form. Millions of small and marginal farmers and agricultural labourers of remote and rural India has really been the basis for 80 per cent of the country's milk production.

Milk as it occurs in the gland tissue of healthy cow is sterile. During the process of milking, bacteria which have reached the lower part of the teat canal are washed down and they contaminate the milk. Moreover contamination during the production and handling of milk, helps various types of micro organisms to gain entry into it. Since milk provides a suitable environment under ordinary atmospheric temperature, these micro-organisms rapidly multiply in it and bring about spoilage. The pathogenic organisms which find their way into the milk remain inert and infect the consumer. At present, milk reaching the consumer in cities passes through many stages from production to processing, thereby affecting its final keeping quality and wholesomeness. In most cases milk is produced under very

unhygienic conditions as a result of which the milk has a very poor keeping quality. The tropical climate prevailing in the country and lack of proper storage and communication facilities aggravate the problem further (Krishnaiah, 1968).

Bacterial growth can be controlled to some extent if the milk after production is cooled rapidly and stored at a low temperature of 5°C. Since low temperature does not destroy the organisms it is essential that milk should be treated in some other way to ensure the destruction of pathogenic types of micro-organisms. To enhance the keeping quality of milk and to render it safe for human consumption, various physical and chemical methods of preservation have come into vogue. Of the various methods, heat treatment of milk is the most popular and effective one. Heat treatment has been recognised to be the most efficient of all the methods of treatment since it destroys the majority of organisms including pathogenic type which gain entry into milk during the various operations through which it passes before reaching the consumer.

The different methods of heat treatment of milk can be classified as (1) pasteurization of milk in which it is heated to a temperature of 63°C for 30 minutes or 72°C for 15 seconds, (2) boiling of milk for varying time intervals and (3) ultra-high temperature processing of milk at 130 to 150°C for 2 to 4 seconds to make it completely sterile.

People discovered, even before the scientific age, that boiling of milk helps to keep it unsoured for a long time. In India by tradition, raw milk is never consumed and it is always heated to boiling point before drinking. This is more or less akin to the conventional pasteurization. Boiling of milk has been found to destroy all the organisms which cause souring and consequent coagulation of milk but also those that are pathogenic (Chaudhuri, 1959).

Boiling of milk, probably the first method involving heat treatment, had been practised in almost all the nations at one time or another in the early stages of dairy development. Boiling is the most common heat treatment of milk followed in India which may be considered to be effective in destroying any pathogen that may be present in milk and practically all the other types with the exception of those existing as spores (Kerala Varma, 1949). In many milk collection centres in India, due to bad communication facilities milk is brought to the collection centres once in a day. In order to improve the keeping quality generally the milk is boiled by the producers and both morning and evening collections are mixed before delivery to the collection centre (Krishnaiah, 1968).

In spite of the advantages of boiling, quite often many samples of boiled milk exhibit moderately high plate counts. This may be attributed to the presence of bacterial spores in

milk which can withstand boiling temperature. Depending on the requirement of oxygen for growth, the spores can be classified into two genera; genus Bacillus comprising of aerobic spore-formers and genus Clostridium which consists of anaerobic spore-forming organisms. Since organisms of genus Bacillus grow well in the presence of oxygen and at room temperature the problem concerning them is more important than that of anaerobic spore-forming bacteria.

Among the aerobic spore-forming bacilli, different species such as Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Bacillus coagulans, Bacillus stearothermophilus etc., are found in milk. Their numbers in milk vary depending upon the source of the sample, extent of contamination from the exposed surfaces of dairy equipments and utensils, season, temperature of incubation of plates, etc.

Eventhough aerobic spore-forming bacilli have been considered non-pathogenic to human beings with the exception of Bacillus anthracis (Wilson and Miles, 1975) they deserve consideration because of bringing about spoilage in pasteurised, boiled and ultra-high temperature heat treated milks. In dairying, the spores formed by them are of considerable importance especially when relatively high processing temperatures are used. High temperatures for processing produce activation of the spore germination and out growth (Roberts, 1961; Black,

1960 and Mitten, 1959) which gradually bring about the spoilage of milk. Several workers (Chung and Cannon, 1971 and Grosskopf and Harper, 1969) have reported the isolation of spore-formers which grow at cold temperatures. Such evidence emphasizes the significance of bacterial spores in milk and milk products.

Besides the satisfactory compositional and physical standards, adequate keeping quality of milk is also important. The milk which is being supplied contains a good variety of organisms which cause spoilage of milk by producing acidity. If the milk is slightly high in its acid content it will curdle or coagulate when boiled. This property is of much importance in a dairy plant because developed acidity has such a powerful influence in lowering the temperature of heat coagulation of casein. The extent of development of acidity which reflects on the keeping quality in terms of the number of hours the raw milk can be kept safe without clotting at the time of heat treatment becomes a matter of vital necessity. Boiling of milk destroys majority of the micro organisms present in milk which ultimately increases the keeping quality.

The pattern of dairying in India is undergoing considerable change in that the raw milk collected from long distances is supplied in metropolitan cities as pasteurised milk in bottles. Majority of the Indian wives practise boiling of pasteurized milk before consumption in order to render it safe.

In the State of Kerala, out of the 0.44 million tonnes of milk produced, only a very limited quantity of milk is subjected to pasteurization and other heat treatments in various dairy plants. But the quantity of sterilized milk produced in Kerala is negligible. This implies that boiling of milk is the only form of heat processing employed extensively in Kerala. Moreover the conditions of storage of boiled milk in Kerala households are most favourable for the growth and development of aerobic spore-forming bacteria present in it.

In view of the above conditions a systematic study of the incidence of micro organisms in boiled milk was considered desirable. Since no systematic work has been done on the incidence and types of aerobic spore-forming bacilli in boiled milk in Kerala State, and only very little work has been done in other parts of India, an attempt has been made to study these aspects in milk collected in this region. In the present work the keeping quality of boiled milk was also studied with a view to evolve a suitable method for increasing the keeping quality of boiled milk.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

There are two forms in which bacteria can occur in milk: the vegetative form and the spore form. The vegetative form, although active in multiplication and metabolism, is not resistant to adverse conditions such as heat and chemicals, whereas the spores are very resistant. Metabolically spores are inactive resting cells which germinate and give rise to vegetative cells only under suitable environmental conditions (Stocker, 1958).

The vegetative form of the bacteria easily get destroyed by heating the milk. The normal pasteurization temperature-time combination of 62.8°C for 30 minutes or 71.7°C for 15 seconds destroys all the pathogenic and a considerable proportion of milk spoilage organisms (Campbell and Marshall, 1975). In spite of the advantages of pasteurization, many samples of pasteurized milk exhibit high plate counts. This may be attributed to the presence of certain types of microorganisms in milk which can withstand heat treatment adopted in pasteurization (Parameswaran, 1966). Depending upon the optimum temperature of growth such heat resistant organisms have been grouped into thermophilic and thermoduric types. The thermophilic bacteria have a growth temperature above 45°C and the thermoduric grow well at normal atmospheric

temperatures (Foster, 1964). This makes the problem concerning the thermoduric organisms more important than that of thermophilic bacteria (Parameswaran, 1966).

The thermoduric bacteria includes various species of Micrococcus, Bacillus, Microbacterium, Lactobacillus and Streptococcus. Their numbers in milk varies depending upon the source of the sample, extent of contamination from the exposed surfaces of dairy equipment and utensils, season, temperature of incubation of plates etc. (Foster et al.1957).

Abnormal heat resistance shown by certain organisms present in milk, help them to survive in pasteurized milk. Gibson and Abd-el-Malek (1940) reported the survival of Streptococci, Micrococci and Corynebacteria and thermoduric types in pasteurized milk. Many of the thermoduric species could withstand temperatures upto 107.2°C in milk for 20 minutes (Procter, 1943). The common species were Streptococcus thermophilus, Streptococcus faecalis, Streptococcus liquifaciens, some Micrococci, Corynebacteria, Sarcina and spores of the genera Bacillus and Clostridium (Kerala Varma, 1949). The predominant microflora in ultra-high temperature pasteurized milk of unsatisfactory quality has been studied by Franklin (1969). Out of 477 isolates the following were identified; B. cereus 130 (27.3%), aerobic spore-formers excluding B. cereus 129 (27.0%), Corynebacteria 103 (21.6%),

Micrococci 70 (14.7%), Streptococci 38 (8.0%) and other 7 (1.4%). Stocker (1958) isolated 250 strains of acid-producing Streptococci giving blue pin-point colonies from china-blue lactose agar plates of pasteurized milk. About 25 per cent of them were destroyed by heating milk at 74°C for 40 seconds; but 72 per cent of these strains survived heating at 72°C for 40 seconds. Many strains survived 77°C when heated in milk in a plate pasteurizer with a holding time of 38.5 seconds. The strains were identified as Streptococcus faecium (80%), S. glycerinaceus (12%), S. thermophilus (8%) and occasionally other cocci.

Pavlas (1962) detected viable tubercle bacilli from a naturally infected milk even after heating at 85°C for 60 seconds. Moureau et al. (1960) found that all of the ten human strains of Mycobacterium tuberculosis suspended in milk (1 mg/ml) were resistant to 70°C for 15 minutes. three strains to 80°C and two strains to 85°C. All of the ten bovine strains were found resistant to 70°C for ten minutes, four strains to 80°C for five minutes and three strains to 85°C for five minutes. Mycobacterium paratuberculosis strains showed higher heat resistance but the number of resistant organisms decreased considerably when the suspension was diluted. The heat resistance of B.C.G. strain and a Moureau strain was extremely poor and only slight growth appeared

following heat treatment at 60°C for ten minutes. No growth was observed after exposure to longer periods or higher temperature.

Generally the predominant types of bacteria in milk with a high thermoduric count were Streptococci and Micrococci, while spore-formers were found to predominate in samples with a thermoduric count of /1,500/ml. Mycobacteria, Actinomycetes and unidentified Gram-positive rods constituted /10 per cent of the total thermoduric microflora (Buchanan and Nelson,1960). Esteves (1962) examined milk containing coliform organisms for thermoduric coliform strains after laboratory pasteurization and found that E. coli var communior and E. freundii were present. Repeated heating of 30 consecutive generations of four strains each of Lactobacillus viridescens, Lactobacillus plantarum, Lactobacillus brevis and atypical Streptobacteria isolated from meat products raised the range of their heat resistance from 60 to 80°C to 75-95°C (Somogyi, 1966).

Procter (1943) found that approximately 90 per cent of the samples contained coliform organisms in 1 ml or 0.1 ml after laboratory pasteurisation in an epidemic due to heat resistant coliform bacteria in milk. He also found that the incidence of thermoduric organisms increased very much since the commencement of the World War II. It was noticed that the bacterial count of pasteurized milk seldom exceeded 2,000/ml

and in winter the count increased to 20,000/ml. Comparison of the thermal death rate curves for one each of pathogenic and non-pathogenic strain of Escherichia coli in the milk of buffaloes with the pasteurization curve indicated that these organisms survive the current pasteurization process used for buffaloe milk (Singh and Ranganathan, 1974). Psychrophilic bacteria isolated from pasteurised milk samples revealed the spore-forming genus Bacillus which occurred frequently and the non-sporing types of the genera Arthrobacter, Microbacterium Streptococcus and Corynebacterium (Washam et al. 1977). Youssef et al. (1971) observed that the psychrophilic organisms originally present in milk resisted laboratory pasteurization at 63°C for 30 minutes and growth was noticed in pour plates incubated at 5°C. Psychrophilic growth due to psychrophilic organisms was seen after seven days in milk samples which have been heated to 82°C for 30 minutes.

In a survey made during 1964-1966 Thomas et al. (1967) found that milk samples collected from bulk supplies had thermoduric counts higher than 1,000/ml, 95.5 per cent having counts of 10,000/ml or less and 4.5 per cent exceeding 10,000/ml. In samples with high thermoduric counts (>10,000/ml) a dominance of Microbacteria and Corynebacteria was noticed. The aerobic spore-forming bacteria constituted 42 per cent of the isolates examined by them. The non-spore forming bacteria

of the genus Microbacterium constituted a high percentage among thermoduric microflora in milk and dairy equipment and they were found to be the most heat-resistant organisms among the non-spore-forming bacteria (Parameswaran, 1966). The heat resistance of Microbacterium lacticum and Microbacterium flavum has been found to 80-85°C for 15 minutes and 75°C for 15 minutes respectively whereas Microbacterium mesentericum was destroyed readily at 70°C (Orla-Jensen,1943). But Breed et al. (1957) reported that Microbacterium lacticum and Microbacterium flavum survived 72°C for 30 minutes and 15 minutes respectively. Speck (1943) studied the heat resistance of Microbacteria at various temperature-time combinations. All the ten cultures used for the study withstood 62.5°C for 30 minutes and 85°C for 2½ minutes, nine cultures survived 71.6°C for 10 minutes and only seven cultures resisted 80°C for 30 minutes. Mckenzie (1945) reported that heat treatment at 80°C for 20 minutes was necessary to eliminate non-spore-forming contaminants from milk. Doetsch and Pelczar (1947) reported the survival of Microbacterium lacticum at 85°C for 2½ minutes. Eventhough a temperature of 80°C for 10 minutes was used by Barber (1962) to eliminate all vegetative cells in the milk samples, Fourie et al. (1972) found that it was not sufficient to kill all vegetative cells in milk.

The group of spore-forming bacteria namely the genus Bacillus are aerobic or facultatively anaerobic, catalase-positive, spore-forming rods. The bacterial spores have found to be highly resistant to heat and other lethal agents. Heating of milk to a temperature of 80°C for 10 minutes has been adopted for the enumeration of spores whereas heating to 100°C for 30 minutes was necessary for the counting of thermo-resistant spores (Galesloot, 1962).

Spore flora of Raw milk.

The raw milk generally contains a considerable number of spores. Mckenzie et al. (1949) found that aerobic spore-formers were regularly present in raw milk in very small numbers Kerala Varma et al. (1950) observed that the spore-forming bacteria are regularly present in milk and they constitute about ten per cent of the microbial population in milk. Martin (1974) also reported the presence of spore-forming bacteria in all raw milk, but usually in small number if the milk is produced under modern sanitary conditions. He could not find any relationship between total bacterial count and the incidence of any particular species of spore-forming organism in milk. Bacillus species accounted for about 95 per cent of the total spore-forming bacteria in milk with Clostridium species comprising the remainder. Franklin et al. (1956) observed a range of 0-700 aerobic mesophilic spore count per

100 ml of raw milk. Galesloot (1962) reported that the spore counts of milk in countries like Netherlands, Australia and Great Britain were 100/ml. Atwal et al. (1974) reported that the total spore-forming bacterial count formed nearly two per cent of the total bacterial count. According to Ridgway (1956) they ranged 1 to 10 per cent of the flora of milk.

In milk having a thermoduric count of less than 1,500/ml, the spore-forming bacteria ordinarily were the predominant thermoduric bacteria (Buchanan and Nelson, 1960). Chung and Cannon (1971) examined milk samples from 18 producers, and found that 83.3 per cent of the sample contained spores in a range of 2 to 900 per ml. Atwal et al. (1974) found an average spore count of 90/ml and mesophilic spore count ranged from 25 to 100/ml in 404 samples of various types of raw milk samples examined. They also found the mesophilic spore count in 100 milk samples collected from a commercial dairy plant ranged from 23 to 1,800/ml; average count ranged from 167/ml in toned milk and 404/ml in raw buffaloes' milk. A high count of spore-forming bacteria in milk with a range of 818-112,800/ml was observed by Kerala Varma et al. (1950). Ionescu et al. (1966) noted that Bacillus cereus was present in numbers upto 10^5/ml in raw and pasteurized milk.

Bhat and Iyer (1955) stated that the groups subtilis, licheniformis, pumilus, cereus, megaterium, firmus, lentus,

circulars and sphaericus may be regarded as aerobic mesophilic spore-forming bacteria in Indian environments. Ethiraj (1976) found that the total spore count of 34 raw milk samples varied from 3,000 to 15,500,000/ml, mesophilic spores from 60 to 3,600/ml, facultative thermophilic spores from 10 to 590/ml and obligatory thermophiles from 0 to 285/ml. He also noticed a general trend for a rise in the various spore counts when there was a rise in the total count in all the 34 samples examined by him.

Seasonal incidence of Spore-formers in Milk.

Regarding the seasonal incidence of spore-formers in milk various workers differ in their opinion. Aerobic and anaerobic spore count of raw milk taken in summer and winter showed a tendency for higher aerobic spore count in winter (1-3,000/ml) than in summer (2-110/ml), whereas anaerobic spore counts were 0-140/100 ml in winter and 0-1,600/100ml in summer (Abo-Elnaga, 1968). Ridgway (1958) found that the spore counts were about 100 times greater in winter when the cows were fed on hay than in summer when they were cut to grass. Galesloot (1951), Ridgway (1954) and Franklin et al. (1956) reported higher spore count in milk during winter than in summer. Ridgway (1954) attributed the reason for this as the contamination of milk during production with spores derived from bedding, fodder etc. Nicholas and Candy (1956)

observed that the incidence of both mesophilic and thermophilic species of spore-formers in commercial 'sterilized' milk in England was higher in winter than in summer. Ridgway (1955) while investigating milk processed at 105.5°C for 35 minutes in a continuous automatic sterilizer, found mean counts of mesophiles of less than 1/100 ml in summer and nearly 5/100 ml in winter. Mean thermophilic counts of about 10/100 ml in summer and 80/100 ml in winter were also observed by him. El-Sadek and Attia (1968) found the average number of aerobic spore-formers in raw milk being higher in summer. However, he found that season had no effect on the spore count of boiled milk. In a regional survey of aerobic mesophilic spore-formers in raw milk Bernhard (1962) found a surprisingly low spore count of 16 spores/ml in winter and 9 spores/ml in summer milk. Atwal et al. (1974) collected 404 samples of various types of raw and pasteurized milk and reported no seasonal variation in mesophilic spore count. Underwood et al. (1974) concluded that the spore count in winter milk was higher than that of summer milk. Ranganathan et al. (1974) studied the incidence of heat resistant spores in milk at the Aarey Milk Colony, Bombay and reported that irrespective of the season, the number of spores in milk varied within the range of 1,000 to 3,000/ml.

Nakanishi and Hyogo (1965) noted that mesophilic counts were found to be minimum in January and highest between July

and September. They have also observed that the counts of B. cereus were highest in warmer months whereas B. subtilis and B. licheniformis occurred more often in cold periods, B. albolactis and B. stearothermophilus were more in May. Milk samples collected from 700 Norwegian suppliers analysed over five separate months, revealed the presence of spore-forming organisms in 36 per cent of cases in March, 39.4 per cent in May, 16.2 per cent in June, 28.4 per cent in September and 5.6 per cent in November (Engan-Skel, 1974). The species of aerobes and their number in winter and summer isolated and reported by Abo-Elnaga (1968) were as follows: B. cereus 40 and 35; B. licheniformis 20 and 25; B. subtilis 11 and 21; B. pumilus 11 and 19; B. megaterium 7 and 12; B. polymyxa 1 and 3; B. coagulans 0 and 2; B. macerans 0 and 1 and unidentified species 1 and 9. Khalafalla et al. (1976) found a seasonal variation in the content of spore-formers in Egyptian buffaloes' raw milk: In summer and winter the aerobic mesophilic spore counts/ml were 42 x 10^2 and 380; aerobic thermophilic 53 x 10^2 and 94, and anaerobic mesophilic 85 x 10^2 and 20 respectively. However, the heat treated (100°C for 60 minutes) milk samples showed no definite seasonal variation. Franklin (1970) has stated that the number of heat resistant spores in bulk raw milk tend to be greater in winter than in summer and at times of peak contamination the level was 1/ml.

Kerala Varma et al. (1950) observed that village produced milk contained about 2,000-5,000 spore-forming bacterial/ml in winter while in summer their number increased to about 10,000-50,000/ml. A bulk milk sample which contained about 2,000 spores/ml showed 8,500 spores/ml at the time of distribution in bottles and about 38,000 spores/ml at the time of distribution in tinned steel utensils. Milk produced in an organised dairy farm contained about 200-1,000 spores/ml in winter and 500-2,000/ml in summer. When the milk was brought to the city for distribution, the number of spore-formers ranged from 10,000-20,000 in winter and 50,000-110,000/ml in summer. On isolation and identification of the organisms it was found that 61 per cent was B. subtilis, 18 per cent B. cereus and 13 per cent B. lentus, B. coagulans and B. sphaericus. Thomas and Druce (1967) also reported the incidence of higher thermoduric colony counts in summer than in winter in farm milk supplies of North America, Netherlands and Britain.

Thomas (1972) reported that the bacterial content of the farm bulk tank milk may be higher in summer than in winter. In Tehran area the summer milk was found to contain more spores than the winter milk (Farkhondeh, 1974). The reasons reported were the dry climate and dusty weather during the summer. Ethiraj (1976) found that the spore counts of raw milk during the period from July to September

were lower than those during the period from October to December. A high incidence of spore-forming bacteria in the pasteurized milk has also been confirmed.

Source of Bacterial spores in Milk.

Spore-forming bacteria are very wider spread in nature and gain entry into milk from many sources during production and processing. The chief sources of contamination of milk in the order of their importance are vessels, exterior of the animal, dust and atmosphere of the byre, the interior of the udder, the milker, the method of milking and flies (Nambudripad, 1950). Galesloot (1962) reported that bacterial spores gain entry into milk mainly by the contamination of milk with particles of dust, soil and manure. Kerala Varma (1949) studied the incidence of aerobic spore-forming bacteria in milk and other farm materials such as feeds, coat of the animals, dung, dust, utensils etc. In concentrate feeds nine out of twelve isolates belonged to B. subtilis, two to B. megaterium and one to other types. In fodder seven out of nine isolates were B. subtilis and two B. megaterium. All the isolates from silage were found to be B. subtilis. In dung six out of nine were B. subtilis, one B. megaterium, one B. cereus and one other types. From dust two out of three isolates were of B. subtilis and one of B. megaterium. In utensils two out of six isolates

belonged to B. subtilis, one to B. megaterium, two to B. cereus and one to other types.

Many workers have concluded that the udder of cows may be the source of bacterial spores in milk. Hoy et al. (1955) found that the teats and udders of the cows get contaminated with spores from bedding, soil, etc. and the spores invaded the udder causing acute mastitis of short duration. Soprey (1963) stated that the important source of bacteria in milk was the udder of the cow and that it contributed from a few inert types to several thousands when there was infection. He also reported that milking utensils, the exterior of the udder, milker and atmosphere of the byre constitute major sources of bacteria in milk. Farm animals and the food they eat may contribute to the spore content of milk (Jayne-Williams and Franklin, 1960a). Ridgway (1958) showed that the bovine faeces contained large numbers of spores. The number of spores in manure and soil during the summer months were found to be 140-4,000/g and 10,000/g respectively (Billing and Cuthbert, 1958). Judkins and Keener (1966) reported that the source for most of the destructive bacteria in milk was the body of the cow which got usually contaminated from the manure and dirt. The organisms usually encountered in the atmosphere of the byre were spore-forming bacteria (aerobic and anaerobic), micrococci, yeasts and moulds.

The contamination of milk with spores at the time of production was found to occur from bedding, fodder etc. (Ridgway, 1954). Ethiraj (1976) found the thermophilic and thermoduric type of the spore-formers gain entry into the milk through dust, soil and manure. Location of the farms in the heart of the city with heavy road traffic contributed to high increase in the spore content of the milk.

Many workers showed that the farm water supplies frequently contained considerable numbers of bacterial spores (Thomas and Roberts, 1946; Thomas and Thomas, 1955 and Thomas et al. 1952). Thomas and Roberts (1946) conducted a survey of the bacteria surviving 63°C for 30 minutes in Welsh waters and found that out of the 342 cultures examined by them 74 per cent had aerobic spore bearing rods, 9 per cent micrococci, 8.2 per cent actinomycets, 3.8 per cent Gram negative rods, 2.6 per cent yeasts, 2 per cent microbacteria and 0.3 per cent streptococci. The waters were derived from piped supplies, pump water from deep and shallow wells and surface waters from rivers, canals and streams. Twenty two of the 116 samples showed thermoduric counts of 101 to 1,000/ml, and only two gave counts greater than 1,000/ml. There was no marked difference in the thermoduric flora of the various types of water. Water from such sources contaminated the dairy utensils and equipments.

Many of the bacteria generally found in utensils were lactic acid producing species and thermoduric species which subsequently contaminated milk (Kerala Varma, 1949). Utensils sterilized in different ways also contributed spores to milk and the count of spores that showed resistance to 100°C for 30 minutes ranged from 0-120/100 ml (Carreira et al. 1955). George et al. (1956) reported that more than half of the unsatisfactorily washed milk cans examined by them contained more than 5×10^4 spores. Hughes and Ellison (1948) observed spores in efficiently washed milk cans. Only five per cent of 895 cultures isolated from milk scale were found to be spore-formers. Leali (1959) found that 32.5 per cent of 367 cultures isolated from pasteurized rinses of milk cans were spore-formers. He also observed that cans cleaned by machine with steam contained more number of spores. Galesloot (1959) came to the conclusion that faulty washing of the milk cans might increase the content of Bacillus cereus spores in milk. Milk cans and milking equipments have been found to contaminate the milk with B. cereus spores especially in summer time (Labots et al. 1965). The unsatisfactorily washed milk cans was found to be the source of large numbers of thermoduric micrococci, corynebacteria and aerobic spore-forming bacilli (Mckenzie et al., 1946; Thomas and Thomas, 1955).

The effects of sterilization of utensils by different methods were investigated by Carreira et al. (1955). They

found that the count of spores able to resist 100°C for 30 minutes ranged from 0-120/100 ml of milk and the method of sterilization of utensils had no influence on the count. Abo-Elnaga et al. (1973) found that the spore-formers constituted two per cent of the total number of bacteria in cans before washing and 2.5 per cent after washing. The spore-formers present in milk pails in winter (December and January) after washing were 8.5 per cent of the total microflora and before milking it reached upto 15 per cent. In spring (March) it was zero in both cases. In summer (May and June) the percentage was 8.3 and 11.2 before milking. The number of sporeeformers present in milk cans during winter after washing was 18.2 per cent of the total microflora and before milking it was 15 per cent. In spring the percentages after washing and before milking were 16.6 and 7.0, and in summer 18.3 and 2.6 per cent respectively.

Mckenzie and Morrison (1945) considered the milking machine as the main source of thermoduric organisms in milk. Thomas and Druce (1967) found that when the sterilization of equipment was effective there was very little difference in thermoduric bacterial content of hand or machine milked supplies. But when cleaning was neglected the thermoduric contamination was very much higher in machine milked supplies. Underwood et al. (1974) indicated that contamination from the

air and milking equipment would contribute $\angle$ 1 spore/ml to milk. Twelve machine milked farm milk samples examined during summer months gave significantly higher thermoduric bacterial counts as compared to farm milk samples obtained by hand milking (Thomas and Evans, 1946). In a comparison of hand milking to machine milking Thomas (1949) found very little or no difference in thermoduric count, provided the sterilization of the machine was efficient. Contrary to this when sterilization was ineffective he observed a tendency for increase in the thermoduric counts in machine milking than in hand milking. Shaw and Nambudripad (1965) also arrived at the same conclusions. Judkins and Keener (1966) reported that the milker or the person who handled the milk after it was drawn, might also contribute microorganism into milk.

Hermier and Bergere (1959) showed the source of contamination of the sterilized milk as the bottle filler, which was difficult to clean. Galesloot (1956) found that steaming 100°C for 30 minutes was not sufficient to destroy contamination of milk from washed bottles, and the filling and capping operations. Washed bottles were shown to be an important source of thermophilic spore-formers by Nicholas and Candy (1956). Galesloot (1962) concluded that the spores of B. coagulans, B. circulans and the thermophilic bacilli contaminated the milk at bottling, either through insufficiently disinfected bottles (B. circulans) or through the

filler (B. coagulans and the thermophilic bacilli).

The higher count of aerobic mesophilic spore-formers in raw milk during winter was attributed to feeding hay to animals (Bernhard, 1962). Billing and Cuthbert (1958) found that the practise of storing hay in or above milking sheds was the reason for the higher spore counts in milk. Engan-Skel (1974) who subjected samples with highest number of spores in milk for more detailed studies observed that the reasons were feeding of poor quality silage and the lower hygienic standards of the cow-sheds, milking techniques and equipment. Atwal et al. (1974) noticed the feed, dung, milk pails, cans and buckets all had higher aerobic mesophilic spore count.

Common Bacillus species present in Milk and its Pattern of distribution.

Grosso and Bergamini (1963) isolated the following species of Bacillus from sterilized milk: B. megaterium, B. cereus, B. subtilis, B. licheniformis, B. pumilus, B. coagulans, B. badius, B. stearothermophilus, B. circulans and B. pulvifaciens. Wide fluctuations had been noticed in the spore count of milk. Galesloot (1962) observed that more than half of the bacterial spores in raw milk were B. licheniformis, such milk also contained as a rule, considerable numbers of spores of B. pumilus and B. subtilis, and in lesser

numbers, spores of many other species of bacillus. El-Sadek and Attia (1968) isolated aerobic spore-formers from raw milk samples and found that 71.8 and 12.9 per cent of the isolates were B. megaterium and B. brevis, while after boiling the incidence was B. brevis 36.99 per cent, B. coagulans 19.8%, B. licheniformis 15.07% and B. megaterium 12.33%. Atwal et al. (1974) found that the predominant species of spore-formers in all types of milk examined were B. subtilis, B. cereus, B. coagulans and B. stearothermophilus. The spore-forming bacteria isolated from raw milk samples by Chung and Cannon (1971) were identified after incubation as B. firmus 46.3 per cent, B. megaterium 23%, B. brevis 15.7% and the remainder B. coagulans, B. polymixa, B. maceran, B. circulans and B. cereus. Khalafalla et al. (1976) found that the aerobic spore-forming bacteria in Egyptian buffaloes' raw milk consisted mainly of B. megaterium (72% of 411 isolates), followed by B. brevis (6%), B. subtilis and B. firmis (5% each), B. licheniformis and B. badius (3.5% each) and other species occurred less frequently.

Fourie et al. (1972) isolated 787 Bacillus species from South African raw milk samples; and found B. licheniformis 21.4%, B. pumilus 23.7%, B. subtilis 20% and B. cereus 12.2%; other bacillus species each constituting greater than 4 per cent of the total number of isolates.

Baoillus stearothermophilus isolates were not found in the samples examined. Martin (1974) isolated the spore-formers in raw milk supplies and identified 297 isolates as B. licheniformis 43.3%, B. cereus 37.4%, B. pumilus 3.4%, B. sphaericus 2.0%, B. cereus var mycoides 1.71%, B. brevis 1.3%, B. laterospores 1.0%, B. circulans and B. stearothermophilus 0.7% and Clostridium (species unidentified) organisms 4.7%. Ethiraj (1976) found that the percentage of incidence of spore-formers in raw milk was B. subtilis 32.62, B. megaterium 19.56, B. coagulans 15.22, B. stearothermophilus 13.04, B. cereus 8.70, B. licheniformis 6.52, B. sphaericus 2.17 and B. pumilus 2.17. Underwood et al. (1974) found B. licheniformis as the predominant organisms in raw milk. Davies (1975) reported that B. subtilis was the most predominant organism in milk. The total number and relative proportions of species isolated from milk of some of the countries were B. subtilis 131 (48.52%), B. licheniformis 44 (16.30%), B. cereus 34 (11.85%), B. lentus 14 (5.15%), B. pumilus 13 (4.81%), B. coagulans 13 (4.81%), B. stearothermophilus 7 (2.56%), B. circulans 4 (1.49%), B. firmis 4 (1.49%), B. brevis 3 (1.13%), B. megaterium and B. macerans 2 (0.76%) each and B. laterosporus 1 (0.37%). According to Mikolajcik (1970) B. cereus and B. licheniformis were the most prevalent species in raw milk. Bernhard (1962) noted that B. subtilis, B. licheniformis, B. coagulans and B. cereus were the most common

aerobic mesophilic spores in raw milk. Ridgway (1958) reported that B. subtilis and B. licheniformis were the species most frequently encountered and B. licheniformis predominated in the raw milk supplies. Janina (1966) found the mean contents of aerobic bacilli in raw and pasteurised milk to be 80 and 200 respectively per ml. B. subtilis constituted 40 per cent, Bacillus cereus 20 per cent and the rest being various bacillus species.

In heat treated milk (100°C for 60 minutes) sample the distribution pattern was noticed with B. megaterium, occurring most frequently (35% of 386 isolated), but very closely followed by B. subtilis (26%), B. coagulans (10%) B. cereus and B. firmis (6% each), B. macerans (5%), B. stearothermophilus (3.5%) and other species less frequently. After the heat treatment B. brevis and B. badius were found to disappear (Khalafalla et al. 1976). Grosskopf and Harper (1969) isolated the psychrophilic spore-forming organism from pasteurized milk and were identified as B. coagulans, B. lentus, B. cereus and B. licheniformis. The predominant types of organisms isolated from pasteurized milk generally comprised of B. subtilis, B. coagulans and B. stearothermophilus (Shroff, 1970). Shroff and Bhat (1955) found B. subtilis as the predominant organisms in pasteurized milk in India. The incidence of spore-formers in boiled milk in India

was investigated by Kerala Varma et al. (1950) who observed that out of 93 cultures isolated, 61 per cent was B. subtilis, 18 per cent B. cereus and 13 per cent B. megaterium. However, differentiation of the species B. subtilis and B. licheniformis was not made. Galesloot (1959) reported B. subtilis species occurring in milk immediately after laboratory pasteurization, but the differential tests carried out later on these cultures showed them to be mainly B. licheniformis.

Atwal et al. (1974) tested milk products for the presence of mesophilic, thermophilic and obligate thermophilic spore-formers and noticed that the numbers of all the three types of spores were highest in spray and roller dried milk powder. Commercial condensed milk samples showed the next higher range of counts for mesophilic spores followed by cheese, ice-cream, and khoa samples which showed higher average.

Heat resistance of Bacillus organisms in Milk.

Many workers have studied the heat resistance of the bacillus organisms at different temperatures for varying time intervals. Wilson and Miles (1975) found that the vegetative forms of the bacilli were killed by moist heat at a temperature of 55°C in one hour. But the spores of the bacilli showed variation in their resistance to heat. Some

like those of B. anthracis were destroyed by boiling for about 10 minutes. The spores of B. subtilis were found to withstand boiling for hours. Most of the spores of the bacillus were killed by steam under pressure at 120°C in 40 minutes but spores embedded in the garden earth were noticed to survive upto six hours (Kurzweil, 1954). The spores of thermophilic organisms abundant in granulated sugar, syrup and spices were found to withstand boiling for 8 to 16 hours. In dry earth anthrax spores have been found to be active even after 60 years (Wilson and Russell, 1964). In canned food thermophilic spore-formers were found to survive for over 100 years (Wilson and Shipp, 1939).

The heat resistance of bacterial spores varies greatly with the conditions during sporulation. Resistance at 100°C may vary from less than a minute to over 20 hours (Frazier, 1976). Galesloot (1962) stated that the anaerobic spores are less heat resistant than aerobic ones. He also reported that pasteurized milk in Holland, contained 20,000-30,000 spore-forming bacteria per ml. Milk samples collected from organised dairy farms when subjected to laboratory pasteurization had a total spore count ranging from 200 to 1,000,000 per ml, mesophilic spore count from 10 to 4,300 per ml, facultative thermophiles from from 0 to 500 per ml and obligate thermophiles from 0 to 125 per ml. In pasteurized milk from commercial dairy plant the total spore count

averaged 11,020,000 per ml, mesophilic count 1,590 per ml, facultative thermophiles 286 per ml and obligatory thermophiles 78 per ml (Ethiraj, 1976).

Boiling of milk was found to destroy all the pathogenic organisms as well as those which cause souring and consequent coagulation of milk (Chaudhuri, 1959). Eckles et al. (1951) reported that temperature of boiling water (100°C) will destroy most organisms in a few seconds. The boiling of milk or heating in flowing steam was found to destroy all microorganisms except the spores of bacteria (Frazier, 1976). The spores of Bacillus anthracis suspended at concentration of 10^2 to 10^4 per ml in raw milk were found to survive heat treatment of 100°C for two seconds but not 110°C for two seconds (Nagasawa et al. 1970). Ridgway (1955) showed that the spores in milk were able to survive 100°C for 15 minutes and 105°C for two minutes and obtained counts of 1-5/100 ml and 200-7,000/100 ml respectively. It was also noticed that when milk heated to 100°C for two minutes it contained 200-700 mesophilic spores per 100 ml.

Burton et al. (1953), Williams et al. (1955), Franklin et al. (1956) and Williams (1958) obtained a spore count of 0-13/100 ml, 0.5-39/100 ml, 0-700/100 ml and 1-600/100 ml respectively for milk heated to 100°C for 30 minutes. By heating milk in Italy to 100°C for 30 minutes, Tentoni (1960)

got a spore count high as 1,000/ml with an average count of 150/ml. In Teheran area, Farkhondeh (1974) found the number of spore-formers resistant to heat treatment at 100°C for 30 minutes was 32/ml. Burton et al. (1953) found the thermophilic content of milk after heating to 100°C for 30 minutes was 1/100 ml. They also reported that the spores surviving 100°C for 90 minutes and 113°C for 35 minutes ranged from 0-13/100 ml and 0-5/100 ml respectively. The average number of resistant spores surviving 100°C for 30 minutes ranged from 1.3 to 10.7/100 ml in samples of milk collected from organised dairy farms and the same was 600/100 ml in the milk of commercial dairy plant (Ethiraj, 1976). The spore-formers present in Egyptian buffaloes' raw milk was found to resist heating at 100°C for 60 minutes. In farm milk Procter (1943) observed that the spores survived laboratory heat treatment of 106°C for 20 minutes. Out of the 699 samples examined 76 per cent had a count upto 100/ml, 11 per cent had counts in excess of 1,000/ml and 5 per cent in excess of 30,000/ml. Speck (1961) concluded that the most difficult group of microorganisms to be destroyed by high temperature pasteurization process would be the spore-formers.

As the spores of the bacillus organisms were not destroyed by heating at 100°C for several minutes ultra-high temperature (UHT) pasteurization process was developed to produce sterilized milk. When this process was developed in

1950 milk was generally heated to a temperature of 88 to 132°C and held at that temperature for a period of two seconds or less: However, new, Federal standards of identity stipulated that a product to be labelled ultrapasteurised, must have been heated to 137.8°C or above and held for at least two seconds. The temperature and time might go as high as 150°C and 9 seconds if sterility was required (Campbell and Marshall, 1975). Prior to the invention of UHT process the procedure adopted to produce sterilized milk involved heating milk to around 110-120°C and holding it at that temperature for 15-30 minutes (Shew, 1977). In the in-container sterilization process for producing sterilized milk the containers were heated to a temperature of 110-120°C and held at that temperature for 20-40 minutes (Burton, 1973).

Martin et al. (1966) studied the effect of ultra-high temperatures of 104.5°C, 121°C and 137.8°C for approximately one second on bacterial spores of the following species: B. licheniformis, B. pumilus, B. circulans, B. sphaericus, P. coagulans, B. laterosporus, B. megaterium, B. cereus and B. cereus mycoides added to the milk. They noticed that the low temperature of 104.5°C produced destruction percentages ranging from none for B. laterosporus to approximately 50 per cent for B. pumilus, 16 per cent for B. licheniformis, 25 per cent for B. cereus and 45 per cent for B. coagulans and B. spaericus. The percentage of the spores of all species

destroyed at a temperature of 121°C and 137.8°C was found to be greater than 97.7 and 99.99 respectively. Their findings showed that as the intensity of UHT treatment increased the number of spores surviving heat treatment decreased. Ogasa et al. (1959) found that UHT treatment at 130-135°C for two seconds decreased the number of heat resistant spore-forming bacteria to one per cent of the original value. Read et al. (1970) stated that in the recent interest of increasing the shelf life of milk products to six months under refrigeration the temperature of pasteurization might have to be increased to 150°C.

Sterility was achieved by heating milk to 135°C for 40 seconds, 130°C for 50 seconds, or 125°C for 90 seconds (Orla-Jensen, 1921). Hucker and Hucker (1929) saw the heat treatment of approximately 110°C for 1-2 seconds would destroy all mesophilic spores and 115°C for 3 minutes was necessary for the destruction of thermophilic spores which were naturally occurring in milk. Jayne-Williams (1959) reported that temperatures in the region of 135°C for a few seconds were sufficient to ensure the sterility of naturally infected milk. Franklin et al. (1958) achieved a 99.99 per cent destruction of B. subtilis spores inoculated into milk at a temperature of 130.5°C in an ultra-high temperature milk sterilizing plant while 135°C was necessary for destruction of spores suspended in water. Eckles et al. (1951) reported a temperature of 235°F to 245°F for 15 minutes was required to destroy

all microorganisms, including the resistant spore-forming type. Frazier (1976) found that the spores of B. subtilis were killed in less than 10 minutes in steam at 120°C, but in anhydrous glycerol 170°C for 30 minutes was required. Burton (1973) stated a temperature in the range of 135°C to 150°C, with holding time of about 1 to 8 seconds produced a level of sterility in milk. The UHT treatment on nine species of bacilli tested was found to effect complete destruction of all except B. coagulans and B. stearothermophilus at 120°C for 10 minutes and a temperature of 130°C for 10 minutes produced 100 per cent destruction of B. coagulans and B. stearothermophilus (Bottazzi and Battistotti, 1975).

The acceleration of inactivation of spores of B. subtilis was more by increased treatment temperature than by increased exposure time (Edwards et al. 1965). Perkin et al. (1977) observed that the heating period had only a negligible effect on treatment temperatures above 135°C. The rate of destruction of spores in milk was found to increase by 10 to 30 times for every 10°C increase in the processing temperature (Burton, 1973). The heat resistance of bacillus species isolated in NIRD/FAO Survey from milk supplies of various countries revealed that a temperature of 130°C for ten minutes was necessary for complete destruction of the

organisms (Davies, 1975). Shew (1977) stated that heating milk to 130-150°C for 2 to 4 seconds was necessary to render it completely sterile so that even the most heat resistant spore-formers were destroyed or at least rendered incapable of growth.

Bacillus stearothermophilus spores inoculated into milk when subjected to heating at 135.5 to 138°C for a holding time of two seconds, caused destruction upto 98.65 per cent only (Franklin et al. 1959). From their experimental data, it appeared that a plant operating temperature of 142°C was necessary to obtain 99.99 per cent destruction of the spores. When approximately 1,000,000 spores per ml were present, Martin and Blackwood (1970) obtained 12.5 per cent destruction while heating for three seconds at 104.5°C and 99.5 per cent at 137.7°C. Speck (1961) observed that a temperature of 140°C for three seconds appears to give sterile milk. A process of pasteurization at 148.88°C with instantaneous holding period known as 'Uperization' was observed to kill all the organisms normally found in milk. It was also found that spores showed resistance to heating at 115.5°C for three hours. The survival of the psychrophilic spore-formers such as B. lentus, B. cereus and B. licheniformis in milk after ultra-high temperature pasteurization was reported by Grosskopf and Harper (1969). A temperature of 146°C for 3.5 seconds including the 'come up'

time was adequate to inactivate spores of B. coagulans even when they were present in large numbers (Senger et al. 1963). Also the same temperature was found to be adequate to inactivate B. stearothermophilus spores if they were present in small numbers. However, Burton (1977) reported that one in 130,000 spores in milk could survive four hours heating at 150°C.

Mikolajcik (1970) reported that the heat resistance of bacillus species in milk varied between the strains studied and the heating conditions imposed. Franklin et al. (1958) found that the temperature required for destruction of B. subtilis spores suspended in water was higher than when they were suspended in milk. Mayon and Jezeski (1977) reported that the B. stearothermophilus vegetative cells sporulated in the presence of milk were more resistant to heat inactivation than spores grown on nutrient agar fortified with manganese sulphate. However, they found no difference in heat resistance between spores derived from vegetative cells grown in milk or nutrient broth when tested at 121°C. The spores of B. stearothermophilus were found to survive heat treatment at 120°C for 35 minutes in phosphate buffer with a pH of 6.95 (Hill and Fields, 1967). Vinton's (1964) research on the viability and heat resistance of spores stored in a refrigerator at 40°F for a period of 20 years showed that the spore count remained approximately at

the same level during storage, but there was a change in thermal resistance. The rough variant of B. stearothermophilus was found to be less heat resistant than smooth variant (Rotman and Fields, 1966).

Plommet (1958) found that domestic boiling of milk resulted in substantial reduction in spore count of B. cereus and B. licheniformis. Galesloot (1956) found that the spores of B. subtilis were the most thermo-resistant of the mesophilic spores present in raw milk. Franklin et al. (1956) found the B. subtilis as the predominant mesophilic spore-former in sterilized milk. Nicholas and Candy (1956) reported that the B. subtilis were the most frequently occurring mesophilic spore-former in sterilized milk in England and B. thermophilus gradually resembled B. stearothermophilus. Ridgway (1958) also observed the predominance of B. stearothermophilus in sterilized milk. B. subtilis was the most heat resistant organism among mesophilic and facultatively thermophilic aerobic spore-forming organisms (Atwal et al. 1974). Fields and Finley (1964) reported the spores of B. stearothermophilus were extremely heat resistant and the vegetative form was capable of growth at 70°C or slightly higher temperature. Janina (1966) found that the thermal stability of B. subtilis strains were generally higher than those B. cereus. Shehata et al. (1977) found that spores of

B. subtilis were the most heat resistant and B. circulans the least heat resistant in milk. However, the spores of B. cereus and B. licheniformis were shown to be the most heat resistant bacillus species in milk by Mickolajcik (1970). Galesloot (1962) observed the spores of B. cereus exhibiting less heat resistance than those of B. subtilis, but the former were found to grow more quickly than the latter.

Factors Favouring Germination of Spores.

Germination of spores occurs when the external conditions become favourable to growth by access to moisture and nutrients. Christian (1931) while working with an aerobic spore bearing bacilli isolated from tainted milk, found that germination appeared to be stimulated by heating the spores to 100°C for 30 minutes. Hiscox (1934) observed that the spores of several species were activated by sub-lethal heating and the facultative anaerobes but not strict aerobes sporulated freely in milk. Evans and Curran (1943) also noticed that heating spores of some species of aerobic spore-bearing bacilli for 10 minutes at 85°C often stimulated germination. Milk heating followed by 3 hours' incubation led to about the same degree of germination as 24 hours incubation without pre-heating. Speck (1961) reported that heat often had an activating effect on spore germination.

Mikolajcik and Koka (1968) showed that the heat treatment of the spores stimulated their germination and outgrowth. Busta and Ordal (1964) concluded that the substantial but sub-lethal heat treatment appeared to render the endospores less dormant, and in turn enhanced the germination process. Ridgway (1958) found B. subtilis to be the highly heat activated type. B. stearothermophilus was found to require an exceedingly high heat activation treatment to attain maximum spore count (Senger et al. 1963).

Mikolajcik and Rao (1974) saw that the outgrowth of B. cereus and B. megaterium spores was best in pasteurized milk and was stimulated by heat activation of 80°C for ten minutes, whilst that of B. licheniformis spores was best in autoclaved milk. Storgards (1957) reported high pasteurizing temperature destroyed acid forming bacteria of milk and thus permitted better growth of spore-formers. Spore-forming bacteria generally brought about negligible change in the raw milk since they proliferated rather slowly in the presence of other fast growing organisms but were believed to stimulate the growth of other species (Ranganathan et al. 1974).

Bottazzi and Battistotti (1975) reported that certain forms of UHT treatment might actually stimulate germination of bacillus spores. Levinson and Hyatt (1970) found that activation as measured by extent of germination was optimal

after heating at 62 to 78°C and the rate of spore germination was maximal after heating at 64 to 68°C. Sterilizing milk in bottles having a partial vacuum in the head space produced conditions inhibitory to the outgrowth of bacillus spores, as compared to the growth in the same milk sterilized in open bottles (Higginbottom and Taylor, 1960). Spores of B. circulans showed greater inhibition in milk sterilized in open bottles than in milk sterilized under partial vacuum. However, they found that the inhibitory effect of partial vacuum on spore germination could be reduced by increasing the size of the inoculum and using an incubation temperature much closer to the optimum growth temperature of the bacillus species. Wilkinson and Davies (1973) noted that the heat treatments for 15 seconds using temperatures between 65-75°C rendered the milk most suitable as a germination medium but temperatures above 80°C were necessary for spore activation. Irrespective of heat treatment, heated spores germinated and outgrew more rapidly than unheated spores thereby indicating that germination was heat activated (Koka and Mikolajcik, 1967).

Martin et al. (1966) found that the peroentage increase in outgrowth of spores heated at 121°C as compared to 104.5°C, ranged from 27 per cent for B. licheniformis to 400 per cent for B. pumilus, with an average increase of 110 per cent for

all species. The increase in outgrowth of spores heated at 137.8°C were 8, 24, 25 and 39 per cent for B. sphaericus, B. circulans, B. laterosporus, and B. megaterium respectively as compared to the outgrowth of these spores after heating at 121°C. Mikolajcik and Chawan (1971) determined the holding time for maximum outgrowth of bacillus spores in infant formulas F_1 (milk protein-lactose-vegetable fat system) and F_2 (soy protein-corn syrup-vegetable fat system) at 110, 115, 121 and 125°C as 33, 10; 8, 5: 3, 3 and 2, 2 minutes respectively. Martin and Harper (1963) reported that the aminoacids L-alanine, L-cysteine and L-valine markedly stimulated the germination of B. licheniformis spores. Levinson and Hyatt (1960) stated that the spores of B. megaterium, irradiated at dose levels sufficient to destroy the capacity for cell division, might retain the ability to germinate in glucose. Hyatt and Levinson (1957) found that a source of sulphur was required for the post germinative development of B. megaterium, but not spore germination. Cobalt (Co^{++}) and Nickel (Ni^{++}) did not affect germination. Manganese (Mn^{++}) stimulated germination, but no effect on subsequent development. Although spores would germinate without the addition of phosphate, added phosphate was required for the transition from germinated spore to vegetative cells. They also found the pH range for optimal germination was lower than that for post-germinative development.

Factors such as the temperature of heat treatment, the

presence or absence of oxygen and temperature of incubation of the pour plates seemed to influence the spore counts. Grosskopf and Harper (1969) studied the psychrophilic spore-formers identified as B. coagulans and found that they failed to grow initially at 32°C but produced spoilage in sterilized milk stored at 2°C. Also the spore-formers survived the UHT pasteurization treatment. Bachmann and Geiges (1970) obtained the maximum counts of B. cereus var albolactis when they were grown in skim milk or coffee cream at an incubation temperature of 30°C for 48 hours. The count gradually declined under anaerobic and semi-aerobic incubation, but remained constant for about 10 days when inoculated aerobically. Roth and Lively (1956) observed that certain aerobic organisms germinate spores under anaerobic conditions. Hyatt and Levinson (1959) also observed germination of B. megaterium under both aerobic and anaerobic conditions, but further development did not take place in the absence of oxygen. Buchanan and Nelson (1960) found that the count was slightly higher when plates were incubated at 35°C, than when an incubation temperature of 32°C was used in samples in which thermoduric organisms were predominantly spore-formers. Huhtanen et al. (1976) compared the effect of incubation temperatures 30 and 32°C for 48 and 72 hours on the bacterial count of raw milk. They found that plates incubated for 72 hours showed significantly higher counts than for 48 hours at both the temperatures.

Good growth of B. coagulans was obtained from 30-35°C and from pH 5 to 7.5. Little difference could be noted at levels between pH 5.5 and 7.0 or at temperatures between 35 and 55°C (Desrosier and Heiligman, 1956). Optimal germination of B. cereus-7 spores in response to L-alanine treatment occurred at 37°C and a pH of 7.0 (Chang et al. 1973).

Factors inhibiting Spore Growth.

Mikolajcik and Kearney (1969) observed that the germination time and spore germination were directly related to pH of the milk; lower the pH the greater the effect. However, outgrowth of B. cereus spores at a pH of 5.5 indicated that the organism had considerable to tolerance for mild acidic conditions. Fields and Finley (1964) stated that the concentration of carbohydrates had a significant effect on spore count of heat shocked B. stearothermophilus and the count got reduced with increased concentration. Suspending spores in reducing agents or in a pH less than 4.5 was found to limit the heat activation of spores (Keynan et al. 1964). Mikolajcik et al. (1973) concluded that vegetative cells of B. cereus failed to survive as the acidity of milk increased, but the spore count remained unchanged. Seaman (1963) found that the bacteria succumb to heat more quickly if they were in an acid or alkaline medium and the greater the divergence from neutrality the more readily they were affected. Acidity was found

to have a greater effect than alkalinity. Mikolajcik and Rao (1974) while studying the proteolytic activity of bacillus spores in raw and heated skim milk found that when heat activated and unheated refractile spores were inoculated into sterilized, pasteurized and autoclaved milk followed by incubation of 37°C all the milk system had a depressing effect on germination of spores. A direct relationship was noticed between heat treatment of milk and rate of proteolysis. A combination of heat, shocking and inducing germination with 0.1 per cent L-alanine resulted in more than 97 per cent destruction of spores in skim milk by pasteurization (Martin,1968).

Mikolajcik et al. (1965) and Hurst (1972) noticed that the lactic acid bacteria hinder development of spore-formers by production of acids, peroxides and antibiotics. Nisin the best known of the antibiotics produced by Streptococcus lactis was found to affect outgrowth of spores but not spore germination. The addition of 200 RU nisin/ml of milk prior to heat treatment at 100°C for 15 minutes was sufficient to increase the keeping quality from two days at 32°C to more than two months (El-Sadek et al. 1976a). Experimental results of Ostwal and Kulkarni (1973) showed that the antibiotics chloromycetin and terramycin exhibited greater inhibitory effects on spore-formers than penicillin and streptomycin. Pre-heat treatment at 90°C for 15 minutes followed by addition of antibiotics proved to be more effective in retarding spoilage

of milk due to spore-formers. Denny and Bohrer (1959) reported that the thermal death rate of the spores of B. stearothermophilus No. 1518 was accelerated by the addition of antibiotics like subtilin, tyrothricin and methylol gramicidin.

In an experiment Mickolajcik and Petricca (1966) studied the rate of outgrowth of B. licheniformis spores in skim milk containing 0-10 mg potassium butyrate, caproate, caprylate and caprate/ml and found that the spore outgrowth was retarded due to the presence of fatty acids. Walker (1964) found that the citrate, phthalate, or ammonium ion in the buffer usually reduced the heat resistance of spores and the survival of spores in the presence of citric acid was always less than in other phosphate containing systems.

Desrosier and Heiligman (1956) observed that additional heating acted as a lethal agent, regardless of the temperature and time, to a spore suspension which is activated to a maximum response. Srivastava (1967) saw the temperature shock produced as a result of intermittent heating and cooling destroyed not only the vegetative forms of organisms but also their spores. Milk samples heated in boiling water continuously for 90 minutes were found to get spoiled within two days of storage in summer whereas those produced in the same pan by intermittent heating and cooling could be stored for

more than two months without bacteriological spoilage as tested organoleptically and by clot on boiling test.

Busta (1966) and Ashton and Busta (1967) found that the growth of B. stearothermophilus was inhibited by raw or mildly heated milk. However, the same conditions could not inhibit the growth of B. subtilis. The inhibitor was found to be in the acid precipitated casein. The inhibition on B. stearothermophilus was partially relieved by addition of calcium and ferric ions. Martin (1968) and Martin and Blackwood (1972) while studying the effects of previous heat shock on the destruction of bacterial spores by pasteurization found that spore destruction by pasteurization was less than 20 per cent in control milk that received no heat shock and to which no L-alanine was incorporated. Cross et al. (1974) found that germination of bacillus in yeast dextrose broth medium was inhibited by bicarbonate at 5, 25, 50 mM concentration.

Defects caused by Bacillus species in Milk.

The spore-forming bacteria play an important role in the spoilage of heat treated milk. Swithinbank and Newman (1903), Buchanan and Hammer (1915), Thomas and Roberts (1946), and Orterholm (1958) observed that the spore-formers could produce ropiness or sliminess in raw and pasteurized milk.

The main species responsible for ropiness or sliminess in milk were found to be B. circulans, B. cereus and B. subtilis.

Eckles et al. (1951) reported that milk will become coagulated occasionally due to the action of a rennet like enzyme produced by the spore-forming organisms of which B. coagulans and B. subtilis were predominant: B. subtilis was responsible for sweet curdling and B. coagulans for coagulation due to production of acid. Galesloot (1953) stated that the sweet curdling caused by B. cereus was the commonest defect in samples free from post-pasteurization contamination. Coagulation in boiled and sterilized milk due to spore-formers was noticed by Thomas et al. (1967). Choudhery and Mikolajcik (1971) concluded that the sweet curd formation was associated with exponential growth phase of the organism and not with spore germination or sporulation. Ranganathan et al. (1974) reported that some of the spore-formers were capable of producing coagulation and proteolysis in milk. The spore-formers were also found to cause spoilage of pasteurized milk in association with other types of microorganism like streptococci even when they were present in small numbers.

Fish et al. (1969) observed B. subtilis causing proteolysis of milk. Mikolajcik and Rao (1968) noticed greater proteolytic activity in autoclaved milk and least in raw milk. Morgan (1943) classified three main types of spoilage in

sterilized milk: bitter flavour caused by B. coagulans, oxidised or 'card board' flavour by B. circulans and sweet curdling by B. subtilis. Cox (1975) stated that the main spoilage problem occurring in pasteurized milk was the one generally referred as 'bitty cream'. Jeanstone and Rowlands (1952) found that 76 per cent of the spore-formers responsible for 'bitty cream' belonged to the species B. cereus. The enzyme proteases produced by the psychrotrophic bacteria which survived heat treatment of 149°C for 10 seconds were found to be responsible for the rapid spoilage of sterile milk with the development of bitter flavour, clearing or coagulation (Adams et al. 1975). In evaporated milk the acid forming bacilli such as B. coagulans produced formation of a curd and a bitter taste, while B. subtilis caused a thinning of the product by digesting the casein (Seaman, 1963).

Franklin (1969) reported that the poor keeping quality of milk, subjected to high pasteurization temperature was due to the destruction of non-spore-forming microflora. Van Den Berg (1962) stated that the spore-formers were responsible for gas formation accompanied by coagulation of the milk and development of disagreeable flavour. A few species of genus Bacillus (eg. B. cereus) were reported to produce some toxins in heat treated milk capable of creating abdominal upsets and nausea in the consumers (Laxminarayana, 1978).

Reasons attributed to Heat resistance of Spores.

Powell (1953) demonstrated the presence of an unique organic component 2,6-dicarboxypyridine (dipicolinic acid - DPA) in the spore cell. All the spores examined since then contained 5-15 per cent of their dry weight as DPA and the homologous vegetative forms had none. Cruickshank et al. (1974) attributed the reason for the marked resistance of spores to several factors in which the spores differ from vegetative cells: the impermeability of their cortex and outer coat, their high content of calcium and dipicolinic acid (DPA), their low content of water and their very low metabolic and enzymic activity. The bacterial spores were found to be almost completely dehydrated and extremely resistant to heat, further dehydration and radiation (Wilson and Miles, 1975).

Martin and Chuang (1971) studied the compositional difference of heat-resistant dormant spores and heat-sensitive vegetative cells for five minerals by atomic absorption spectrophotometry and for fatty acid composition by gas chromatography of the lipid fraction. They found that the dormant spores contained considerably more calcium, manganese, magnesium and zinc than vegetative cells. Calcium was approximately 900 times greater in dormant spores but no significant difference was noticed in iron content. Martin (1976) found that the total

lipid approximated 2.89 per cent of the dry weight of vegetative cells and 2.09 per cent of the dry weight of the spores; the spores contained more long and branched chain fatty acids with odd number of carbon atoms than did the vegetative cells.

Woese and Forro (1960) reported that the spores differ from vegetative cells in their ribonucleic acid - deoxyribonucleic acid (RNA-DNA) ratios, synthetic capacities, presence of absence of certain enzymes and other constituents and state of the RNA. Murrell and Warth (1965) have suggested that the heat resistance results from a complex interaction among several spore components rather than a simple dependence of the calcium and DPA contents.

Since vegetative cells were found to contain much less calcium and totally lacking in DPA, Frobisher et al. (1974) proposed that these substances play an important part in the heat resistance of bacterial spores. Wilson and Miles (1975) attributed the resistance of spores to heat and dehydration with the synthesis of unique metabolite calcium dipicolinate which arises from the lysine biosynthetic pathway, as an alternate and product of the condensation of pyruvic acid and aspartate B-semialdehyde. Berg and Grecz (1970) linked the high content of dipicolinic acid in bacterial spores with their exceptional hardiness to physical and chemical stresses.

Davies et al. (1973) stated that calcium dipicolinate evidently played a role, by some unknown mechanism and its content markedly influenced heat resistance of the spores. The spores of B. cereus-1 lacking dipicolinic acid showed a statistically significant reduction in resistance to ultraviolet and radiation as compared with spores having high DPA content. El-Bisi et al. (1962) reported the release of all the DPA into the surrounding medium, while the spores were autoclaved or germinated. Rode and Foster (1960) opined that the death of the spores preceded the release of DPA.

Wilson and Miles (1975) found the resistance to radiation was due to a Keratin-like protein surrounding the cortex of the spore and having unusually higher number of disulphide bonds. The heat resistance of bacterial cells and spores was found to be related to the type and melting point of protoplasmic lipids (Gaughram, 1947). Vinter (1969) suggested the involvement of cystine-rich structures, presumably in the spore coat, in the protection mechanism of spores against radiation. Davies et al. (1973) reported that the various enzymes in spores were much more thermostable than the corresponding enzymes in vegetative cells. It appeared that the striking thermostability of spores depended on their internal environment rather than on intrinsic properties of their proteins. They also found that dehydration and ionic conditions were undoubtedly the major factors in stabilizing spore proteins.

Williams and Hennessee (1956) reported that the B. stearothermophilus spores heated in disodium phosphate buffer showed an apparent increase in the resistance and it increased with the decreasing molal concentration of phosphate over the range of M/15 to M/120. Bausam and Matney (1965) found that the vegetative cells of B. subtilis and B. licheniformis grown above 48°C were more thermally resistant than cells of the same strains grown below 40°C. Aoki and Slepecky (1974) reported that the spores grown on media containing increasing amounts of Mn^{2} + showed greater resistance to ultraviolet light irradiation than the spores grown on low Mn^{2}+ containing media. Eventhough, analysis of the metal and DPA content of the spores harvested from media containing various concentrations of Mn^{2}+ revealed very little difference in the Ca^{2}+, Mg^{2}+ or DPA content of the spores, a greater heat resistance was noticed in the spores of the Mn^{2}+ enriched medium. The spores of B. coagulans var thermoacidurans of high heat resistance were obtained from media enriched with Mn^{2}+ and Ca^{2}+ (Amaha and Ordal, 1957). It was found that B. megaterium spores formed in the presence of high Mn^{2}+ in the medium exhibited greater resistance to ultraviolet and X-ray irradiation than spores formed in a medium of low Mn^{2}+.

Hanson et al. (1972) recently isolated mutants of B. cereus producing thermoresistant spores lacking dipicolinate

and having low levels of calcium. Spores were found to retain their resistance to heat and ultraviolet light when the spore coats were removed by chemical treatment or mutants without coats were obtained. The resistance of the spores was found to be restricted to areas other than the spore coat. The DNA and RNA molecules located in the core were found to exhibit different degrees of thermostability (Cassier and Ryter, 1971).

Factors Influencing heat resistance of Spore-forming Bacteria.

Factors such as composition of media, pH, concentration and age of spores and temperature were found to influence the heat resistance of spore-forming bacteria. The heat resistance of spores of B. subtilis having an initial resistance of six minutes when exposed to 105°C was found to change when the media in which the spores formed were altered (Williams, 1929). The spores could be killed more easily by heat in an acid medium than in an alkaline medium, and the greatest resistance was noticed when the medium was near its neutral point (Weiss, 1921). It was reported that greater the concentration of spores in the medium the more difficult it was to destroy them (Watkins and Winslow, 1932). Spores suspended in a medium containing fat were found to be protected from heat and such spores seemed to survive only in the

absence of moisture in fat (Ragacheva, 1939). Old spores were found to be more resistant than young spores by Magoon (1926). Spores formed at temperatures near the optimum temperatures for growth were more resistant than those formed at temperatures below the optimum (Theophilus and Hammer, 1938).

Keeping Quality of Milk.

Besides satisfactory compositional and physical standards milk should have adequate keeping quality. The keeping quality of a sample of milk is the period in hours which lapses from its production until it is considered unsuitable for consumption either because it curdles on boiling or develops an undesirable odour or flavour. Throughout this period the milk is held at a constant temperature. The chief factors influencing the keeping quality of raw milk are the bacterial flora, the mineral salt balance, season of the year, temperature and degree of agitation during transit and the development of physical and chemical reactions. In general the keeping quality can be correlated with the bacterial population of milk.

The keeping quality of raw milk produced under the farm conditions and stored at room temperature was reported to be 8 hours (Moorthy, 1966). According to Sinha and Nambudripad (1973), even if the milk is produced under clean

conditions and stored in clean sanitised containers it can keep only for 16 to 18 hours at 30°C. The rate at which the spoilage takes place depends upon the extent of microbial load as well as the temperature at which the milk is held subsequent to production. If milk is cooled immediately after production, the keeping quality of milk can be considerably increased. Kerala Varma et al. (1959) reported that pasteurization brought about destruction of more than 90 per cent of the bacterial population of milk, thereby enhanced its keeping quality. For enhancing the keeping quality, however, chief reliance was placed on the maintenance of a high standard of quality in the raw milk and the efficient cooling and refrigerated storage of the pasteurized milk. They found that the average keeping quality of raw milk sample collected from three different milk plants were 6 hours 30 minutes, 7 hours 15 minutes and 9 hours 30 minutes. Ranganathan et al. (1974) found that during the summer months quality of milk at the time of reception is so poor that the milk cannot be expected to remain in liquid condition for more than one hour. The milk supply of such a quality when heat treated will keep for 6 to 8 hours at normal handling temperature.

Anantakrishnan et al. (1946) found that the milk samples kept at a temperature of 130 ± 40°F did not curdle upto 48 hours. They also stated that boiling of milk for

5 to 10 minutes increased the keeping quality by another 2 to 3 hours. The keeping quality of milk heated for 16 seconds at various temperatures from 162 to 180°F ranged from 17 to 22 hours (Kerala Varma et al. 1959). Burgwald and Josephson (1947) reported that milk of good quality could be expected to retain excellent bacteriological and flavour qualities for at least four days during the summer months and six to seven days during the winter months provided the refrigeration temperatures are maintained near 40°F. Boyd et al. (1955) reported that when the storage temperature was lowered from 40 to 33°F the keeping was found to be extended 11 to 14 days. Investigations carried out at the National Dairy Research Institute, Karnal, indicated that market milk supplies in India generally contained many thermoduric flora and that pasteurized milk underwent spoilage within 12-20 hours at atmospheric temperatures of 30-37°C (Iya,1962). Ethiraj (1976) observed that the keeping quality of pasteurized milk from organised dairy farm varied from 18.3 to 23.3 hours when stored at room temperature. In the case of commercial dairy plant the keeping quality was found to be 9.3 hours.

Franklin et al. (1958) reported that pasteurization at 206°F for three seconds was much superior bacteriologically to that at 176°F for 16 seconds and it increased the shelf life to 30 days when the milk was stored at 45°F. Milk

pasteurized at 220°F for two seconds was claimed to keep well in excess of three weeks.

Thomas et al. (1967) reported that increased relative incidence of milk spoilage strains of aerobic spore-formers in the thermoduric microflora could pose a new problem in the deterioration of pasteurized milk. Franklin (1969) observed that although other thermodurics were present to the same extent as B. cereus immediately after pasteurization, the latter species developed to predominate in most of the milks at the end point of keeping quality.

Ogasa et al. (1959) reported that the UHT treated milk showed no spoilage for seven days in the refrigerator or for one day at room temperature. Milk treated at 75°C for 15 minutes was safe only for four days in refrigerator and less than one day at room temperature. Moreover they found that all samples treated at 75°C for 15 minutes become spoiled after 8-10 days of refrigerated storage. The spoiling of the samples which had received UHT treatment was less than 50 per cent even after 20 days of storage.

MATERIALS AND METHODS

MATERIALS AND METHODS

A total of 48 samples of raw milk, 16 each from three different sources such as the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households were collected and used for the study.

Collection of Samples.

The collection of samples of milk was done from November 1977 to February 1978. A quantity of 250 ml of raw milk was collected for each sample in sterile glass bottles with all precautions to avoid contamination at the time of collection. The procedures laid down in Indian Standard Institute (IS: 1479, Part I, 1960) for the collection of samples for bacteriological purposes were adopted. The samples were stored at a temperature of 4.5°C till taken out for the various tests. The samples were examined within two hours from the time of collection.

The tests given under were carried out in the samples collected.

Aerobic Spore Count.

The aerobic spore count of the milk samples collected was determined by subjecting the samples to laboratory boiling and plating them in agar plates.

Five ml quantities of well mixed samples of milk were pipetted into previously sterilized 6" x 5/8" pyrex glass test tubes of uniform thickness. The tubes were closed with sterile non-absorbant cotton plugs and placed in an electrically operated thermostatically controlled boiling water bath to which some salt was added for maintaining the temperature a little over 100°C. The level of water was maintained at least one inch above the level of the milk in the test tube. A test tube containing 5 ml of milk with a thermometer inserted in it was placed in the water bath to record the correct temperature of the bath and it served as the control. The period of holding was recorded from the time the temperature in the control tube reached 100.5°C. All the tubes were held in the water bath for one minute. At the end of one minute holding period, the tubes were removed from the water bath and rapidly cooled to a temperature below 30°C using tap water and with intermittent shaking.

Plating.

Plating was done according to the standard procedure (APHA, 1953) using plate count agar and one in ten dilution of laboratory boiled milk. The plate count agar having the following composition was used for the plate count.

Plate count agar

Bacto - Yeast extract	2.5 g
Bacto - Tryptone	5.0 g
Bacto - Dextrose	1.0 g
Bacto - Agar	15.0 g
Distilled water	1000 ml

Final pH 7.0 ± 0.1

The plates were incubated at 37°C for 48 hours and then the total number of colonies were counted.

Isolation of the Colonies.

Isolations from the colonies were made from the counted plates. The number of colonies in a plate ranged from one to twenty four. For purposes of isolation a minimum of one and a maximum of six colonies from each plate were utilised.

The selected colonies were transferred to Nutrient Broth (Cowan, 1974) having the following compositions.

Nutrient Broth

Proteose peptone	10 g
Beaf extract	10 g
Sodium chloride (Nacl)	5 g
Distilled water	1000 ml

After filtering, the pH of the nutrient broth was adjusted to 7.2 to 7.4. The medium was dispensed in test tubes which were then autoclaved at 115°C for 20 minutes. After attaining the room temperature the tubes containing nutrient broth was inoculated and incubated at 37°C. At the end of 24 hours' incubation, characters such as turbidity, formation of surface scum, heavy flocculant or membranous deposit and motility were studied. From the broth tubes the cultures were streaked on Nutrient Agar (1.5% agar added to nutrient broth) slants. The slants were incubated at 37°C and at the end of 48 hours the morphological characters were studied.

Preliminary Studies.

Morphological Characters.

Smears prepared from the selected colonies were stained by the Gram's method. Morphological characters like shape, size and arrangement of cells, Gram's reaction, presence or absence of spores, shape and arrangement of the spores, and swelling of the bacillary body were noted.

Catalase test.

The ability of the organism to produce the enzyme catalase was tested by placing a drop of three per cent hydrogen peroxide over the portion of growth taken from nutrient agar slants and observing effervescence.

Motility.

Motility was tested by microscopic examination of a fresh cell suspension of the culture by hanging drop method.

Sporulation.

Smears stained by the Gram's method was examined for sporulation. In doubtful cases the period of incubation was extended for another 72 hours, stained and examined for the presence of spores.

Identification.

After the preliminary tests, all the spore-forming, motile, Gram-positive and catalase-positive organisms were tested further. The selected colonies were transferred to tryptone dextrose agar slants in duplicate. One set of cultures was stored in the refrigerator and the other was used for further identification by conducting biochemical tests as per Kerala Varma (1949), Cowan (1974) and Wilson and Miles (1975). The following tests were carried out on the isolates.

Citrate Utilization Test.

Citrate utilization by the isolated organisms were

studied using the Christensen's citrate medium having the following composition (Cowan, 1974).

Sodium citrate	3.0 g
Glucose	0.2 g
Yeast extract	0.5 g
Potassium dihydrogen phosphate	1.0 g
Sodium chloride	5.0 g
Agar	20.0 g
Distilled water	1000 ml
Phenol red (0.2% solution)	6 ml

The pH of the medium was adjusted to 6.8 to 6.9 and sterilized at 115°C for 20 minutes.

(Note: Since hydrogen sulphide production was not determined, cysteine, sodium thiosulphate and ferric ammonium citrate were not included in the medium used).

Four plates of the citrate medium were prepared. The plates were streaked with test cultures and incubated at 32°C for 24 hours. Development of magenta colour indicated utilization of citrate.

Hydrolysis of Starch.

The hydrolysis of starch was tested using starch agar medium (Nutrient broth containing 1.5% bacteriological agar

and 1% soluble starch). Starch agar plates were streaked with test cultures and incubated at 32°C for 24 hours. Gram's iodine was layered on the surface of the plates. A clear zone around the line of streaking indicated the hydrolysis of starch, the rest of the area being dark blue in colour.

Liquefaction of Gelatin.

Gelatin agar having the following composition was used for testing liquefaction of gelatin.

Gelatin	4 g
Distilled water	50 ml
Nutrient agar	1000 ml

The gelatin was mixed with nutrient agar and autoclaved at 15 lbs pressure for 20 minutes. This medium was used for streaking the test cultures. After incubation at 32°C for 24 hours the liquefaction of gelatin was tested by layering mercuric chloride solution having the following composition.

Mercuric chloride	12 g
Distilled water	80 ml
Hydrochloric acid (conc.)	16 ml

A clear zone around the colonies indicated liquefaction of gelatin.

Production of Indole.

The medium used for the test was Dunham's solution having a composition given hereunder.

Bacto tryptone	10 g
Sodium chloride	5 g
Distilled water	1000 ml

The solution was filtered and the pH was adjusted to 7.0. The medium was dispensed in 5 ml. quantities in test tubes and autoclaved at 15 lbs pressure for 20 minutes. After cooling the medium to room temperature the tubes were inoculated with the test cultures and incubated at 32°C for 48 hours. To each of the tubes 0.5 ml of Kovacs' reagent (Para dimethyl amino benzaldehyde 5 g; amyl alcohol 75 ml and hydrochloric acid conc. 25 ml) was added and the contents mixed well by shaking. The tubes were then allowed to stand for one minute. A red colour in the reagent layer was taken as the indication for production of indole.

Reduction of Nitrates.

The composition of nitrate broth used in the experiment was as follows:-

Dunhams' solution	100 ml
Potassium nitrate	0.2 g
Distilled water	1000 ml

The solution was filtered and the pH was adjusted to 7.0. The clear solution was dispensed in 5 ml quantities in test tubes and autoclaved at 15 lbs pressure for 20 minutes.

After cooling the solution to room temperature, it was inoculated with the test culture. The tubes were then incubated at 32°C for 48 hours. Reduction of nitrate was tested by adding a mixture containing 1 ml each of the test solutions A and B having the following composition.

Test solution A

Alpha-naphthalamine	5.0 g
Acetic acid (5N)	1000 ml

The solution was mixed well and filtered.

Test solution B

Sulphanilic acid	8.0 g
Acetic acid (5N)	1000 ml

The solution was mixed well and filtered.

The development of red colour after adding the reagent indicated reduction of nitrate to nitrite.

A pinch of zinc powder was added to the negative tubes and gently shaken. If the colour did not develop, it was

concluded that the organisms had reduced the nitrate beyond nitrite level such as ammonia or nitrogen.

Voges-Proskauer Reaction.

The Voges-Proskauer reaction was studied using the medium having the composition given hereunder.

Bacto peptone	10 g
Glucose	5 g
Distilled water	1000 ml

The pH was adjusted to 7.6 after filtering the medium. The medium was taken in 5 ml quantities into tubes and sterilized at 115°C for 10 minutes using solid bottomed container.

The tubes were cooled to the room temperature, inoculated with test culture and incubated at 32°C for 48 hours. Acetyl methyl carbinol production was tested by adding 0.6 ml of 5 per cent α-naphthol in ethanol solution and 0.2 ml of 40 per cent aquous solution of potassium hydroxide. The tubes were shaken gently, kept in a slanting position and examined after 15 minutes. The development of a strong red colour was taken as the positive reaction.

Fermentation of Sugars.

The fermentation of sugars such as glucose, mannitol and arabinose was studied using proteose peptone broth having

the composition given below as the basal medium.

Bacto peptone	10 g
Meat extract	3 g
Sodium chloride	5 g
Distilled water	1000 ml
Brom cresol purple (0.2% solution)	10 ml

After dissolving the solids in water the indicator was added. The pH was adjusted to 7.1 to 7.2. Sterilization was carried out at 115°C for 20 minutes. The appropriate sugar was added aseptically at one per cent level. The contents were mixed and distributed in 5 ml quantities into sterile tubes containing inverted Durham's tubes and steamed for 30 minutes.

After cooling the tubes to room temperature, they were inoculated with the test cultures. The tubes were incubated at 32°C for 48 hours and the production of acid and gas was noted. The negative tubes were incubated upto one week and examined again for acid and gas.

Seven per cent Sodium chloride Medium.

Nutrient broth containing seven per cent sodium chloride was used as the medium for determining the growth. The medium was dispensed in 5 ml quantities into test tubes and autoclaved at 15 lbs pressure for 15 minutes.

The tubes after attaining the room temperature were inoculated with the test cultures and incubated at 32°C for 48 hours. At the end of 48 hours incubation the tubes were examined for the presence of growth.

Casein Hydrolysis.

Casein hydrolysis by the isolates was studied using casein agar (milk agar) having the following composition.

Skim milk	500 ml
Nutrient agar, double strength	500 ml

Skim milk obtained after separating the fresh whole milk was sterilized at 115°C for ten minutes and, cooled to about 50°C. The double strength nutrient agar melted and cooled to 50-55°C was added to this, mixed well and distributed in petridishes.

The casein agar plates were inoculated with the organisms, incubated at 37°C for 48 hours and examined. A clear zone around the colonies was taken as the positive reaction.

Urease Activity.

The urease activity of the isolated cultures was examined using Stuart, Van Stratum and Rustigian (SSR) medium having the following composition (Cowan, 1974):

Potassium dihydrogen phosphate	9.1 g
Disodium ortho hydrogen phosphate (anhydrous)	9.5 g
Yeast extract	0.1 g
Urea	20.0 g
Phenol red (0.2% solution)	5.0 ml
Distilled water	1000 ml

The solids were dissolved in water without heating and the pH was adjusted to 6.8. The medium was sterilized by filtration and distributed in 5 ml quantities in sterile tubes observing aseptic conditions.

The tubes were inoculated with the test cultures and incubated at 37°C for 48 hours. At the end of the incubation period the tubes were examined for hydrolysis. The development of red colour was taken as an indication of the positive reaction.

Lecithovitellin Reaction.

For studying the above reaction of the isolated cultures lecithovitellin agar having the following composition was used:

Lecithovitellin solution (egg-yolk saline 5% W/V egg yolk)	100 ml
Nutrient agar	900 ml

The nutrient agar was melted and cooled to about 55°C. The lecithovitellin solution (previously sterilized by filtration through a bacteria-proof filter) was added aseptically mixed and poured into plates.

The plates inoculated with the organisms were incubated at 37°C for 48 hours and examined for (i) growth (ii) opalescence within the medium and (iii) 'Pearly layer' formation over and around the colonies. The positive reaction was constituted by (ii) and (iii).

Keeping Quality of Milk Samples.

From each of the well mixed sample of milk collected from different sources, a quantity of 5 ml was transferred to each of 64 numbers of previously sterilized test tubes. From this a total of 32 tubes containing the raw milk were placed in boiling water bath for one minute and after cooling the tubes were kept for storage as follows: Six at 37°C in an incubator, six at room temperature and 20 at 4°C. The remaining 32 tubes were also distributed as indicated above but without any heat treatment.

The keeping quality of the milk samples, both raw and boiled was examined at intervals of four hours of storage upto a period of 24 hours and subsequently at intervals of 24 hours. The keeping quality was assessed by the physical examination

and clot on boiling test and tubes showing clot formation were removed as positive. The time required for the samples of milk stored at different temperatures to give the clot formation was noted as the keeping quality of the sample.

RESULTS

RESULTS

The incidence and distribution of aerobic spore-formers in 48 samples of boiled cow's milk collected from three different sources are presented in Tables 1 to 9.

The aerobic spore count per ml in the samples of milk collected from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households around Trichur Town and subjected to boiling are shown in the Table 1. The average spore count in the samples of milk collected from the farm, milk supply union and individual households was 70, 87 and 50/ml respectively. Three of the samples collected from households and one from the farm had the lowest count of 10/ml.

The geometric mean and the range of spore count for milk samples of different sources are presented in Table 2. The geometric mean of the spore count per ml of the samples from the farm was 53.71 as against 81.75 and 35.89 obtained for the samples from milk supply union and individual households respectively. The spore count for samples from the farm ranged from 10-190/ml; whereas the range was 40-155 for samples obtained from milk supply union and 10-150/ml for those collected from individual households. The samples

collected from the farm revealed the maximum spore count of 190/ml as against the count of 155 and 150/ml obtained for the samples collected from the milk supply union and individual houses respectively. None of the samples collected had a spore count less than 40/ml from the milk supply union.

The analysis of the variance table (Table 3) for the spore count in boiled milk from the three different sources indicated that the effect of the sources were significantly different ($P < 0.01$). The pairwise comparison showed that there was a highly significant difference ($P < 0.01$) between the samples collected from the milk supply union and those from individual households. But no significant difference was noticed between the samples obtained from the farm and those of the milk supply union or individual households.

The frequency distribution of the samples based on the aerobic spore count per ml of milk is shown in Table 4. Out of the 48 milk samples studied 17 samples had a spore count of less than 50/ml; 21 samples 50-100 spores per ml; five samples 100-150 spore per ml and five samples have 150 spores per ml. Of the 17 samples having a spore count of less than 50/ml seven were of the University Livestock Farm, Mannuthy, one from the Co-operative Milk Supply Union, Trichur and nine from the individual households. Among the 21 samples having a count of 50-110 spores per ml, five samples were from

the Livestock Farm, 11 from Co-operative Milk Supply Union and five from the individual households. Of the five samples having a count of 100-150 spores per ml, two each were from the Livestock Farm and Co-operative Milk Supply Union, and one was from the individual households. The five samples of milk having a count above 150 spores per ml were contributed two each by Farm and Co-operative Milk Supply Union and one from individual households.

The incidence and distribution of the different types of the aerobic spore-formers isolated from the 48 samples of boiled milk are presented in Table 5. The total of 162 isolates were identified as Bacillus subtilis 85 (52.47%); Bacillus cereus 23 (14.20%), Bacillus pumilus 20 (12.35%), Bacillus licheniformis 15 (9.26%), Bacillus megaterium 11 (6.79%), Bacillus alvei 6 (3.70%) and Bacillus firmis 2 (1.23%). The characters of these organisms are presented in Table 7. The photographs of the different organisms isolated from boiled milk are presented in Plate Nos. I-III.

In order to identify the species of the organisms, all the 162 isolates were subjected to cultural, morphological and biochemical tests. The cultural, morphological and biochemical characters of the bacillus species isolated from boiled milk are presented in Table 6. The details of the characters studied and tests carried out for distinguishing the various species of bacillus organisms are given in

Table 7. The number and types of the various species of spore-formers isolated from the samples of milk collected from the different sources are presented in Table 8. Out of the 162 isolates selected for identification of the species of the bacilli, 56 were from samples obtained from the University Livestock Farm, 62 from the Co-operative Milk Supply Union and 44 from individual households.

From the 56 isolates that were selected from the samples of milk collected from the University Livestock Farm, the following species were identified: B. subtilis 27, B. cereus 8, B. pumilus 5, B. licheniformis 6, B. megaterium 7, B. alvei 2 and B. firmis 1. The 62 isolates from milk samples of the Co-operative Milk Supply Union were identified as B. subtilis 34, B. cereus 9, B. pumilus 6, B. licheniformis 7, B. megaterium 3, B. alvei 2 and B. firmis 1. The organisms identified from the 44 isolates of the samples of milk collected from the individual households were B. subtilis 24, B. cereus 6, B. pumilus 9, B. licheniformis 2, B. megaterium 1 and B. alvei 2.

The statistical analysis of the data presented in Table 8 was done by applying Chi square Test. The table value of Chi square allowing ten degrees of freedom at 5 per cent level was (18.307) found to be greater than the calculated value (9.396). This indicated that there was no significant

difference between the samples collected from the various sources with regard to the types of bacillus organisms present in them.

The percentage of incidence of the different types of bacillus organisms in boiled milk samples are given in Table 9. The incidence (percentage) in the samples of milk collected from the Farm, Co-operative Milk Supply Union and individual households for the different species was as follows: B. subtilis 48.214, 54.839, 54.545; B. cereus 14.286, 14.516, 13.636; B. pumilus 8.929, 9.677, 20.455; B. licheniformis 10.714, 11.290, 4.545; B. megaterium 12.500, 4.839, 2.273; B. alvei 3.571, 3.226, 4.545; and B. firmis 1.786, 1.613.

Keeping Quality.

The keeping quality of samples of raw milk collected from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households around Trichur Town, scored at 37°C, room temperature (29°C) and refrigeration temperature (4°C) is presented in Tables 10 to 12. The keeping quality of the samples from the same sources stored at 37°C, room temperature and refrigeration temperature after boiling is shown in Tables 13 to 15.

The keeping quality of the samples of raw milk collected from different sources and stored at 37°C is presented in Table 10. All the samples collected from different sources had a keeping quality of less than 12 hours. Out of the total of 48 samples 34 showed a positive reaction for clot on boiling test at the end of eight hours of storage. The remaining 14 samples proved positive for clot on boiling test at the end of twelve hours of storage. Of the 34 samples having less than eight hours of keeping quality, 11 were from the University Livestock Farm, Mannuthy, 16 from Co-operative Milk Supply Union, Trichur and seven from individual houses. With regard to the 14 samples having a keeping quality of more than eight hours but less than 12 hours, five were from the University Livestock Farm, Mannuthy and nine belonged to individual households. None of the 16 samples of milk collected from the Co-operative Milk Supply Union, Trichur had a keeping quality of more than eight hours.

Regarding the keeping quality of raw milk samples stored at room temperature of 29°C (Table 11) 20 samples got clotted on boiling at the end of eight hours of storage. Twenty eight samples had a keeping quality of more than eight hours but less than 12 hours. None of the samples indicated a keeping quality of more than 12 hours. Of the 20 samples that had a keeping quality of less than eight hours, four were from the University Livestock Farm, Mannuthy and 16 from the

Co-operative Milk Supply Union, Trichur. All the samples from the individual households had a keeping quality of more than eight hours but less than 12 hours. Out of the 28 samples that gave a positive reaction for clot on boiling test at the end of 12 hours of storage, 12 were from the University Livestock Farm, Mannuthy and the remaining 16 from individual households.

The keeping quality of samples of raw milk stored at refrigeration temperature (4°C) is presented in Table 12. Forty one out of the 48 samples, gave a positive result for clot on boiling test at the end of five days of storage. Seven samples showed clot on boiling at the end of six days of storage. None of the 48 samples had a keeping quality of more than six days. Of the 41 samples having a keeping quality of five days, 14 were from the University Livestock Farm, Mannuthy, 16 from the Co-operative Milk Supply Union, Trichur and 11 from the individual households. Among the seven samples having a keeping quality of six days, two were of the University Livestock Farm, Mannuthy and five from individual households.

The keeping quality of samples of boiled milk stored at 37°C is given in Table 13. None of the samples had a keeping quality of more than 12 hours. Only four out of 48 samples had a keeping quality of less than eight hours.

These four samples were from the Co-operative Milk Supply Union, Trichur. The remaining 44 samples of boiled milk showed positive reaction for clot on boiling test at 12 hours of storage. This indicated that the keeping quality of those samples was more than eight hours but less than 12 hours. Of the 44 samples of boiled milk that had a keeping quality of 8-12 hours, 16 were from the University Livestock Farm, Mannuthy, 12 from the Co-operative Milk Supply Union, Trichur and 16 from the individual houses.

The keeping quality of boiled milk stored at room temperature (29°C) is presented in Table 14. All the samples had a keeping quality of not less than eight hours. But none of the samples had a keeping quality of more than 20 hours. The number of samples which had a keeping quality less than 12, 16 and 20 hours were 38, 8 and 2 respectively. Out of the 38 samples which had a keeping quality of less than 12 hours, 13 were from the University Livestock Farm, Mannuthy, 14 from the Co-operative Milk Supply Union, Trichur and 11 from the individual households. The number of samples collected from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households that showed a keeping quality of not less than 16 hours was 1, 2 and 5 respectively. Two of the samples collected from the University Livestock Farm, Mannuthy had a keeping quality of more than 16 hours but less than 20 hours. None

of the samples of boiled milk from the Co-operative Milk Supply Union, Trichur and individual households had a keeping quality of more than 16 hours.

The keeping quality of boiled milk stored at refrigeration temperature (4°C) is presented in the Table 15. All the samples had a keeping quality of more than 12 days and the maximum keeping quality was found to be 16 days. The number of samples that gave a positive reaction to COB test at the end of 13, 14, 15 and 16 days was 19, 20, 6 and 3 respectively. Out of the 19 samples having a keeping quality of 13 days, two were from the University Livestock Farm, Mannuthy, 10 from the Co-operative Milk Supply Union, Trichur and 7 from the individual households. Among the 20 samples having a keeping quality of 14 days, seven belonged to the University Livestock Farm, Mannuthy, six to Co-operative Milk Supply Union, Trichur and seven to individual households. Four samples of University Livestock Farm, Mannuthy and two of the individual households had a keeping quality of 15 days. All the three samples which showed a keeping quality of 16 days were from the University Livestock Farm, Mannuthy.

The samples of raw and boiled milk collected from the three different sources giving a positive reaction to clot on boiling (COB) test on storage at 37°C, 29°C and 4°C is represented in Figs. 1, 2 and 3 respectively.

TABLES

Table 1. Average spore count per ml of boiled milk samples from different sources.

Sample number	Source		
	Co-operative Milk Supply Union	University Live-stock Farm	Individual households
1	55	25	10
2	135	30	25
3	135	65	30
4	120	190	30
5	65	100	70
6	90	110	35
7	80	20	105
8	40	55	70
9	60	170	60
10	85	45	85
11	90	10	150
12	65	85	65
13	155	80	15
14	60	30	10
15	75	65	25
16	85	40	10
Total	1395	1120	795
Average	87.19	70.00	49.69

Table 2. Aerobic spore count per ml in boiled milk from different sources.

Sl.No.	Source of the sample	No. of samples	Spore count per ml	
			Geometric mean	Range of count
1	University Livestock Farm, Mannuthy	16	53.71	10-190
2	Co-operative Milk Supply Union, Trichur	16	81.75	40-155
3	Individual households	16	35.89	10-150

Table 3. Analysis of Variance Table for spore count in milk.

Source	df	SS	MSS	F
Source of collection	2	1.0229	0.5115	5.2896**
Error	45	4.3521	0.0967	
Total	47	5.3750		

** Significant at 1% level.

Mean: Farm 1.7301, Milk Supply Union 1.9125, Individuals 1.5550.

Pairwise comparison:

Between sources $T_1 - T_2 = 1.7301-1.9125 = 0.1824$

$T_1 - T_3 = 1.7301-1.5550 = 0.1751$

$T_2 - T_3 = 1.9125-1.5550 = 0.3575**$

Table 4. Frequency distribution of samples based on the spore count per ml of milk.

Sl. No.	Source of sample	No. of samples	Less than 50 spores per ml	50-100 spores per ml	100-150 spores per ml	Above 150 spores per ml
1	University Livestock Farm, Mannuthy	16	7	5	2	2
2	Co-operative Milk Supply Union, Trichur	16	1	11	2	2
3	Individual households	16	9	5	1	1
	Total	48	17	21	5	5

Table 5. Incidence and distribution of different types of spore-formers in boiled milk samples.

Sl.No.	Species of organism	Number	Percentage
1	Bacillus subtilis	85	52.47
2	Bacillus cereus	23	14.20
3	Bacillus pumilus	20	12.35
4	Bacillus licheniformis	15	9.26
5	Bacillus megaterium	11	6.79
6	Bacillus alvei	6	3.70
7	Bacillus firmis	2	1.23
	Total	162	100.00

Table 6. Morphological, cultural and biochemical characters of bacillus species isolated from samples of boiled milk.

Sl.No.	Characters	Number of isolates	
		Positive	Negative
1	Gram reaction	162	0
2	Motility	162	0
3	Spore shape (oval)	162	0
4	Spore position:		
	Central/subterminal	162	0
	Terminal	0	162
5	Swelling of bacillary body	6	156
6	Growth in 7% Nacl.	156	6
7	Utilization of citrate	154	8
8	Carbohydrates, acid from:		
	a) Glucose	162	0
	b) Arabinose	133	29
	c) Mannitol	130	32
	d) Xylose	133	29
9	Voges-Proskauer reaction	160	2
10	Starch hydrolysis	142	20
11	Nitrate reduction	136	26
12	Indole production	6	156
13	Gelatin hydrolysis	162	0
14	Casein hydrolysis	162	0
15	Urease	134	28
16	Lecithovitellin reaction	23	139

Table 7. Characters studied and tests carried out to distinguish the different species of bacillus organisms.

Sl. No.	Characters/Tests	B. subtilis	B. cereus	B. pumilus	B. licheni-formis	B. mega-terium	B. alvei	B. firmis
1	Gram reaction	+	+	+	+	+	+	+
2	Motility	+	+	+	+	+	-	+
3	Spore shape	x	x	x	x	x	x	x
4	Spore position	U	U	U	U	U	U	U
5	Swelling of the bacillary body	-	-	-	-	-	+	-
6	Growth in 7% Nacl.	+	+	+	+	+	-	+
7	Utilization of citrate	+	+	+	+	+	-	-
8	Carbohydrates: acid from							
	a) Glucose	+	+	+	+f	+	+	+
	b) Arabinose	+	-	+	+	d	-	d
	c) Mannitol	+	-	+	+	d	-	+
	d) Xylose	+	-	+	+	d	-	d
9	Voges-Proskauer reaction	+	+	+	+	+	+	-
10	Starch hydrolysis	+	+	-	+	+	+	+
11	Nitrate reduction	+	+	-	+	d	-	d
12	Indole production	-	-	-	-	-	+	-
13	Gelatin hydrolysis	+	+	+	+	+	+	+
14	Casein hydrolysis	+	+	+	+	+	+	+
15	Urease	d	d	-	d	d	-	-
16	Lecithovitellin reaction	-	+	-	-	-	-	-

d positive in 3%, unknown 7%
f gas may be produced on suitable medium.
U central spore
x spore oval

Table 8. The number and types of aerobic spore-formers isolated from boiled milk.

Sl.No.	Source of the sample	No. of samples	Total no. of isolates	Number of aerobic spore-formers						
				B. subtilis	B. cereus	B. pumilus	B. licheniformis	B. megaterium	B. alvei	B. firmis
1	University Livestock Farm, Mannuthy	16	56	27	8	5	6	7	2	1
2	Co-operative Milk Supply Union, Trichur	16	62	34	9	6	7	3	2	1
3	Individual households	16	44	24	6	9	2	1	2	-
	Total	48	162	85	23	20	15	11	6	2

Table 9. The incidence (percentage) of various types of aerobic spore-formers in boiled milk.

Sl.No.	Source of the sample	No. of samples	Total no. of isolates	Percentage of aerobic spore-formers							
				B. subtilis	*B. cereus*	*B. pumilus*	*B. licheniformis*	*B. megaterium*	*B. alvei*	*B. firmis*	Total
1	University Livestock Farm, Mannuthy	16	56	48.214	14.286	8.929	10.714	12.500	3.571	1.786	100
2	Co-operative Milk Supply Union, Trichur	16	62	54.839	14.516	9.677	11.290	4.839	3.226	1.613	100
3	Individual households	16	44	54.545	13.636	20.455	4.545	2.273	4.545	-	100

Table 10. Keeping quality of samples of raw milk stored at 37°C.

Sl. No.	Source of the sample	No. of samples	No. of sample showing positive COB Test at the end of		
			4 hrs.	8 hrs.	12hrs.
1	University Livestock Farm, Mannuthy	16	-	11	5
2	Co-operative Milk Supply Union, Trichur	16	-	16	-
3	Individual house-holds	16	-	7	9
	Total	48	-	34	14

Table 11. Keeping quality of samples of raw milk stored at room temperature (29°C).

Sl. No.	Source of the sample	No. of samples	No. of sample showing positive COB Test at the end of		
			4 hrs.	8 hrs.	12 hrs
1	University Livestock Farm, Mannuthy	16	-	4	12
2	Co-operative Milk Supply Union, Trichur	16	-	16	-
3	Individual households	16	-	-	16
	Total	48	-	20	28

Table 12. Keeping quality of samples of raw milk stored at refrigeration temperature (4°C).

Sl. No.	Source of the sample	No. of samples	No. of samples showing positive COB Test at the end of					
			1 day	2 days	3 days	4 days	5days	6days
1	University Livestock Farm, Mannuthy	16	-	-	-	-	14	2
2	Co-operative Milk Supply Union, Trichur	16	-	-	-	-	16	-
3	Individual households	16	-	-	-	-	11	5
	Total	48	-	-	-	-	41	7

Table 13. Keeping quality of samples of boiled milk stored at 37°C.

Sl. No.	Source of the sample	No. of samples	No. of samples showing positive COB Test at the end of		
			4 hrs	8 hrs	12 hrs
1	University Livestock Farm, Mannuthy	16	-	-	16
2	Co-operative Milk Supply Union, Trichur	16	-	4	12
3	Individual households	16	-	-	16
	Total	48	-	4	44

Table 14. Keeping quality of samples of boiled milk stored at room temperature (29°C).

Sl. No.	Source of the samples	No. of samples	No. of samples showing positive COB Test at the end of				
			4 hrs	8 hrs	12 hrs	16 hrs	20hrs
1	University Livestock Farm, Mannuthy	16	-	-	13	1	2
2	Co-operative Milk Supply Union, Trichur	16	-	-	14	2	-
3	Individual households	16	-	-	11	5	-
	Total	48	-	-	38	8	2

Table 15. Keeping quality of samples of boiled milk stored at refrigeration temperature (4°C).

Sl. No.	Source of the samples	No. of samples	No. of samples showing positive COB Test at the end of						
			11 days	12 days	13 days	14 days	15 days	16 days	17 days
1	University Livestock Farm, Mannuthy	16	-	-	2	7	4	3	-
2	Co-operative Milk Supply Union, Trichur	16	-	-	10	6	-	-	-
3	Individual households	16	-	-	7	7	2	-	-
	Total	48	-	-	19	20	6	3	-

ILLUSTRATIONS

Fig. 1. NUMBER OF SAMPLES OF RAW AND BOILED MILK STORED AT 37°C SHOWING POSITIVE REACTION TO COB TEST.

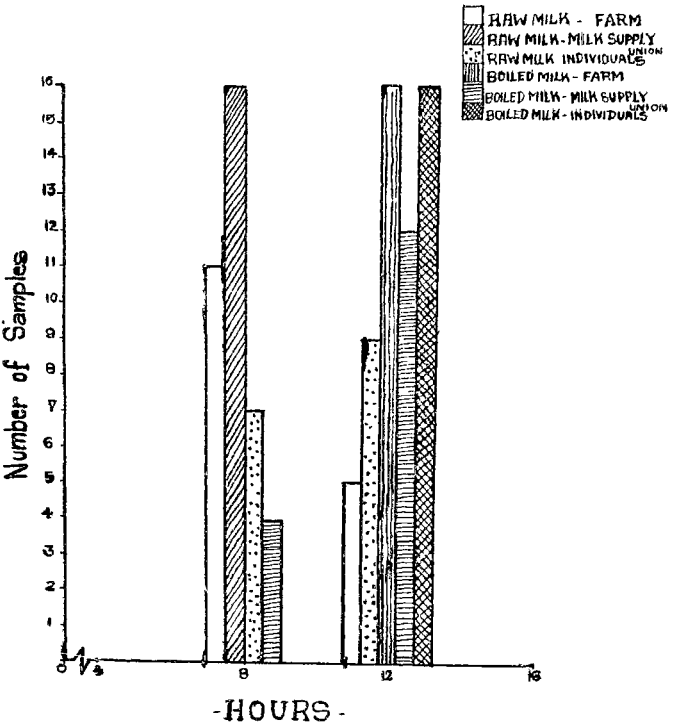


Fig. 2 - NUMBER OF SAMPLES OF RAW AND BOILED MILK STORED AT ROOM TEMPERATURE (29°C) SHOWING POSITIVE REACTION TO COB TEST.

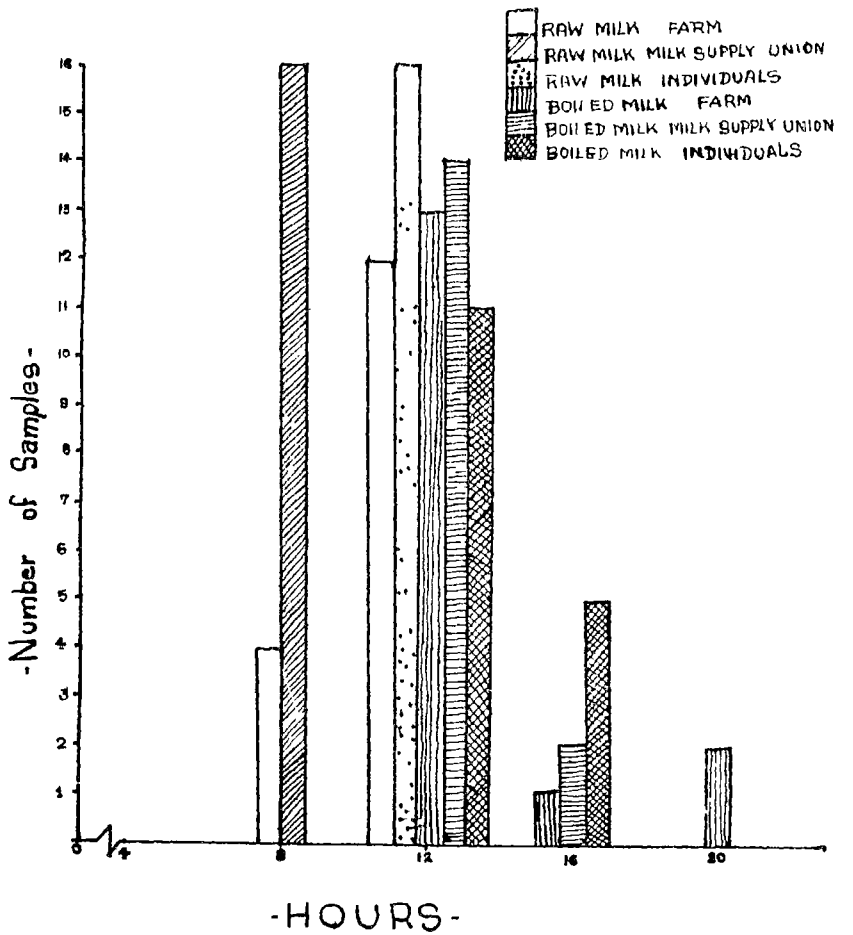


Fig. 3 NUMBER OF SAMPLES OF RAW AND BOILED MILK STORED AT 4°C SHOWING POSITIVE REACTION TO COB TEST.

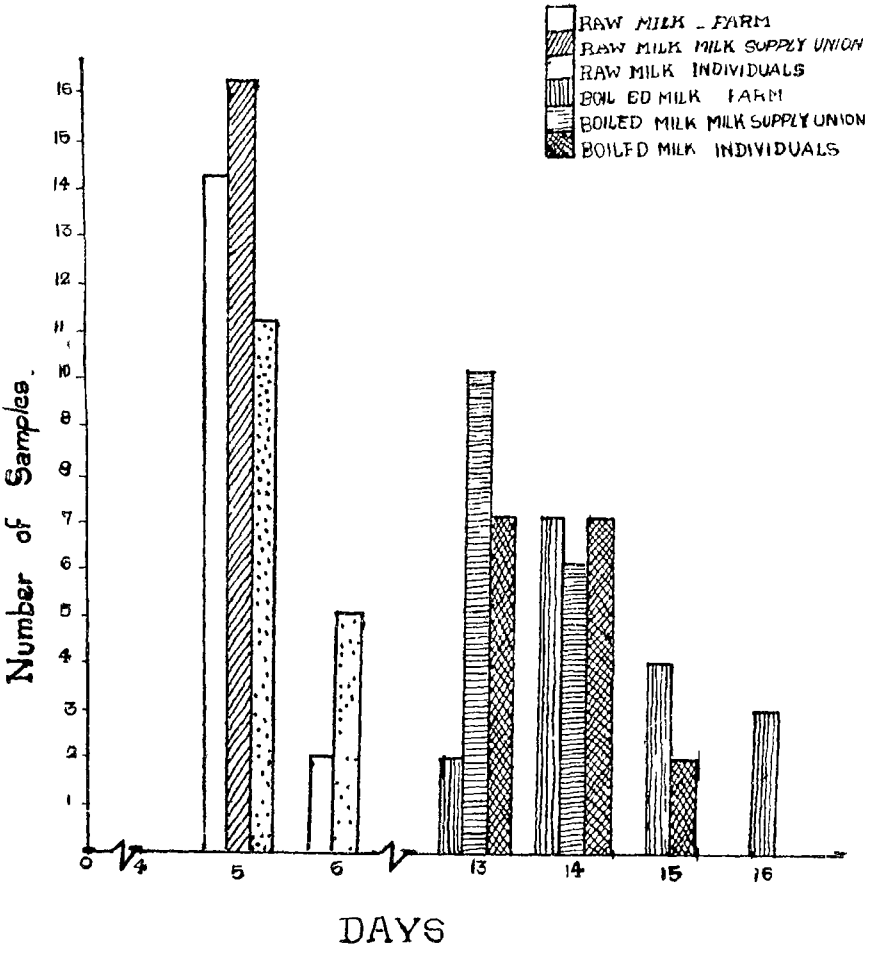
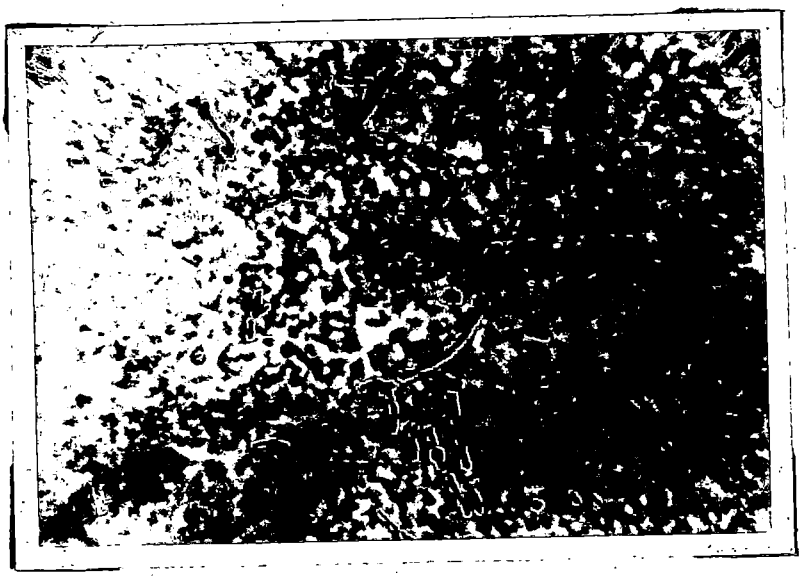


PLATE-I

Bacillus subtilis: Small rod shaped organisms occurring singly and in short chains. (1000x).

Bacillus cereus: Large rods occurring singly and in short chains. (1000x).



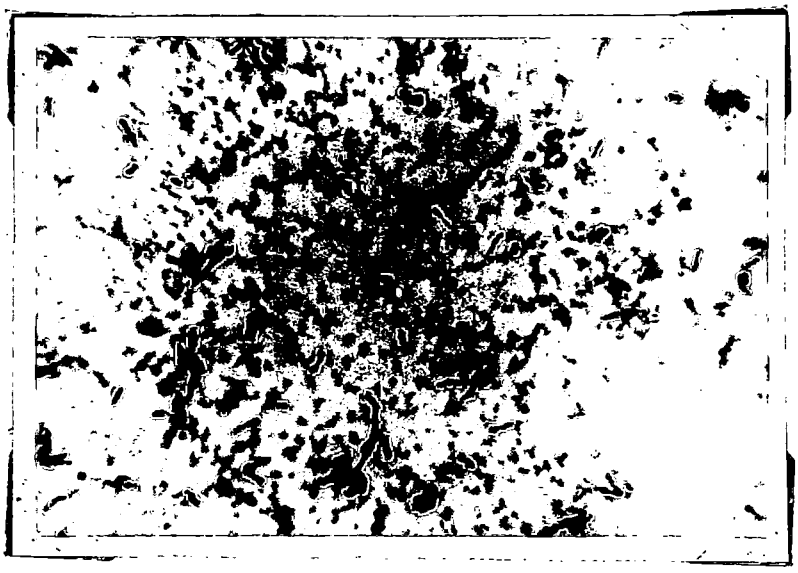


PLATE -II

Bacillus pumilus: Small rods occurring singly and in short chains. Closely resembles Bacillus subtilis. (1000x).

Bacillus licheniformis: Small rod shaped organisms occurring singly. Ellipsoidal spores are also seen. (1000x)

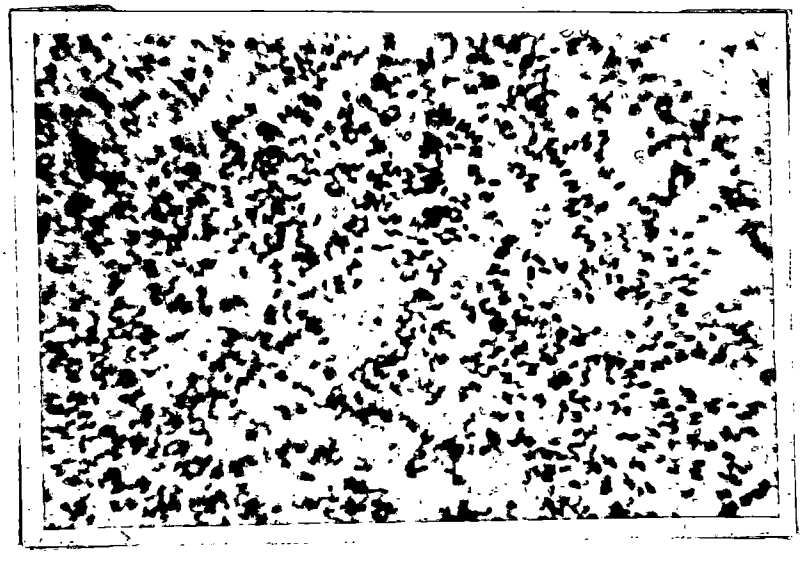
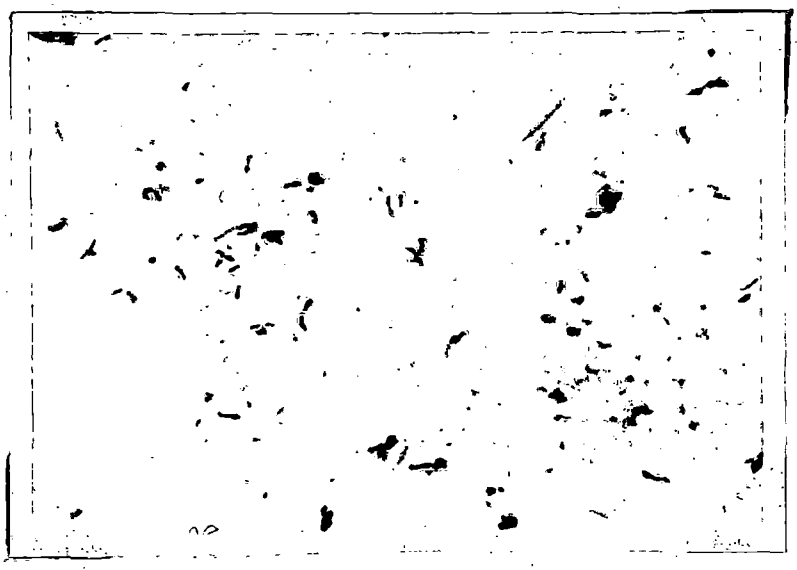


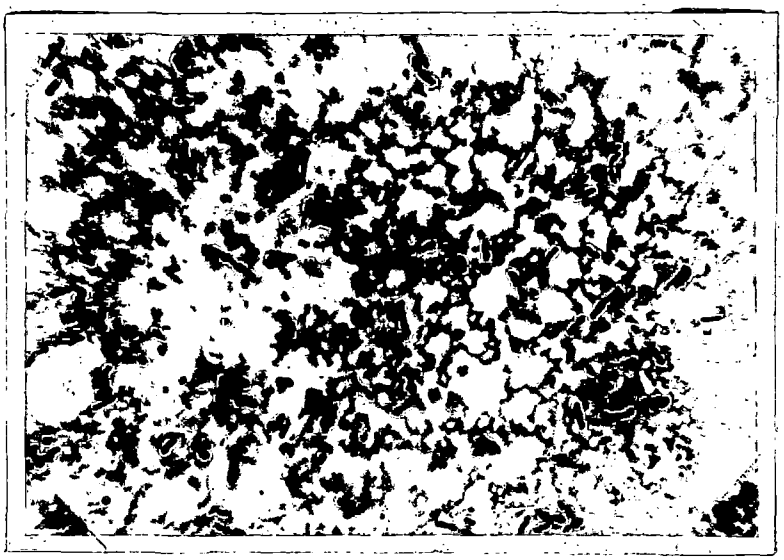
PLATE-III

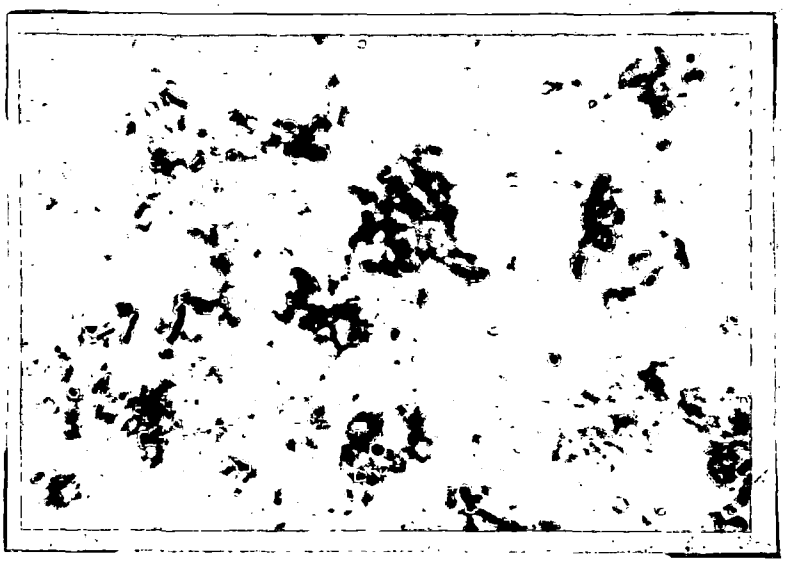
Bacillus megaterium: Large rods occurring singly and in pairs. In the background spores are also seen. (1000x).

Bacillus alvei: Rod shaped organisms, frequently occurring in chains. Spores are also seen. (1000x)

Bacillus firmis: Rod shaped organisms with poorly rounded ends, occurring singly or in pairs. (1000x)







DISCUSSION

DISCUSSION

In dairy industry heat treatment of milk at higher temperature has been receiving considerable importance in recent years. The bacterial spores present in milk are resistant to high temperature heat treatments. Indeed these high temperature treatments result in activation of the spore germination. Many published reports are available on the survival of bacterial spores in pasteurized milk, boiled milk, sterilized milk and ultra-high temperature treated milk. The number of heat resistant spores present in milk has been found to be quite variable due to differences in the time-temperature combination, incubation temperature, duration of incubation and the type of media used etc.

In the present study, the aerobic spore count of a total of 48 samples of milk collected at the rate of 16 samples each from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and from individual houses around Trichur Town were determined after boiling the samples of milk for one minute. It was observed that at 37°C for 48 hours (Table 1) the spore count ranged from 0-190/ml of boiled milk. Franklin *et al.* (1956) observed only a range of 0-700 aerobic mesophilic spores per 100 ml of raw milk. The high spore count in the present study can be attributed

to the heat activation of the spores stimulating their germination and outgrowth at boiling temperature. The findings were in agreement with those reported by Christian (1931), Hiscox (1934), Evans and Curran (1943), Speck (1961) and Mikolajcik and Koka (1968).

Atwal et al. (1974) obtained a spore count of 182 to 570/100 ml of milk after steaming at 100°C for 30 minutes. In the present study boiling was only for a short period of one minute which might have rendered the milk a good germination medium for spores as compared to heating at 100°C for 30 minutes. More types of aerobic spore-formers might have grown in milk boiled for one minute. Mikolajcik and Rao (1974) found that pasteurized milk was the best medium for outgrowth of B. cereus and B. megaterium whereas autoclaved milk favoured the growth of B. licheniformis only.

The average spore count of the samples of milk collected from the Farm, Co-operative Milk Supply Union and individual households was 70, 87 and 50/ml respectively (Table 1). The average spore count of the 48 samples studied was 69/ml. This was found to be less than the average spore count of 90/ml reported by Atwal et al. (1974). Galesloot (1962) also reported an average count of 100/ml as the maximum spore count of milk in countries like Netherlands, Australia and Great Britain. Considering the average number of spores obtained

for the samples collected from the different sources and the maximum number of spores reported by others it can be stated that the samples studied were of satisfactory bacteriological quality with regard to the content of spores.

It was observed that the samples of milk collected from individual households contained the lowest average spore count of 50/ml as compared to the count of 70 and 87/ml obtained for the samples collected from the farm and Milk Supply Union respectively. The reason for the lowest spore count in individual household samples could be attributed to the use of limited number of utensils for milking and subsequent handling of milk. Exposure of milk to a larger surface area of utensils, and the greater extent of handling of the milk after production could be the reasons for the increased spore count in the samples collected from the milk supply union. Moreover the samples collected from the Milk Supply Union were from the pooled milk supplied by a large number individual producers of milk in the villages who do not possess sufficient knowledge about clean milk production. Out of the 48 milk samples, 21 had a count ranging from 50-100 spores per ml (Table 4). Among the 21 samples, 11 samples were from the Co-operative Milk Supply Union. The higher spore count (50-100 spores/ml) in the majority of the milk samples of the Co-operative Milk Supply Union might be due to the reasons stated above. Engan-Skel (1974) reported a significant drop in spore-forming

organisms in milk when measures to raise hygienic standards were practised.

As regards to the samples collected from the University Livestock Farm, the number of utensils used for milk production and the degree of subsequent handling were much greater than those for milk production in the individual houses. These factors would have been responsible for the higher spore counts in the samples of the University Livestock Farm. Mckenzie et al. (1946) and Thomas and Thomas (1955) reported that milk cans washed unsatisfactorily were the source for a large number of aerobic spore-forming organisms in milk. Labots et al. (1965) also reported that contamination of milk with bacterial spores occurred from the cans and milking equipment. Abo-Elnaga et al. (1973) observed that the spore-formers from milking pails and milk cans reached upto 15 per cent of the total microflora before milking.

Again, the lack of proper knowledge on the part of the villagers about clean milk production might be the reason for the increased spore counts of 40 and above per ml in the individual samples collected from the milk supply union and the average count of 87/ml for them. This view has been expressed by Nambudripad (1950) who reported that the source of contamination of milk were vessels, exterior of the animal, dust and atmosphere of the byre, interior of the udder, the

milker, method of milking and flies. Also since some of the individual houses located near the road side supply milk to the Co-operative Milk Supply Union there might be an increase in the spore content of the milk samples of the Milk Supply Union. Ethiraj (1976) reported that location of the farms in the heart of the city with heavy road traffic might also increase the spore content of the milk.

The samples collected from the University Livestock Farm, Mannuthy generally had a higher spore count with the maximum of 190/ml in one sample. During the period of collection of samples from the farm the animals were stall fed with silage. This might be the reason for the higher spore count in the samples collected. Engan-Skel (1974) found highest number of spores in milk when animals were fed on silage of unsatisfactory quality and when cow shed, milking techniques and equipment etc., were of lower standard of hygiene. Farkhondeh (1974) reported that in the Teheran area animals were usually fed in the stable and that it was an important factor for high spore count in the raw milk.

Statistical analysis of the results presented in Table 1 showed that there were significant differences ($P < 0.01$) among the samples collected from the three different sources viz., Farm, Co-operative Milk Supply Union and individual houses with regard to the spore count. When a

pairwise comparison of the samples was made no significant difference was noticed between the samples collected from the farm and those of individual households. Similarly the samples collected from farm did not significantly differ from those of the milk supply union. But significant differences ($P < 0.01$) were found to exist between the samples collected from the milk supply union and individual houses.

The results presented in Table 5, showed predominance of the species B. subtilis. This was followed by B. cereus, B. pumilus, B. licheniformis, B. megaterium, B. alvei and B. firmis in the order of their occurrence. This finding was similar to that observed by Kerala Varma et al. (1950). The predominance of B. subtilis organisms in milk samples has been reported by many workers (Kerala Varma, 1949; Shroff, 1970; Janina, 1966; Davies, 1975; Ethiraj, 1976 and El-Sadek et al. 1976b). Also B. subtilis seemed to be the predominating organism in the milk samples collected from all sources and subjected to boiling for one minute. Atwal et al. (1974) also found the predominance of B. subtilis in all the types of milk examined by him.

Among the 162 isolates subjected for identification of the species of bacilli, 85 isolates (52.47%) were identified as B. subtilis, 23 isolates (14.20%) as B. cereus, 20 (12.35%) as B. pumilus, 15 (9.26%) as B. licheniformis, 11 (6.79%) as

B. megaterium, 6 (3.70%) as B. alvei and 2 (1.23%) isolates as B. firmis. In the present work B. subtilis and B. cereus together constituted about 67 per cent of the total isolates. Kerala Varma (1949) found that B. subtilis and B. cereus together accounted for 73 per cent of the total spore-formers present in boiled milk. But subsequently Kerala Varma et al. (1950) observed that B. subtilis and B. cereus together constituted 79 per cent of the spore-formers isolated (total 93 isolates) from boiled milk in India. As reported by Davies (1975) the relative proportion of bacillus species (B. subtilis and B. cereus) isolated from milk of all countries in NIRD/FAO Survey, was 60.37 per cent. Ethiraj (1976) found that both B. subtilis and B. cereus together constituted about 41 per cent of the total spore count in heat treated milk. However, El-Sadek et al. (1976b) reported that B. subtilis and B. cereus accounted for 70 per cent of the aerobic mesophilic spore-formers in Egyptian raw milk.

The differences in the percentage of occurrence of B. subtilis and B. cereus might be due to the differences in the initial spore count, extent of contamination, temperature and time of heat processing, temperature and period of incubation etc. Martin et al. (1966) found that heating milk at 137.8°C increased the percentage of spore outgrowth than that heated at 104.5°C and 121°C. Huhtanen et al. (1976) found that plates

incubated for 48 hours at 32°C had a higher count than those incubated at 30°C. They also found that incubation for 72 hours gave higher counts than that for 48 hours.

The number and types of aerobic spore-formers isolated from boiled milk samples of different sources are presented in Table 8 and the percentage of their incidence is given in Table 9. These tables revealed that there was very little variation in the number, incidence and percentage of the various species of spore-formers in the samples from different sources. Statistical analysis of the data recorded for the samples from the different sources revealed that the samples were not significantly different from each other with regard to the number and species of the spore-forming organisms.

Table 9 showed that in the samples collected from the Farm, B. subtilis predominated and accounted for 48.214 per cent of the total isolates. This was followed by B. cereus 14.286 per cent, B. megaterium 12.5 per cent, B. Licheniformis 10.714, B. pumilus 8.929 per cent, B. alvei 3.571 per cent and B. firmis 1.786 per cent. The predominance of B. subtilis with 54.839 per cent of the total isolates were seen in milk samples collected from the Co-operative Milk Supply Union. B. cereus accounted for 14.516 per cent, B. licheniformis 11.290 per cent, B. pumilus 9.677 per cent, B. megaterium 4.839 per cent, B. alvei 3.226 per cent and B. firmis 1.613 per cent. Eventhough B. subtilis was the predominating

organism with 54.545 per cent of the total isolates observed in individual household samples B. pumilus which accounted for 20.455 per cent become the next predominating type. However, B. cereus accounted to 13.636 per cent of the total followed by B. licheniformis and B. alvei 4.545 per cent each and B. megaterium 2.273 per cent.

In the present work, differences were noticed in the percentage of incidence of the different organisms isolated from milk samples of different sources. These observations were similar to those made by many workers like Kerala Varma (1949), Atwal et al. (1974), Davies (1975), Ethiraj (1976) and El-Sadek et al. (1976b). Kerala Varma (1949) observed the predominance of B. subtilis followed by B. cereus and B. megaterium. The same finding was noted in the samples collected from the University Livestock Farm, Mannuthy. Davies (1975) pointed out that the bacillus species isolated from milk in all countries by NIRD/FAO Survey were predominantly B. subtilis followed by B. licheniformis, B. cereus, B. pumilus, B. firmis, B. brevis and B. megaterium. Atwal et al. (1974) also reported the incidence of Bacillus species in milk in order of predominance as B. subtilis followed B. cereus, B. coagulans and B. stearothermophilus. Ethiraj (1976) studied the spore-formers in milk samples and reported that the predominating organism was B. subtilis followed by B. megaterium

B. coagulans, B. stearothermophilus, B. cereus, B. licheniformis, B. sphaericus and B. pumilus.

Galesloot (1962) observed that more than half of the bacterial spores in raw milk belonged to B. licheniformis. El-Sadek and Attia (1968) found that B. megaterium and B. brevis constituted 71.8 and 12.9 per cent of the isolates in raw milk. But when milk was boiled the percentage of B. brevis and B. megaterium changed to 36.99 and 12.33 respectively. Of the total spore-forming bacteria in milk, 46.3 per cent was observed to be B. firmis (Chung and Cannon, 1971). Khalafalla et al. (1976) reported that 72 per cent of the aerobic spore-forming bacteria in Egyptian buffaloes' raw milk belonged to B. megaterium. Fourie et al. (1972) found the predominating type as B. pumilus (23.7%) in South African raw milk followed by (21.4%) B. licheniformis.

The reasons for these variations in the type of bacillus organisms and their percentage of incidence in milk could be attributed to source of bacillus spores in milk and to the extent of contamination from the sources. Galesloot (1959) found that faulty washing of the milk can, increased the content of B. cereus spores in milk. Kerala Varma (1949) stated that different species of aerobic spore-formers were found in different sources of contamination.

Keeping Quality of Boiled Milk.

The data presented in Table 10 revealed that among the 16 samples of raw milk collected from University Livestock Farm, Mannuthy and stored at 37°C, five had a keeping quality of more than eight hours whereas none out of the 16 samples from the Co-operative Milk Supply Union, Trichur, had a keeping quality of more than eight hours. However, nine out of the 16 samples collected from individual houses had a keeping quality of more than eight hours. This indicated that the milk samples collected from individual households were having a better keeping quality on storage at 37°C than that of the samples collected from the Milk Supply Union and milk samples from farm were better as compared to those collected from the Co-operative Milk Supply Union.

The keeping quality of raw milk samples stored at room temperature presented in Table 11 showed that the milk samples collected from the Co-operative Milk Supply Union were having a poorer keeping quality as compared to those from the University Livestock Farm and individual houses. All the samples collected from the Co-operative Milk Supply Union and kept at room temperature (29°C) got spoiled within eight hours of storage. The milk samples from individual houses had a better keeping quality than those from University Livestock Farm. All the samples from individual houses had a

keeping quality of more than eight hours when stored at room temperature.

With regard to the keeping quality of raw milk at refrigeration temperature of 4°C (Table 12) the samples from the Co-operative Milk Supply Union were found to have the minimum keeping quality. The samples collected from the farm had a better keeping quality than those of the Co-operative Milk Supply Union, but less as compared to the samples from individual households. The milk samples from individual houses were found having the maximum keeping quality when stored at 4°C.

The main reason for the differences in keeping quality of the milk samples collected from different sources and stored at the same temperature could be attributed to the differences in the bacteriological quality of the samples prior to storage. In this context the bacteriological quality of milk implied the types and magnitude of bacterial population a sample had, depending upon the conditions under which the milk was produced (Marutiram and Singh, 1968).

It was observed from the data in Tables 10 to 12 that when the temperature of storage was increased the keeping quality of milk decreased. This decrease in keeping quality was found to be proportional to the increase in the temperature of storage. The findings were in agreement with those

stated by Sinha and Nambudripad (1973) who reported that in warm temperature the rate of spoilage was proportionate to the temperature at which the milk was held.

The data presented in Tables 10 to 12 indicated that the keeping quality of milk samples from all the three sources was lower at 37°C as compared to the storage temperature of 29°C or 4°C. A slight increase in keeping quality of milk samples from all sources was observed when they were stored at room temperature. But when the samples were stored at refrigeration temperature (4°C) the keeping quality was found to increase by 10 to 12 times than the samples stored at 37°C or at 29°C. The reason for the differences in keeping quality at the different temperatures could be attributed the changes brought about in milk due to the growth and multiplication of the bacterial flora present in milk. The mesophilic bacteria have the optimum growth temperature in the range of 20 to 40°C whereas thermoduric bacteria grow well at 30-32°C (Foster et al. 1957), and both are responsible for spoilage of milk at 37°C and at room temperature (29°C). But at the refrigeration temperature of 4°C the psychrophilic microorganisms only grow in milk. Therefore the changes that could be produced in milk stored at 4°C are very much limited which ultimately result in the increased keeping quality of milk.

The milk samples collected from the Co-operative Milk Supply Union were found having the lowest keeping quality when stored at 37°C, 29°C and 4°C as compared to the samples from University Livestock Farm and individual houses. The lowest keeping quality exhibited by milk samples from the Co-operative Milk Supply Union might be due to the higher spore-count which in turn was a reflection of the hygienic methods adopted for production and collection of milk.

It was generally observed that in individual households the quantities of milk produced were small (2-3 litres) and sufficient precautions were taken to prevent bacterial contamination of milk from various sources. On the other hand in the University Livestock Farm, the quantities of milk produced were considerably larger (700-750 litres) and due to the larger number and greater volume of the equipments used for handling milk the chances of contamination were comparatively greater. The milk received at the Co-operative Milk Supply Union was from a large number of milk societies which in turn received their supply of milk from a considerably large number of producers in the villages. In the various opera ions of production of milk at the village level and subsequent collection and transport the chances of contamination of milk were quite enormous. These factors could be responsible for the higher spore-formers to be present in milk samples collected from the Co-operative Milk Supply Union.

Smith (1920) also noticed higher thermoduric counts in cow milk coming in contact with more exposed surfaces of dairy utensils and equipments.

Regarding the keeping quality of boiled milk stored at 37°C (Table 13), only four samples out of 48 had a keeping quality of less than eight hours. The rest of the samples had a keeping quality of more than eight but less than 12 hours. The milk samples collected from the Co-operative Milk Supply Union were found to have a lesser keeping quality as compared to the samples from the farm and individual households. When the storage was at room temperature the milk samples collected from the farm were found to have a better keeping quality as compared to that of samples from the Co-operative Milk Supply Union and individual households. In general all the samples had a better keeping quality when stored at room temperature as compared to the storage temperature of 37°C.

When samples of boiled milk were stored at room temperature it was found that out of 48 samples 38 had a keeping quality of 8 to 12 hours. The keeping quality ranged from 12-16 hours in eight samples and two samples had a keeping quality of 16-20 hours (Table 14). Kerala Varma and Laximinarayana (1947) reported that the keeping quality of boiled milk under Indian household conditions varied generally

between 16 to 18 hours and the spoilage was caused by the activity of any type of spore-forming aerobes present in it. But Kumar et al. (1975) found that pooled raw milk that had been given one instant boil, stored upto 72 hours at temperatures of 18-22°C. Ogasa et al. (1959) found the keeping quality of milk treated at 75°C for 15 minutes to be 4 to 6 days at 2-12°C and 0 to 1 day at 18-30°C. The ultra-high temperature treated bottled milk was found to keep well for 8 to 10 days at 2-12°C and 1 to 2 days at 18-30°C.

The storage of boiled milk samples at 4°C revealed that the keeping quality was 13 to 16 days for the samples collected from the Farm, 13 to 14 days for those from the Co-operative Milk Supply Union and 13 to 15 days for individual household samples. The reasons for increased keeping quality at refrigeration temperature might be due to the slow bacterial growth in heat treated milk at low temperature. Similar observations have been made by Sherman et al. (1941) who reported that the bacterial growth in pasteurized milk was much slower at 0°C than in raw milk. Knaysi (1948) reported that the vegetative growth of B. subtilis stopped at 8°C and sporulation at 10°C. Grosskopf and Harper (1969) found that fluid pasteurized (62.5°C for 30 minutes) milk, aseptically packaged and stored at 4°C had a shelf life of four weeks and subsequent spoilage of milk was due to psychrophilic spore-forming organisms. Boyed et al. (1955) also

reported that when storage temperature was lowered from 40 to 33°F the keeping quality was extended from 11 to 14 days.

In general, the results indicated that boiling of milk for one minute enhanced the keeping quality at all the incubation temperatures studied. This was presumably due to destruction of majority of the microflora present in milk. In the present study all the colonies that developed in the tryptone dextrose agar plates prepared from samples of boiled milk were found to be spore-forming bacilli. Kerala Varma (1949) also reported that boiling of milk may be considered to be effective in destroying all types of microorganisms with the exception of those existing as spores.

Eventhough boiling of milk destroyed all microorganisms except those present in its spore form, the keeping quality of boiled milk samples stored at 37°C and 29°C in the present study was found to be slightly better as compared to the samples of raw milk stored at the same temperatures. This might have been due to the activation of spores present in milk by the heat treatment. Black (1960) observed that the spore-formers in some raw milk become active at somewhere above 210°F resulting in spoilage. Mitten (1959) stated that sterilization required temperatures of 285°F for 15 seconds and occasionally surviving bacteria grew very rapidly after the third or fourth day, some of them

at storage temperature of 45 to 55°F. He also noticed rapid germination and outgrowth of spore-formers following heat treatment.

The keeping quality of raw milk stored at 4°C was found to be 5 to 6 days. The findings were in agreement with Burgwald and Josephson's (1947) statement that milk of good quality could be expected to retain excellent bacteriological and flavour qualities for at least four days during the summer months and 6 to 7 days during winter months if refrigerator temperature are maintained near 40°F.

The samples of boiled milk stored at 4°C were found to keep well for a period of 13 to 16 days. Therefore, the keeping quality of boiled milk stored at refrigeration temperature could be more than twice that of raw milk stored at the same temperature. Sherman et al. (1941) reported that the keeping quality of pasteurized milk, if not recontaminated, was two or three times as that of raw milk. The keeping quality of boiled milk stored at 4°C observed in the present study was found to be less than that reported by Glazier (1963) who found that pasteurization at 206°F for three seconds increased the shelf life to 30 days when stored at 45°F. Grosskopf and Harper (1969) also reported that fluid pasteurized milk stored at 4°C had a shelf life of four weeks. The reduced keeping quality of the boiled milk at refrigeration

temperature could be due to the increased rate of proteolysis caused by organisms at the increased temperature of processing. Similar findings were reported by Mikolajcik and Rao (1974) who found a relationship between heat treatment of milk and rate of proteolysis, the more intense the heat treatment the more rapid the rate of proteolysis. Moreover there was a time lapse of 2 to 4 hours between the time of production of milk and collection of samples for study. During this period since the milk was at atmospheric temperature some multiplication of the organisms would have occurred resulting in an increase in the bacterial load. This might also be a contributory factor for not obtaining a high keeping quality for boiled milk stored at refrigeration temperature as reported by other workers. Sinha and Nambudripad (1973) have also stated that the rate at which the spoilage took place depend upon the extent of microbial load as well as the temperature at which the milk was held subsequent to production. Hadland and Hoye (1974) also reported that the general keeping quality of pasteurized milk was estimated as acceptable for about 14 days after raw milk storage of only one day at 2°C before pasteurization; after four days storage of raw milk at 2°C, the keeping quality was reduced to 6 to 8 days. The decrease in keeping quality was still more marked after increasing raw milk storage at 6°C for more than one day.

Methods suggested for Increasing the Keeping quality of Boiled Milk.

The milk subjected to heat treatment at higher temperatures than those normally used for pasteurization processes have been stated to be a good medium for the growth of heat resistant spore-forming bacteria present in milk. It has also been found that 94.9 per cent of the spores germinate in boiled milk (Koka and Mikolajcik, 1967). The spoilage in heat treated milk was not due to the development of lactic acid, but due to the rennin like enzyme secreted by the spore-forming bacteria (Ethiraj, 1976). El-Sadek et al. (1973) found that strains belonging to B. cereus, B. circulans, B. magaterium, B. polymyxa, B. pumilus and B. subtilis were capable of producing milk clotting enzymes. Adams et al. (1975) reported that most of the raw milk supplies appeared to contain either heat resistant proteases or bacteria capable of producing them. They found that all the psychrotrophs obtained from raw milk produced proteases that survived 149°C for ten seconds.

The poor keeping quality of milk subjected to high pasteurization temperature was stated to be due to destruction of non-spore-forming microflora, so that the B. cereus had no competetion for growth (Franklin, 1969). As the spore-forming organisms are not destroyed by boiling of milk,

and some of them survive even in sterilized milk it is necessary to minimise the contamination of milk by spores of the organisms during production and subsequent handling of milk. But it is very difficult to avoid such contamination in dusty weather and dry climatic conditions as these spores get into milk from sources such as dust, utensils, body surface of the cow, dung and water supply. Since the milk for a major dairy has to be collected from hundreds of sources, the problem of contamination of milk with spores of bacteria become all the more important. The time-temperature conditions of heat treatment of milk are also important in that they influence spore germination and subsequent growth of vegetative cells.

The keeping quality of boiled milk obtained in the present study was 8 to 12 hours at 37°C, 12 to 20 hours at room temperature (29°C) and 13 to 16 days at refrigeration temperature (4°C). These findings tend to show that when the storage temperature of milk after boiling was reduced, there was a corresponding increase in the keeping quality. From the findings of the present study and those of the studies made earlier by several workers it can be stated that the following procedures will aid in the increased keeping quality of boiled milk:

1. Practising all methods necessary for clean milk production.

2. Cooling the milk to refrigeration temperature soon after production.

3. Avoiding unnecessary handling of milk after production.

4. Immediate cooling and storage of boiled milk at refrigeration temperature.

5. Avoiding contamination of boiled milk.

SUMMARY

SUMMARY

The purpose of the present study was to determine the count of aerobic spore-forming organisms present in boiled milk and to identify their species. The keeping quality of milk boiled for one minute was also determined at storage temperatures of 37°C, room temperature (29°C) and refrigeration temperature (4°C). The keeping quality of boiled milk was compared with that of the raw milk stored at the temperatures mentioned above.

A total of 48 samples of raw milk, 16 each from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households were collected and used for the study. The collection of samples of milk for the study was made from November 1977 to February 1978.

The aerobic spore count of the 48 samples of milk collected was determined after subjecting them to laboratory boiling and plating in agar plates. At the end of an incubation period of 48 hours at 37°C, the colonies developed in the agar plates were counted. Among the colonies that developed, a total of 162 were isolated at random and subjected for identification of the different species of the organisms. For the purpose of identification of the different species of the organisms, morphological, cultural and biochemical

tests were performed. The following were the different species of the organisms and the number of isolates from which such species were identified: Bacillus subtilis 85, Bacillus cereus 23, Bacillus pumilus 20, Bacillus licheniformis 15, Bacillus megaterium 11, Bacillus alvei 6, and Bacillus firmis 2.

No significant difference was noticed in the incidence and percentage of the different species of the sporeformers in the samples of milk collected from the various sources. Statistical analysis of the results regarding the spore count showed no significant difference between the samples collected from the farm and those from individual households or Co-operative Milk Supply Union. But significant difference was found to exist between the samples collected from the Co-operative Milk Supply Union and those of individual households.

The keeping quality of samples of raw milk collected from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households was determined at a storage temperature of 37°C, room temperature (29°C) and refrigeration temperature (4°C). The keeping quality of the samples of milk after subjecting them to boiling for one minute was also determined at the temperatures specified above. The keeping quality of raw milk samples was

found to be lower at 37°C as compared to that at the storage temperature of 29 and 4°C. Even at 29°C none of the samples had a keeping quality of more than 12 hours. But when the samples were stored at 4°C the keeping quality was found to increase by 10 to 12 times more than that of the samples stored at 37°C or 29°C. The keeping quality of boiled milk at different storage temperatures was found to be generally greater than that of raw milk.

The samples collected from the Co-operative Milk Supply Union, Trichur were found to have the lowest keeping quality both in raw as well as boiled form at the storage temperatures studied. The keeping quality of the samples from University Livestock Farm, Mannuthy was better than those of the Co-operative Milk Supply Union. Two out of the 16 samples from this source on boiling exhibited a much higher keeping quality (16-20 hours) on storage at room temperature. Three of the samples of raw milk collected from the University Livestock Farm, Mannuthy, on boiling indicated the maximum keeping quality of 16 days on storage at refrigeration temperature. In general, the samples collected from the individual households had the highest keeping quality.

The keeping quality of boiled milk obtained in the present study was 8 to 12 hours at 37°C, 12 to 20 hours at

room temperature (29°C) and 13 to 16 days at refrigeration temperature (4°C). These findings tend to show that when the temperature of storage of milk after boiling was reduced, there was a corresponding increase in the keeping quality. Adopting procedures necessary for clean milk production, cooling the milk to refrigeration temperature soon after production, avoiding unnecessary handling of milk, immediate cooling and storage of boiled milk at refrigeration temperature and avoiding contamination of boiled milk will generally help in increasing the keeping quality of boiled milk.

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STUDIES ON MICROFLORA IN BOILED MILK

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ABSTRACT OF A THESIS
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ABSTRACT

A study was carried out to determine the total spore count, the incidence and distribution of the different types of the aerobic spore-formers present in milk boiled for one minute. The keeping quality of raw as well as boiled milk stored at 37°C, 29°C and 4°C was determined and compared. The samples of milk required for the study were collected from the University Livestock Farm, Mannuthy, Co-operative Milk Supply Union, Trichur and individual households around Trichur Town.

A total of 48 samples of milk were collected. The spore count in the samples was determined after subjecting them to boiling for one minute. The spore count for the samples of milk collected from the Farm ranged from 10 to 190 per ml whereas the range was 40 to 155 per ml and 10 to 150 per ml for those obtained from Co-operative Milk Supply Union and individual households respectively. The average spore count of the 48 samples studied was 69 per ml. A total of 162 isolates were identified as Bacillus subtilis 85, Bacillus cereus 23, Bacillus pumilus 20, Bacillus licheniformis 15, Bacillus megaterium 11, Bacillus alvei 6 and Bacillus firmis 2. The samples from all the sources revealed

the predominance of Bacillus subtilis and it accounted for more than 52.5 per cent of the isolates.

Out of the 48 samples of raw milk stored at 37°C, 34 had a keeping quality of less than eight hours and the remaining between 8 and 12 hours. When raw milk was stored at room temperature (29°C) 20 had a keeping quality of less than eight hours, and it was 8 to 12 hours for 28 samples. At refrigeration temperature of 4°C, 41 samples remained good for five days and in the other seven samples the keeping quality was for six days.

When the keeping quality of boiled milk stored at 37°C was studied, 4 out of 48 had a keeping quality of less than eight hours and the remaining 44 samples had 8 to 12 hours of keeping quality. At room temperature (29°C) the keeping quality was 8 to 12 hours for 38 samples as against the same of 12 to 16 hours and 16 to 20 hours for 8 and 2 samples respectively. When the samples of boiled milk were stored at refrigeration temperature (4°C), 19 out of 48 samples had a keeping quality of 13 days. The keeping quality was found to be 16 days for three of the samples stored at 4°C. Some of the procedures necessary to improve the keeping quality of boiled milk have been suggested.