STUDIES ON LACTOBACILLUS THERMOPHILUS

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In a review of the literature on the subject of thermophilic and thermoduric bacteria, Robertson (4) stated, "Only one non-spore-forming thermophile, *Lactobacillus thermophilus* Ayres and Johnson, has been isolated." Although thermophilic bacteria have been studied extensively in recent years, it appears that *Lactobacillus thermophilus* has not been encountered since the original isolation. It is therefore pertinent that an additional isolation of the organism be reported and a more complete description presented. As in the original isolation the organism was secured during an investigation of high plate counts with "pin point" colonies from pasteurized milk.

PRELIMINARY OBSERVATIONS

In the routine bacteriological analyses of the Corvallis milk supply, there was noticed a higher bacterial count in the pasteurized milk from one of the local plants, than in the raw milk delivered to the plant. The 1:100 dilution plates on the pasteurized milk were crowded with minute colonies. Daily platings were then made and the plates were incubated at 37° C., with a duplicate set at 50° C. Every sample of the pasteurized milk tested showed thermophilic bacteria which were present in widely varying numbers. Growth at 37° C. was poor in that the colonies were small and sometimes did not appear. At 50° C. the colonies were larger, and by the use of milk powder agar, distinct colonies averaging 0.8 mm. in diameter were obtained. The milk revealed rod forms on direct examination. At the time the preliminary work was being carried on, it was reported from the milk plant that the milk after pasteurization had an acidity of 0.25 per cent calculated as lactic acid, that patrons had complained of a peculiar flavor in the milk, and that a slight odor could be detected in the pasteurizing vat.

DESCRIPTION OF LACTOBACILLUS THERMOPHILUS

A number of cultures of the organism were isolated from colonies on the milk powder agar plates incubated at 50° C. Stock cultures were maintained by making daily transfers in litmus milk, incubating at 50° C. for approximately twenty hours. The same time and temperature were used throughout this work unless otherwise stated.

Morphology

Form and size. Non-spore-forming rods, averaging 1.0 by 3.5 microns in milk culture. Variations from 2.5 to 6 microns in length are observed. In broth, the cells are somewhat longer. The rods are straight or slightly curved.

Arrangement. Single cells and short chains predominate in milk cultures. In broth, chains occur most frequently, some being very long.

Motility. Non-motile.

Staining reactions. Gram positive, but commonly gram negative in milk culture. The cells are not stained uniformly with methylene blue.

Cultural Characteristics

Agar colonies. On a satisfactory medium (such as milk powder agar), surface colonies are slightly raised, glistening, translucent, circular with a curled edge and vary from 1 to 2 mm. in diameter. Sub-surface colonies are filamentous. Their diameter and density of structure depends upon the medium.

Agar slant. Filiform, glistening, translucent, flat or slightly raised growth on milk powder agar.

Broth. Slight growth in plain broth. In broth rich in peptone, good growth takes place. No surface growth, no sediment, moderate clouding.

Litmus milk. Acid with partial reduction of the indicator.

Biochemical Features

Indol—not produced.

Nitrates—not reduced.

Action on carbohydrates and alcohols. Glucose, galactose, levulose, mannose, lactose, maltose, sucrose, raffinose, soluble starch and dextrin fermented with the production of acid, but no gas. Arabinose, xylose, glycerol, rhamnose, salicin, insulin, and mannitol not fermented.

Oxygen relationships. Organism facultative; in agar-shake cultures the best development of colonies takes place near the surface of the medium.

Temperature relationships. Slight or no growth at 30° C. Weak growth at 35° C. (On plates at this temperature only a small part of the total inoculated will grow compared with duplicates at 50° C.) Optimum 50° to 60° C. Maximum at approximately 62° C. A milk culture is rendered sterile on heating at 70° C. for thirty minutes.

Total and Volatile Acid

The total and volatile acids were determined in milk cultures of the organism after incubation for 48 hours at 55° C. The total acid was determined by titrating 10-gram samples with 0.1 N. NaOH using phenolphthalein as an indicator, and calculating the acidity as lactic acid. A large number of determinations have all shown from 0.35 to 0.42 per cent. The volatile acid was determined by steam distillation of a 250-gram sample of the milk, after acidifying with 15 ml. of N. H_2SO_4. One liter of distillate was collected and titrated with 0.1 N. NaOH using phenolphthalein as an indicator. The following milliliters of 0.1 N. NaOH, 14.5, 15.6, 10.9 and 13.0 indicate that comparatively little volatile acid was produced, as the figures for sterile milk treated in the same manner are from 3 to 6.

Determination of the Non-Volatile Acid

It is apparent that the non-volatile acid makes up most of the total acid. Zinc salts were prepared from the residues remaining after the steam distillations, using the method by Hammer (3). The results obtained in the study of these salts show that the non-volatile acid formed is largely or entirely *d* lactic acid.

Comment upon the Description

The organism described is practically identical to *Lactobacillus thermophilus* Ayres and Johnson, and may be considered as the same species. From the additional characteristics recorded in the description, it would seem that the possible similarity between *Microbacterium lacticum* Orla-Jensen and *Lactobacillus thermophilus* Ayres and Johnson, as was suggested by Robertson (5), does not hold true.

FACTORS AFFECTING THE GROWTH OF LACTOBACILLUS THERMOPHILUS

The conditions of the Standard Plate Count are not particularly favorable for the growth of *Lactobacillus thermophilus.* The temperature of 37° C. is near the minimum for the organism and at two or three degrees lower (temperatures which exist in many routine incubators) normal development does not take place. Secondly, the organism does not grow in slightly acid media. With other conditions favorable, a pH of 6.0 in agar media, determined colorimetrically, prohibits growth. Many batches of agar used in routine work, but not carefully adjusted to a pH of 6.2 or above, have failed to grow the organism. Thirdly, standard nutrient agar is not a very satisfactory medium for the growth of *Lactobacillus thermophilus.* Surface colonies do not appear, nor can appreciable surface growth

be induced by streaking (this is characteristic of many lactobacilli), although on a more nutritive medium surface colonies do appear. The sub-surface colonies on standard nutrient agar are filamentous, and of very thin veil-like structure averaging 0.4 mm. in diameter, and though not "pin-point" in size, are not readily seen in some cases.

A preliminary study was made of the nutritive requirements of *Lactobacillus thermophilus* in regard to the type and amount of peptone. By substituting Bacto-Proteose peptone in standard nutrient agar, in place of Bacto-Peptone, in the usual 0.5 per cent concentration, luxuriant growth is obtained. Surface and sub-surface colonies are from one to two mm. in diameter. Almost as good growth can be obtained by increasing the concentration of Bacto-Peptone to two or three per cent. In regard to the minimal peptone concentrations, 0.1 per cent Bacto-Proteose peptone in washed agar without beef-extract supports the growth of distinct colonies, whereas 0.3 per cent Bacto-Peptone is required to grow visible colonies. Here again, any considerable variation from the standard methods formula might not allow growth of *Lactobacillus thermophilus*. The required nutritive substance is present in peptone, particularly proteose-peptone. The organism is not saccharophilic as are the thermo-tolerant "pin-point" streptococci. The addition of diluted sterile milk to an agar plate culture increases the colony size of *Lactobacillus thermophilus,* but sterile lactose has little or no effect.

VIABILITY OF LACTOBACILLUS THERMOPHILUS

In maintaining stock cultures, it was found that cultures of the organism rapidly became sterile. Milk cultures which had been incubated at 50° C. for 12 to 24 hours and then held at room temperature were sterile in from four to ten days. Cultures containing calcium carbonate remain viable only somewhere longer. Broth cultures and colonies are usually sterile in a week or less. Storing cultures at ice-box temperatures lengthens the period of viability.

THE DEVELOPMENT OF LACTOBACILLUS THERMOPHILUS IN THE PASTEURIZING PLANT

The organism was regularly present in the pasteurized milk from one plant during a period of several weeks. The raw milk received at the plant did not reveal the organism either by plating or direct microscopical examinations. As the plant equipment has often been reported as a source of thermophiles, this possibility was investigated. The pasteurizing vats of the Burrell Spray type were in good condition and attempts to find the organism in the cleaned vats just before use were unsuccessful. Swabbings from the sides and corners of the vat as well as drip water in the bottom did not contain thermophilic bacteria. The failure to isolate the organism

in the raw milk and in the equipment seemed inconsistent with the daily appearance of large numbers of the organism in the pasteurized milk. However, a check-up on the plant operations did explain the problem. The milk was being standardized before pasteurization to the desired butterfat content with the pasteurized cream of the previous day. It was a case of daily inoculation as the data in the table show.

Thermophilic bacteria in milk shown by platings at 50° C.

Mixed raw milk in vat before heating	0 in 0.01 cc.
Raw milk after addition of cream	40,000 per cc.
The same, after heating to 143° F.	60,000 per cc.
" , after holding 45 minutes	100,000* per cc.
The cream used to standardize	1,000,000* per cc.

* Approximate numbers due to low dilutions used.

Since discontinuing the addition of pasteurized cream to standardize the milk, weekly platings of the pasteurized milk have been continuously free from *Lactobacillus thermophilus*. The trouble was therefore due to re-pasteurization, often recognized as a source of high counts of thermophilic bacteria. The unusually large numbers in the cream, which gave a sizable inoculation to each vat of milk in the standardizing process, can be explained. The cream was obtained by separating the pasteurized milk at the end of each day's run, during which the vats were in operation from four to six hours, and not sterilized between batches. Pasteurization was performed at 143° F. for 45-minute periods. A further factor favorable to the development of the organism was the limited capacity of the separator which delayed the eventual cooling of the warm milk from the last vat. As Ayres and Johnson (1) have pointed out, the organism is readily killed by efficient steaming of the equipment, and for this reason equipment infections of this organism are not so likely as in the case of the spore-forming thermophilic bacteria.

THE SOURCE OF LACTOBACILLUS THERMOPHILUS

The organism was not isolated from the raw milk delivered to the plant. Six weekly platings from each of twenty-five shippers' milk were consistently free from the organism in the 0.01 cc. amounts tested. Attempts to stimulate growth by a prior four-hour incubation of the milk at 50° C. were not successful. More recently, four attempts to isolate the organism from the pasteurized milk and also from the raw milk from seven shippers to the Iowa State College pasteurizing plant, were unsuccessful. This evidence agrees with the statement of Ayres and Johnson, "It seems probable that the organism may be generally present in raw milk, but in small numbers, which may increase under suitable conditions. The source of the

organism is therefore not clear." These authors did isolate the organism from raw milk but their data show 10 shippers' milk free from thermophiles in the amounts tested, and that the high counts were due to a plant infection.

A similar organism, probably the same species, as judged by colony formation, morphology, and heat resistance, was recently the cause of trouble in another Oregon pasteurizing plant and it was traced to the milk from one shipper.

A close scrutiny of the bacteriological analyses of the milk supply of Corvallis for more than a year and of a much greater number of analyses of the Portland milk supply during the entire years 1926–27, has failed to reveal any outbreaks of thermophilic bacteria as shown by the Standard Plate Count, other than in the two cases mentioned. During this time, more than a hundred cases of high counts in pasteurized milk showing typical "pin-point" colonies have been investigated. In almost every case streptococci were the cause of the trouble. Studies on these organisms in regard to morphology, heat resistance, poor growth on standard nutrient agar and their saccharophilic nature have agreed with the results of Fay (2). It is a simple matter to differentiate the filamentous colony of *Lactobacillus thermophilus* from the more typical lens-shaped "pin-point" colonies characteristic of the thermo-tolerant streptococci.

As a by-product of this work it appears that under the conditions of the Standard Plate Count, spore-forming thermophiles have not been the cause of high-counts in the very large number of routine milk analyses observed. Although these organisms are wide-spread in nature they are not common in raw milk in large numbers as hundreds of platings on raw milk at 50° C. have revealed few and usually no thermophilic bacteria of any kind in the 0.01 cc. amount tested.

SIGNIFICANCE OF LACTOBACILLUS THERMOPHILUS

In the present investigation the organism appeared as a result of re-pasteurization, which is prohibited by most city milk ordinances. In the work of Ayres and Johnson, failure to sterilize the pasteurizing vat resulted in high counts of the organism. Hence, in these cases the presence of the organism has been indicative of faulty plant procedure. Cultures of the organism have been consumed without harmful results.

SUMMARY

An additional isolation of *Lactobacillus thermophilus* is reported. The organism agrees in almost every respect with the original description. Additional characteristics are recorded. The organism grows poorly on the nutrient agar used in routine milk platings, and the indistinct filamentous

colony may be "pin-point" in size. The colony can easily be distinguished from the lens-shaped "pin-point" colonies of the thermoduric streptococci. Much better growth is obtained when Bacto-Proteose peptone is substituted for ordinary peptone.

As in the original isolation *Lactobacillus thermophilus* is found to be the causative organism of high bacterial counts in a pasteurized milk supply. It would appear that the organism is not of common occurrence in milk. The particular growth requirements and the relatively short period of viability characteristic of the organism may also explain why it has not been isolated more frequently.

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