APPLICATIONS AND LIMITATIONS OF QUALITY TESTS FOR MILK AND MILK PRODUCTS. A REVIEW

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SUMMARY

Changes in milk production and handling practices of recent years have altered the types and behavior of bacteria in milk. There is a need for reappraisal of bacteriological tests currently in use. With growth of bacteria in raw milk virtually eliminated by efficient cooling, tests applied to the freshly taken sample are not always effective in detecting faulty production practices. Encouraging the growth of contaminants prior to testing increases the utility of the tests.

Longer refrigerated storage of pasteurized products is focussing attention on post-pasteurization contamination with psychrophiles. Here again, tests on freshly taken samples are much less useful than those made after these organisms have been encouraged to develop in the product.

Tests for specific groups of bacteria as indices of carelessness in production and processing probably will grow in importance. Coliform tests will have greater usefulness as efficient cooling hinders the growth of these organisms in raw milk, and as their significance in pasteurized milk is more fully appreciated. More emphasis on udder health may also be expected.

The term quality, as applied to milk and its products, embraces a variety of aspects. These include such diverse properties as freedom from dirt, antibiotics, off-flavors, pathogenic organisms, and abnormal numbers of body cells; evidence of cleanliness and care in production and handling as indicated by microbiological analysis; possession of desirable flavor and aroma, and of adequate amounts of those constituents of nutritional importance. Thus the meaning of quality differs from product to product. With milk itself, the bacteriological aspect has received the greatest attention. With butter and cheese, on the other hand, flavor is of far more importance. With ice cream, we are interested in both of these aspects as well as in composition. In dealing with the subject of quality tests, it was considered preferable to take each test in turn and consider its application, where appropriate, to milk and its various products. This review will not attempt to deal with the chemical and nutritional aspects of quality as these are extensive enough to warrant separate treatment.

MICROBIOLOGICAL TESTS

Interest in bacteriological testing of milk stemmed from the discovery that bacteria in milk could cause disease and spoilage. The first systematic investigation of a milk supply was that of Sedgewick and Batchelder in Boston in 1892 (144). From a public health standpoint the importance of bacteriological examinations was quickly recognized and gradually such examinations have become a regular practice. The need (135) for uniform procedures led the American Public Health Association in 1905 to appoint a committee to standardize methods. Studies by this and subsequent committees have resulted in successive editions of

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Standard Methods for the Examination of Dairy Products (2), the 10th edition of which is currently being revised. In Europe there has also been interest in standardizing methods internationally; more recently, the International Dairy Federation has been active in this sphere (77).

With marked advances in the eradication of bovine tuberculosis and brucellosis, together with widespread pasteurization of milk, interest in bacteriological testing of raw milk has largely shifted from the disease aspect. Nowadays, such examinations are more concerned with obtaining an estimate of the degree of care taken in the production and handling of milk on the farm. Nevertheless, it should be remembered that conditions which allow the entrance and multiplication of large numbers of microorganisms may also allow the entrance and multiplication of pathogenic types.

The disease aspect is more important with pasteurized products, however. Recontamination with pathogens can have serious consequences. Direct testing for pathogens is impracticable, but testing to detect any recontamination after processing is now common.

High bacterial populations in milk may result from heavy initial contamination, as from neglected milking machines, from growth in the milk, or from both. Over the years, cooling procedures have greatly improved and, with the farm bulk tank, microbial growth is virtually eliminated. Consequently, cooling may mask careless production practices. This is causing concern to many milk sanitarians and there is a definite feeling that a reappraisal of testing methods and standards is desirable. Attempts to cope with this situation by promulgating stiffer standards—e.g., 50,000 per ml. (36)—are scarcely adequate, and involve setting up two different standards for acceptable milk.

Long ago it was recognized that the more carefully milk is produced, the less important cooling becomes. This is because the udder flora, which comprise the bulk of the bacteria in cleanly produced milk, grows best at body temperature and very slowly at temperatures below 15° C. (59° F.). In Britain, the importance of this has long been recognized. Official procedures (113) for testing raw milk call for it to be held at atmospheric shade temperature (averaging slightly under 60° F.) for 12 or 18 hr. before starting the test. Obviously, this is less satisfactory than a fixed temperature and would be out of the question where wider extremes of temperature are encountered. Recently, it has been suggested (90) that samples be given a preliminary incubation (P.I.) at 12.8° C. (55° F.) for 18 hr. before testing. At this temperature the udder flora fail to multiply. When counts made before and after P.I. are compared, little or no increase is found in carefully produced milks; others, even though initially low, may have increased over one hundred-fold. This procedure thus tends to show up those milks where cooling masks faulty production practices, and rightly puts the emphasis on clean methods rather than on efficient cooling. A somewhat similar procedure has been used with bulk tank milk in Scotland (30).

While bacteriological testing is essential in a quality control program, it is not enough in itself. Regular farm inspection is also considered essential. The work of the fieldman or sanitarian can be made much more effective by an adequate program of bacteriological testing. Both are indispensable.
QUANTITATIVE TESTS FOR TOTAL BACTERIA

The Standard Plate Count (S.P.C.). This method of assessing the bacteriological quality of milk was the first laboratory test devised for this purpose. Before pasteurization came into widespread use, there was a tendency to regard the plate count as a partial index of the freedom of the milk from pathogens. This view has not completely disappeared. Medical authorities in Britain still insist on 37° C. incubation for the plate count method, on the grounds that pathogenic bacteria will be more readily detected at this temperature. (It is usually recognized that the S.P.C. is of no value for this purpose.) Nowadays, it is more generally accepted that the chief value of the standard plate count (S.P.C.) on raw milk is as an indication of the sanitary conditions of production and handling.

Although generally conceded to be the most precise method for assessing the bacterial population, the S.P.C. is not without limitations. No one medium incubated for a short time at a given temperature will bring out all the bacterial types present. Furthermore, colonies may represent single organisms or clumps of hundreds. The degree of experimental error inherent in the method is considerable (175), so that in comparison with chemical tests the plate count is a rather elastic yardstick by which to measure the bacterial content. This, unfortunately, is not always realized by administrators, and unwarranted significance is sometimes attributed to small differences in counts. There is also a tendency to consider the S.P.C. the sole criterion of hygienic quality, and to regard it as the standard against which all other tests for milk quality must be compared. Standardization of plating media, in order to ensure uniform productivity from different batches of media, has yet to be accomplished, although a synthetic reference medium has been developed (131).

Despite these limitations, the S.P.C. is generally conceded to be the most suitable method of examining raw milk with a low (e.g., less than 200,000 per milliliter) bacterial content. As quality improves, it is tending to replace other types of test. Some authorities consider it too laborious and expensive (59, 75); the United States appears to be the only country able to afford it for routine control of raw milk. Even here there is interest in simpler and cheaper procedures, such as the roll-tube method (5, 34, 75) and others (2, 67), especially for unofficial control purposes. In Britain, although the methylene blue test is the official test for routine control (113), the plate count is preferred for advisory purposes (19).

As a result of the studies at the Geneva Agricultural Experiment Station (130), the desirability of an incubation temperature lower than 37° C. gradually has been accepted. Dairy bacteriologists generally prefer 30 or 32° C. for a 48-hr. count. Some would like incubation for 72 hr. for pasteurized products (77). Public health laboratories, on the other hand, have generally preferred the higher temperatures used in their diagnostic work. To try to satisfy both groups, in 1948 incubation either at 32 or at 35° C. was made optional (2). S.P.C. at 32° C. is less likely to disagree with results of dye reduction tests, according to Harris et al. (65). Evidence continues to accumulate that 35° C. is too high
for certain types of bacteria (10, 11, 49), but there is still considerable resistance to adopting a lower temperature.

While the plate count is generally conceded to be the most suitable test for measuring the bacteriological quality of pasteurized products, about all it really tells when applied to freshly pasteurized products is how many thermoduric bacteria were present in the milk before pasteurizing. To this extent, a high count is useful in indicating neglected milking equipment, especially milking machines, the chief source of these organisms. In Europe, however, the primary interest appears to be in the keeping quality of the pasteurized milk or cream. Instead of making tests on the fresh samples, it is customary to hold samples at 5 °C for 24 hr., then at 17 °C for another 24 to 48 hr., before testing (16, 18, 125). After such treatment, a coliform test or a methylene blue reduction test is preferred to a plate count as correlating more closely with actual keeping quality at 17 °C.

While the same incubation period is specified in Standard Methods for both raw and pasteurized milks, the Committee set up by the International Dairy Federation (77) recommended an extra 24 hr. (72 hr.) for pasteurized milk. This is in recognition of the fact that many organisms which survive this treatment exhibit a long lag phase, resulting in failure to form discernible colonies in 48 hr. This is even more true with milk powder samples; after 72 hr., counts are often sharply increased, and colony size is much more satisfactory for counting (86). It is believed that Standard Methods should be revised to call for 72-hr. incubation for dry milks; the Committee of the International Dairy Federation (77) has recognized the need and has recommended incubation at 30 °C for five days.

In passing, it should be emphasized that a low standard plate count on a fresh sample is no guarantee of adequate keeping quality (87, 126). Milk may be a week or more old before it is consumed, and during that period has generally been exposed to temperatures which permit the growth of psychrophiles and some mesophiles. A freshly taken sample may show an extremely low S.P.C.—mostly thermodurics—yet, within a week at refrigeration temperature a slight contamination with psychrophiles may result in hundreds of millions per milliliter. As an index of plant sanitation, the S.P.C. made on samples after holding for five days at 7.2 °C. (45 °F.) is most effective (87); this procedure is much more sensitive than the coliform test, either before or after refrigerated storage. Samples with low counts after such treatment should have excellent shelf-life.

Other cultural methods. The S.P.C. requires considerable glassware, equipment, and media, and is expensive. Consequently, there is considerable interest in simplified methods, even where these may be less precise.

The Burri slant method (2, 28) employs a calibrated loop with which to deposit and spread 0.001 ml. milk over the surface of a hardened agar slope in a test tube. Although less accurate than the S.P.C., it is a useful screening test, especially where, as on the farm, no laboratory facilities are available. Modifications include the oval tube method for determination of thermoduric organisms (2, 119). The "Seeing Is Believing" test of Jamieson (2, 78), an offshoot of
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the Burri slant method, can be used to advantage for swab tests on utensils, equipment, etc.

The roll-tube method has been proposed from time to time. With the development of improved apparatus, especially the Astell spinner, interest has revived, and favorable results have been reported (5, 34, 75, 140). The method is said to be used fairly extensively by milk plants in Britain. Heinemann and Rohr (67) have described a simplified procedure employing a loop and a screw-capped bottle which appears to have promise.

The membrane filter (MF) technique for total count yielded lower counts and was more cumbersome to carry out than the plate count method, according to Norwegian workers (57).

Surface plating techniques have been advocated from time to time. The latest of these is that of Mallmann and Broitman (105). None of them appears to have found favor for routine analytical work.

**Direct microscopic count.** This method (D.M.C.), described by Breed (2, 24) in 1911, has outstanding value in furnishing a bacteriological estimate within a few minutes. It also enables a count to be made of body cells (leucocytes, lymphocytes, etc.); this is especially valuable in indicating mastitis symptoms. With high-count milk the method, in the hands of trained technicians, can often furnish a clue as to whether utensil contamination or growth is responsible, provided the sample is from an individual can. It is also possible to preserve samples for later testing by adding formaldehyde (100). Despite these advantages, its popularity for controlling fluid milk supplies has been declining in recent years. In part, this is due to the trend to lower count levels, especially with farm bulk tank milks, necessitating the examination of many fields for quantitative determinations and, in part, to the realization that the D.M.C. grades high-count milk more leniently than does the S.P.C. (81). Black (21) reported the S.P.C. to give higher counts than the D.M.C., with counts exceeding 50,000 per milliliter; below this level, the reverse was true. The author has confirmed this. Another factor has been the growing recognition that many laboratories using the method lacked trained personnel and adequate equipment, especially illumination (21), and that often far too few fields were being examined (101). The effect of fatigue on qualified, conscientious technicians also has been belatedly recognized (99). The growing difficulty of obtaining suitable personnel, and their reluctance to undertake this work, must also be considered. While the D.M.C. is largely being superseded by the plate count for market milk, it is still a very valuable supplementary test here, and has considerable control value with the higher-count milk used in manufactured dairy products.

While the D.M.C. has been recommended in place of the S.P.C. for the control of pasteurized milk (111, 167), it has not been generally adopted for this purpose. More recently it is coming into use in the control of skimmilk powder (54). Here it gives valuable evidence of the past history of the product not obtainable from the viable count. Much more emphasis on adequate equipment and training and close adherence to *Standard Methods* is essential, however, if the method is to yield reliable results (54).
A modification of the D.M.C. for making counts of thermoduric bacteria was proposed (106), but does not appear to have been adopted. Poor agreement with the plate count has been reported (51).

"Little Plate" method. An attempt was made by Frost (56) to combine the advantages of the plate and direct microscopic counts. His little plate method involved growing the bacteria in a thin layer of nutrient agar on a microscopic slide, incubating in a moist chamber for 8-16 hr., then drying the film, staining and examining under a low-powered objective. The method is cheap and results are available earlier than with the S.P.C. Modifications have been published from time to time, but the method has failed to gain much support. Interest in its use as a screening procedure has recently been reported (44). It has also been proposed for making counts of bacteria, molds, and yeasts in butter (82, 114).

The methylene blue reduction test. The methylene blue reduction test (2), introduced in Denmark and Sweden around 1912, is probably the most extensively used bacteriological test, being widely employed in Europe, Australia, and New Zealand, as well as in North America. Its great advantages are its simplicity, cheapness, reproducibility, and rapid detection of poor-quality milks. Technically trained personnel are not necessary, and several hundred samples can be tested simultaneously. Thus, more frequent testing is possible.

Until recent years there has been good correlation between reduction times and plate counts, especially where the creaming error was minimized by periodic inversion of the dye-milk mixture during incubation. Wilson's (175) extensive studies were so favorable to the modified methylene blue test that in 1937 it officially replaced the plate count for the examination of graded raw milks in England and Wales (113).

Several factors have tended to distort the relationship between counts and reduction times. More productive media and lower incubation temperatures have increased the levels of plate counts. The proportion of thermoduric organisms has increased (152); these are slow reducers. Antibiotics in milk tend to slow down reduction. Psychrophiles, which sometimes comprise a high percentage of the flora (90), fail to grow at 35-37 °C. Finally, with more efficient cooling, the bacteria are extremely dormant at the start of the test and substances inhibiting bacterial growth are conserved. The resulting prolonged lag phase so delays reduction that some high-count milks escape detection.

The creaming error, caused by the sweeping of varying proportions of the bacterial population to the surface with the rising fat globules (84, 175), has already been referred to. Wilson (175) recommended inversion of tubes every 30 min., to redistribute the bacteria and thus minimize this error, but hourly inversion is equally effective (83) and more convenient. The value of this practice is greatest with low-count milks; for milks reducing in 3 hr. or less, it is less important (83).

The practice in Britain of aging or storing raw milk samples at atmospheric shade temperature for 12 or 18 hr. before testing has already been mentioned. This stems from the 1920's, when regulations (112) governing the conduct of Clean Milk Competitions called for holding samples for around 24 hr. at atmos-
pheric shade temperature before subjecting them to analysis by the plate count. The weakness of this procedure lies in the wide range of temperatures encountered even in Britain. Thus, although the stipulated reduction time for the summer months is only 4.5 hr. as against 5.5 hr. in winter, summer samples show a much higher percentage of failures to conform than do winter samples. Furthermore, several workers have reported the grading to be too lenient in the winter months, a high percentage of samples with high bacteria counts not being detected (103, 166, 175). Storage at a definite temperature, e.g., 12.8° C. (55° F.) would seem more desirable; during cold weather it would allow the growth of saprophytic contaminants and result in shorter reduction times. Comparison of results from one season to another, and one year to another, also would be facilitated.

On this continent, the methylene blue test is rarely applied to pasteurized milk or cream. In Britain, where the interest is predominantly in keeping quality, Provan and Rowlands (136) recommended that the modified methylene blue or the resazurin test replace the plate count. After being held at 18° C. for 18 to 24 hr., the sample is incubated with the redox indicator at 37° C. for 0.5 hr.; samples failing to reduce the dye by this time are considered to have a keeping time in excess of one day at 18° C. Unfortunately, the official regulations (53) which followed specified holding at atmospheric shade temperature (not exceeding 18.2° C.) in place of 18° C., as recommended; methylene blue, rather than resazurin, was designated as the indicator, although Provan and Rowlands had favored the latter. In Britain, this type of test is still applied to ice cream also, but its suitability appears dubious. As Humphriss (76) states, “After all, the Public Health Officers, the manufacturer and the consumer are concerned with the wholesomeness of the product as eaten, and not after incubation for three-quarters of a day at warm room temperature.”

As previously mentioned, some of the more progressive dairies in Europe use the methylene blue reduction test as a measure of keeping quality of pasteurized products (16, 18, 125). Their practice of holding samples at 5° C. for 24 hr. (to facilitate detection of oxidized flavor), then at 17° C. for 24 or 48 hr. before testing, avoids the variation in storage temperature of the British method and allows more satisfactory comparison of results from various periods. However, the coliform test is considered to be superior to the dye reduction test for this purpose (18, 125), particularly since rapid dye reduction is sometimes due to aerobic spore-formers which survive pasteurization (125).

For milk for manufacturing purposes, where standards are not as high as for market milk, the methylene blue reduction test is still widely favored in most countries. Unless and until standards for such milk are made much more stringent, it will doubtless continue in popularity. However, as cooling of such milk becomes more effective, its usefulness in its standard form will probably decline.

The resazurin test. As milk quality improves, reduction time increases, increasing the experimental error and making it difficult to complete testing in the ordinary working day. The introduction of the dye resazurin as a redox indicator to replace methylene blue (132) offered the advantage of earlier indication of Eh change, and aroused considerable interest. This was heightened
by the discovery (137) that resazurin was affected by the presence of excessive numbers of leucocytes etc. and thus could indicate the presence of abnormal (mastitic, late lactation, etc.) milk. This advantage is not possessed by methylene blue and is largely dissipated on prolonged storage. A number of modifications have been described, but only three of these have come into general use.

The 1-hr. test (137), modified from the original test by using 37° C. incubation in place of room temperature, was studied by various workers. In this test the degree of color change from the original blue through pink to complete decolorization is measured. While useful in reflecting abnormal udder conditions, it had limited value except where little quality-improvement work had been attempted; well-cooled milks containing excessive numbers of dormant bacteria often escaped detection (68, 69, 91). Where samples are aged before testing, as is officially required in Britain, this weakness applies only during the colder months. The pink test (84) which followed employed the full pink color as the end-point; this was reached in three-fourths of the time required to reduce methylene blue, but unfortunately the ability to detect abnormal milks was lost. A modification, somewhat more involved, has been proposed by Hempler (69), whereby abnormal secretion may be detected by a reading at 1.5 hr. and bacterial numbers by readings after 3.5 and 5 hr.

The triple reading test (2, 91) was developed to avoid the disadvantages of the 1-hr. and the pink tests. It employs a single end-point (Munsell 5P7 4) with readings after 1, 2, and 3 hr. This end-point is reached in one-half the time required for methylene blue reduction. Reduction beyond this end-point within the first 2 hr., followed by slow subsequent change, suggests reduction by leucocytes. The triple reading test has been recognized as a standard method (2) since 1948, and is widely used in Canada for market milk supplies. In order to distinguish milk of exceptionally good quality from that of only satisfactory quality, Hempler (68) has suggested a slightly stiffer grading with the triple reading test. He comments that the triple reading test, in addition to saving considerable time, "reflects the sanitary quality of the milk on a far broader basis than does the methylene blue test." Overby (127), after a study of various tests for the bacteriological quality of milk, also gave the preference to the triple reading test.

Of recent years, especially where farm bulk tanks are common, there have been criticisms that the resazurin triple reading test fails to detect an appreciable percentage of high-count milks. The organisms are so dormant that reduction is delayed appreciably. Here, too, preliminary incubation (90) is most useful in overcoming this dormancy, as well as in encouraging the growth of saprophytic contaminants. The poor agreement between S.P.C. and resazurin reduction time noted on fresh samples largely disappeared following P.I., and there was close agreement between gradings by these two tests (90). Unfortunately, reduction due to leucocytes is largely dissipated during P.I.; it is necessary to use a direct microscopic examination (2) or the Whiteside Test (117, 171), to detect abnormal milk.
Other forms of the resazurin test are employed in Britain. A 10-min. rejection test is applied to milk of doubtful quality (14). This test was selected during the war years as being the most satisfactory of eight methods compared for detecting milk unsatisfactory for pasteurizing, plate counts being of the order of many millions per milliliter (14). [In other countries, reliance is placed more upon a keen nose on the inspection deck; this appears to be a more delicate test (36).] Where cans are owned by the producer, it is also difficult to visualize cans of milk being held for over 10 min. awaiting results of the test.

In Scotland, a weekly temperature-compensated resazurin test is also officially required (42); the reduction time for acceptable milk varies with the mean atmospheric shade temperature during the preliminary holding period from 2 hr. at 40 °F. to 15 min. at 61 °F. and over. Where excessive leucocyte activity is suspected, a methylene blue test at 37.5 °C. also is run. In Britain, grading is done using a Lovibond tintometer and standard color disks ranging from six for initial color to zero for complete decolorization. This method is considered to be more cumbersome and time-consuming than that using Munsell color standards (85) in test-tubes.

In any modification of the resazurin test, it is well to remember that 8% of the male population is color-blind; also that good illumination, preferably from a daylight type fluorescent lamp (40, 91), is highly important.

Golding’s modification (61), wherein dye is added to sterile vials and dried down, is particularly useful for plants lacking adequate laboratory facilities, and for use by fieldmen testing suspected quarters for mastitis (108).

The use of another oxidation-reduction indicator, triphenyltetrazolium chloride, has been suggested (118). Unfortunately, it is extremely sensitive to light. Its usefulness appears to be confined largely to heavily contaminated milks, although it has been advocated for use in the detection of antibiotics and other inhibitory agents in milk (120), and in a keeping quality test for pasteurized milk (26, 41).

**QUANTITATIVE TESTS FOR SPECIFIC TYPES OF MICROORGANISMS**

*Thermoduric bacteria.* These organisms are sufficiently heat-resistant to survive ordinary pasteurizing temperatures and thus may be responsible for counts in excess of the legal limit for pasteurized products. They enter milk chiefly from the surfaces of inadequately cleaned milking and handling equipment and thus are an indication of insanitary conditions. Some sanitarians believe the thermoduric count is more useful here than the standard plate count (15). While the thermoduric count is officially determined by plating a suitable dilution of raw milk after being subjected to laboratory pasteurization (2), less expensive modifications have been sought. These include the oval tube method of Myers and Pence (119), streaking measured loopfuls of laboratory pasteurized milk on the surface of poured plates (2); the agar strip method (153), a version of the oval tube method, developed in Britain (it is preferred over the roll-tube method (5), as it requires no special equipment (47); counts by both methods were lower than by the standard plate count, but not enough to invalidate their
successful use]; and inoculating melted agar in a test-tube or small bottle, holding at pasteurizing temperature for 30 min., then spreading the inoculated medium in a thin layer. The Astell version (5) of the roll-tube method has already been mentioned.

Incubation at 32°C is preferable to that at higher temperatures; 37°C is too high for some thermoduric species. British workers (37) recommended incubation at 30°C, for four days, but subsequently (47) reduced this to three days. While the results from these simplified methods are more variable than those by the official plating method, they are quite adequate for screening out those milks containing excessive numbers of these organisms. A standard of 10,000 per milliliter, commonly employed, seems unduly lenient.

Thermophilic bacteria. Organisms capable of growing at holder pasteurization temperatures were a serious problem when batch pasteurization was common. With the trend toward higher temperatures with HTST and UHT pasteurization, the interest has diminished. These organisms are detected by incubating plates at 55°C for 48 hr. (2), by direct microscopic examination of smears (2), or by a dye reduction test (methylene blue or resazurin) incubated at 62–63°C. (94).

Psychrophilic bacteria. For the purpose of this review, psychrophilic bacteria are those able to grow actively at temperatures below 7.2°C (45°F). It should not be overlooked, however, that their optimum growth temperatures are generally around 25 to 30°C; not one of 722 cultures grew at 35.5°C. (49). With milk being held longer at refrigeration temperatures from cow to consumer, opportunities for the growth of psychrophiles have increased greatly. Many of them are proteolytic and lipolytic, and are capable of inducing flavor and other defects in milk and milk products on refrigerated storage.

The current official procedure (2) for counting psychrophiles calls for incubation at 5°C for seven days. Some claim that this low temperature is too selective. Milk and its products are frequently exposed to temperatures above 5°C; some species capable of inducing spoilage can grow actively in this range. After seven days at 5°C, colonies are frequently so tiny as to be difficult to recognize; counts after ten days are frequently many times larger, while some workers (23) found 20 days necessary to obtain maximum counts. In Denmark, Hempler (70) prefers 7°C for ten days;¹ this should be much more satisfactory.

Spoilage in pasteurized milk, cream, and cottage cheese is generally due to psychrophilic growth. Pasteurization destroys these organisms; their presence in processed products represents recontamination. Examination of the fresh sample is not always helpful, since the numbers present may be small (126). A more useful procedure is to hold samples at 7.2°C (45°F) for five days, then subject them to the S.P.C. at 32°C. (87). This gives any psychrophiles present an opportunity to grow, and counts will often be well into the millions. This is regarded as a much more satisfactory procedure than making a psychrophilic count on the fresh sample.

¹ This will probably be specified in the forthcoming 11th edition of Standard Methods.
Coliform bacteria. The presence of coliform bacteria in milk and its products does not have the same public health significance as does their presence in water supplies. Hence, in routine control work there is no point in attempting to differentiate between so-called fecal and nonfecal types.

For the routine examination of raw milk, interest in North America has been largely confined to certified milk (1), although one state (123) has a standard requiring freedom from coliforms in 1/100 ml. portions. It has generally been felt here that high counts represented growth more than direct contamination. With the widespread adoption of farm bulk tanks, growth will no longer be a factor, and more interest in the use of this test may be expected. The survey of milk supplies in eight U.S. cities reported in 1953 (38) showed some correlation between udder cleanliness and coliform content. The results of this survey (log. average coliform count 5,810 per milliliter) suggest there is room for considerable improvement.

In Britain, various authorities (62, 148, 165) have asserted that the coliform test gives the best indication of cleanliness of milk production; high coliform counts most frequently arise from neglected milking machines (148). The possibility that high counts may come from udders infected with coliform organisms must not be overlooked (164).

In pasteurized products, the use of the coliform test to detect recontamination has been more generally accepted. Stemming largely from the work of McCrady and his coworkers (109), the great value of this test, to both sanitarians and management, has steadily received wider recognition. The excuse that coliforms in pasteurized milk may not be heat-resistant was discredited by Buehbinder and Alf (27); the presence of coliforms in a pasteurized product definitely indicates recontamination and a potential health hazard. Pasteurized products, handled in clean, sanitized equipment, should be entirely free from coliform organisms. That this is possible is shown by the record of the Trifolium Dairy in Copenhagen (125); in 1955, 82.4% of samples were coliform-negative in 1-ml. portions after samples had been held at 17° C. (62.6° F.) for 24 hr. before testing. This procedure is employed by other European dairies. Encouraging results have also been reported by the Quebec Ministry of Health (7), where a limit of 50 coliforms per 100 ml. is in effect. The standard of not more than 10 per milliliter currently in vogue in the United States (168) is so lenient as to be of limited value; the presence of any coliforms should call for immediate investigation.

Two distinct types of test procedure, a plating technique and a tube dilution technique, are officially approved (2) in North America. Divergent opinions have been expressed concerning their relative merits. Where standards are stiff, as in Quebec (< 50 per 100 ml.), the dilution technique is preferred as larger volumes of milk can be tested (7). Where standards are more lenient (168), the plating technique is generally favored. Two shortcomings of the latter are (a) difficulty in deciding whether small red colonies should be counted, and (b) atypical colonies on overcrowded plates.

When applied to products containing other sugars in addition to lactose, e.g., ice cream, positive results must be confirmed to avoid misleading results.
False positive results have been reported where sweetened and unsweetened fresh fruits (bananas, peaches, strawberries) are added to the mix (12).

While 35°C is currently the accepted incubation temperature on this continent (2), there is evidence that higher counts would be obtained with a lower incubation temperature. In Britain, the Coli-Aerogenes Sub-Committee of the Society of Applied Bacteriology, impressed with the evidence of numerous 37° negative strains, has recommended incubation at 30°C (139). Belgian workers (174) report that over 50% of strains positive at 30°C were negative at 35°C.

A unique procedure, the Baeto-strip method (55), has recently been developed for determining coliform counts. A definite volume of milk is absorbed by a sterile strip of filter paper impregnated with a suitable medium; the strip is returned to its plastic pouch and incubated 8–10 hr. The incubation period is inconvenient, and counts are generally lower than those by the regular plating technique (89). The tendency for colonies developing from motile strains to coalesce is a further drawback (60). Nevertheless, the method could be quite useful where no laboratory facilities exist.

The millipore filter technique has been advocated for the determination of coliforms in pasteurized and certified milk (48), but not enough information is available to date to judge of its value here. The use of a nonionic agent to facilitate filtration of 10 ml of milk or 1 ml of cream has been suggested (50).

Proteolytic bacteria. Organisms capable of proteolyzing casein are frequently responsible for undesirable flavors in dairy products. Surface taint of butter caused by Pseudomonas putrefaciens (43) is an example. The presence of most proteolytic organisms can be detected by plating on an agar medium containing 5–10% sterile skim milk, and incubating at approximately 21°C for three or more days. Colonies arising from proteolytic types will be surrounded by clear zones, where the protein has been digested by the enzymes they have secreted. A clear zone may also surround certain nonproteolytic bacteria which produce slight acidity; by flooding the plate with dilute acids, these zones around non-proteolytic colonies will disappear.

Lipolytic bacteria. Organisms attacking fats are often also proteolytic and also psychrophiles. This makes them particularly important in butter, cream, and cottage cheese, which are frequently held refrigerated for extended periods. The free fatty acids liberated from fats by hydrolysis contribute pungent flavor and odor to the product; these may be desirable in certain cheeses, but in other products they generally cause defects.

The enumeration of lipolytic bacteria is based upon detection of free fatty acids liberated from fat added to the culture medium; Nile blue sulfate is the indicator normally used. It stains normal fat globules pink; when the fat is hydrolyzed by lipolytic colonies, the globules stain blue. Care must be taken to avoid the toxic effect of an excessive amount of dye (96). One per cent of sterilized stained fat is added to the tempered medium just before pouring; the fat should be in the form of a fine emulsion. Tributyrin is frequently preferred to natural fat as it is more easily hydrolyzed; however, not all organisms hydrolyzing tributyrin also hydrolyze butterfat.
Molds and yeasts. Proper pasteurization of milk or cream destroys molds and yeasts; thus, their presence in a product indicates recontamination (22). Mold and yeast counts on butter are commonly run to check on plant sanitation; trouble with mold growth on butter is much less frequent when counts are low. The medium currently accepted here as standard (2) is potato dextrose agar, acidified to pH 3.5 before pouring to inhibit bacterial growth. Olsen and Bonner (124) recently reported 100 p.p.m. aureomycin even more useful for this purpose, yeast counts frequently being much higher than with the standard medium. Unpublished results from the writer’s laboratory have confirmed this.

Mold and yeast counts are also employed by progressive cottage cheese manufacturers and sanitarians as indices of plant sanitation. A standard of not over 10 per milliliter is currently being met in some areas.

Bacto-strips (55) are also available for determining the mold and yeast count on equipment surfaces, in the air, etc. Good agreement with results from the conventional technique for determining air-borne contamination has been reported (89).

Mold mycelia count. When cream is stored on the farm at unsuitable temperatures for too long periods, growth of Geotrichum candidum takes place. Although the mold is killed by pasteurization, dead mycelia pass into the butter in appreciable amounts. They can be counted by a method (2) similar to that used to detect mold filaments in tomato products. This method can, therefore, tell much more about the lack of care the cream has been subjected to than can a viable count on the butter; it is frequently employed by food and drug officials and may be used as the basis for seizure and confiscation of butter.

Fermentation test. Milk for cheese-making is often tested by the methylene blue test, then incubation continued overnight in the fermentation test (153). It is believed by some that the type of curd formed indicates the types of bacteria present in the milk, particularly those causing gassy defects in cheese-making. However, the more commonly held view is that it is better to discriminate against milk heavily contaminated with bacteria; with lower-count levels the lactic acid bacteria in the starter have little difficulty in establishing dominance.

Keeping-quality tests. In Britain considerable emphasis is still placed upon keeping-quality tests for raw milk. Since almost all milk is pasteurized, and since there is usually little correlation between keeping quality before and after pasteurization, this interest seems rather archaic. Various methods have been tried out; of these, the clot-on-boiling (C.O.B.) test (45) is considered the most accurate, although it reflects chiefly souring. In this test, portions of the sample are maintained at 18° C.; morning and evening a fresh portion is immersed in boiling water and examined for clotting; this continues until clotting occurs. The method appears too cumbersome and time-consuming for routine testing.

As already indicated, the dye reduction tests are used to assess probable keeping quality of pasteurized milks in Europe. In the United States, TTC (26) and neotetrazolium (41) have been suggested for this purpose. In Europe, the coliform test after preliminary incubation is considered to be a more reliable
indication of keeping quality (18, 126). In all of these procedures, the pasteurized sample is maintained at a temperature—usually 17–18 °C. (62.6–64.4 °F.)—favorable for the growth of saprophytic contaminants for a period before testing. The S.P.C. on pasteurized samples held at 7.2 °C. for five days gives a good indication of the extent of recontamination, especially with psychrophiles responsible for spoilage during refrigerated storage.

**Antibiotics.** The presence of antibiotics in milk, either residual from therapy or by deliberate addition, can influence the results of bacteriological examinations (52, 92, 173), in addition to causing trouble in the manufacture of products dependent upon the lactic fermentation and the possible hazard to those individuals acutely sensitive to penicillin. Where the results of bacteriological tests are unexpectedly good, it may be wise to test for the presence of antibiotics.

Unfortunately, there is no rapid test whereby milk containing antibiotics can be detected and rejected. The incorporation of a suitable dye as a tracer has been reported on favorably (64, 149), but the method still lacks official sanction. This would appear to be the most satisfactory solution of this problem; producers would be much less prone to ship milk containing residual antibiotics if they knew that detection was simple. However, this would not preclude the possibility of the deliberate addition of antibiotics as preservatives.

A number of different tests have been reported for the detection of antibiotics in milk. All of these are based upon interference with bacterial growth and activity. One of the simplest is a starter activity test (146), patterned after that introduced by Horrall and Elliker (73); here, the extent of acid development when inoculated with a lactic starter and incubated for several hours is compared with that of a control. Care must be taken, however, to exclude the action of naturally occurring inhibitory substances. Greater sensitivity can be obtained by using *Streptococcus thermophilus* in place of the common starter streptococci (17). Another form of test utilizes a redox indicator to reflect interference with bacterial growth when incubated at a suitable temperature; triphenyl tetrazolium chloride is the indicator commonly recommended (120), although methylene blue (58, 143) and resazurin (115) have been used. The disk assay method has also been studied extensively. In its standard form (2), it is most useful for detecting the presence of penicillin; the test organism, *Bacillus subtilis*, is less sensitive to other antibiotics (88). An interesting modification of this method has recently been described (145), wherein the agar layer is flushed with resazurin; the completed test takes considerably less time than the standard disk assay method.

**Hydrolytic rancidity.** With the spread of bulk handling of milk there has been heightened interest in the occurrence of hydrolytic rancidity. A useful method for estimating the degree of its development has been developed by Minnesota workers (162).

**ESTHETIC CONSIDERATIONS**

**Sediment.** In addition to the microbiological tests already discussed, certain tests are commonly conducted which are concerned with esthetic considerations. Tests for sediment in milk (2) are usually applied to incoming raw milks. Dirt
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has no place in milk; hence, its presence indicates carelessness in milking or handling. Milk containing in excess of 2.5 mg. per pint as determined by the off-the-bottom type of tester is customarily rejected. Unfortunately, a relatively clean disk may simply represent a good job of filtering on the farm, rather than care in production.

Different types of apparatus for sediment testing may give quite different results. Preliminary studies (80) have shown that the type operated by compressed air may collect appreciably less sediment on the disk than do certain other types.

There is also a tendency to consider that milk containing appreciable sediment is also high in bacteria. This is not necessarily so. Milk may contain appreciable amounts of sediment, yet show a reasonably low count; on the other hand, milk free from sediment may have picked up a heavy load of contamination from unclean equipment surfaces etc., or appreciable growth may have taken place.

Sediment tests are occasionally run on bottled milks. In most well-operated plants, however, the milk is subjected to filtration or clarification there in addition to straining on the farm, so the amount of sediment present is usually extremely small. Sediment tests for churning cream are also made by enforcement agencies; these require special facilities whereby the entire can of cream may be filtered (66). The visual mold test for cream (2, 129) might also be regarded as a form of sediment test. It is a useful additional criterion of churning-cream quality (122).

Milk for cheese-making may contain appreciable amounts of sediment, which is concentrated approximately elevenfold during the making process. The U. S. Food and Drug Administration has taken the lead in attacking this problem. In Canada since 1954, every vat of Cheddar cheese offered for grading by the federal grading service (111,884 in 1957) has been tested for extraneous matter, using essentially the procedure developed by Thibodeau (161). In 1957, federal grade standards (29) were amended to require a satisfactory extraneous matter test to qualify cheese for First Grade. This has greatly improved the picture, but it is suspected that it represents more effective dirt removal at farm and factory rather than cleaner milk production.

Decomposition. Tests for decomposition also may be regarded as concerned with esthetic considerations. Here tests for water-insoluble acids (WIA) and butyric acid (BA) in butter come to mind (71). Lipolysis in churning cream caused by growth of mold or lipolytic bacteria is reflected in higher values for these compounds in butter, and such evidence may be used by enforcement agencies to take legal action.

The souring of milk and cream also may be regarded as a form of decomposition. The titratable acidity test (2, 153) really measures the buffer capacity of the product. It is still widely used in the grading of churning cream; for market milk on this continent it has all but disappeared, but for manufacturing milk it is still used as a basis for rejection. Its great advantage is the speed with which it can be carried out. Care must be used when applying this test to milk.
with high solids-not-fat; cases are known where even certified milk with counts under 10,000 per milliliter has been threatened with rejection because the titratable acidity was high (153). The effect of dilution of the milk or cream with water (2) in reducing the titratable acidity is not generally recognized (154).

The alcohol test (153), still used mainly to determine the suitability of milk for condensing, is primarily a reflection of acid development. Milk giving a positive reaction is usually within a few hours of curdling; on the other hand, some milks quite satisfactory for pasteurization may react positively (32).

Lactic acid is the chief product of bacterial growth in most milk samples; a rapid test for its detection and measurement would be most helpful. Based on the method of Ling (102), Clegg and coworkers (133, 158) have described a rejection test which can be completed in 5 min.; a value of 0.03% is recommended as the lowest value of apparent lactic acid at which milk could be rejected. However, even a 5-min. delay on the receiving platform would disrupt operations in many plants. A test of this type might replace the titratable acidity test in the examination of nonfat milk solids (29); at present careful neutralization makes possible the use of substandard raw material without detection.

Mastitis. While mastitis is primarily a problem of animal health, and occasionally is a factor in the transmission of human pathogenic organisms, the inclusion in the milk supply of the secretion from infected udders also may be regarded as an esthetic problem. Regardless of the use to which it is to be put, no milk can be considered to be of satisfactory quality if drawn from diseased udders. Milk is customarily defined, in part, as the product of the uninterrupted milking of one or more healthy cows, yet the average leucocyte count on samples in the 1953 U. S. survey (38) was 680,000 per milliliter. McKenzie (110) has recently reported that where the leucocyte count exceeded 100,000 per milliliter, the solids-not-fat content was correspondingly reduced. Certainly, a count on herd milk in excess of 500,000 per milliliter strongly suggests an appreciable number of infected udders in the herd.

Various procedures have been suggested for the detection of milks carrying excessive numbers of leucocytes. The direct microscopic examination of smears (2), using a low-powered objective, can yield very useful information in the hands of a trained worker. The ability of resazurin to reflect the weak reducing activity of leucocytes (137) can be used to indicate those herds in need of examination; in either the triple reading test (2, 91) or Hempler's pink test (68, 69), rapid, early color change, followed by a lag for an hour or more, should be regarded with suspicion. Tests can be carried out at the farm, also, preferably using Golding's dried vial technique (108).

A simple rapid procedure, which is rapidly gaining in popularity, is the Whiteside test (117, 171). It is effective in showing differences in herd milks, and can be used as a platform test; it can also be used by the fieldman, or by the farmer himself, to check individual cows or quarters. It has considerable educational value, since the farmer can readily observe how milk from normal and from infected animals differs in appearance. Favorable reports have appeared from various countries; one from Sweden (3) states that the Whiteside test was
more valuable than the catalase or the toxin–blood agar tests, and recommends its adoption. The California Mastitis Test (CMT) (142), developed from the Whiteside test, is designed primarily for checking individual quarter samples; it is much more sensitive than the strip-cup test, and is the basis of a promising mastitis control program (72).

The tests mentioned so far are indirect tests, useful for directing attention to certain milk supplies. A search for specific causative organisms also can be made; information from such tests is a valuable guide in treatment of mastitis. Samples should be taken aseptically from individual quarters. The simplest procedure is probably the Hotis test (74), especially good for detecting Streptococcus agalactiae. Use of blood agar, either as streak or pour plates, is considered the most reliable method; the type of colony and hemolysis aids in differentiating various common mastitis organisms. The CAMP test (31) is very useful in identifying S. agalactiae by a typical hemolytic reaction. Fuller information on various aspects of mastitis, including testing, is given in Plastridge’s review (134).

HEALTHFULNESS

Over the years, milk and its products have been involved in the spread of a number of diseases of human as well as of bovine origin (2). In North America and some European countries, marked progress in the elimination of bovine tuberculosis and brucellosis has sharply reduced the hazard to those still consuming raw milk, while the pasteurization of all but a small fraction of the milk supply has reduced the amount of milk-borne disease to insignificant proportions (39, 150).

Nowadays, routine laboratory testing of raw milk supplies in regard to safety is largely confined to the milk ring test (2) as a screening test to detect brucellosis. This simple, inexpensive test is so sensitive that when applied to pooled herd samples a single infected animal in a herd of 40 can be detected (79). Like all biological tests, it has its limitations. False positive tests occasionally occur in milk from noninfected cows, especially fresh cows or those suffering from mastitis; infected nonlactating animals escape detection, whereas milk from a small percentage of infected animals gives a negative reaction. Despite these limitations, the test has been of inestimable value in programs for the elimination of brucellosis (79).

Phosphatase test. Although it was accepted that efficient pasteurization made milk safe, health officials had no assurance that the procedure had been properly carried out. With the development of the phosphatase test by Kay and Graham (95), a routine analytical method first became available that could give fairly substantial assurance. Underpasteurization, or recontamination with as little as 0.1–0.2% of raw milk or cream will result in a positive reaction. It is still possible for small amounts of underpasteurized milk to be mixed with fully pasteurized milk and escape detection, but judging by the greatly diminished number of outbreaks of milk-borne disease (39, 150), the public health hazard is slight.
Various modifications of the original phosphatase test have been developed; two of these, the New York State Department of Health and the Sanders and Sager methods, are accepted here as official methods (2), whereas the Scharer method is only a screening procedure. A modification developed by Aschaffenburg and Mullen (9) is simple and rapid enough for a routine plant test, yet compares in sensitivity with the Kay-Graham test (95). Directions must be followed carefully, to avoid false positive reactions. Flavors and coloring compounds in ice cream which possess phenolic rings may give false positive reactions. False positive reactions also may result from growth of certain species of microorganisms which produce phosphatase (13, 63). Means of differentiating microbial phosphatases from milk phosphates have been described (98). More recently, reactivation of phosphatase in milk or cream processed at higher temperatures (176) has caused some concern to control officials. More emphasis upon keeping samples cold until tested would go a long way toward the solution of this problem, although an intensive study of reactivation is undoubtedly needed.

Cheese made from raw milk, especially Cheddar and its modifications, has been responsible for numerous outbreaks of enteric disease. Regulations usually require that Cheddar cheese sold when less than 60 days old be made from pasteurized milk. The Sanders and Sager method (141) is a modification of the phosphatase test expressly designed to overcome the strong buffering capacity of cheese and the presence of interfering substances. That of Kosikowski (97) is another. Phosphatase tests for cheese are fully discussed in a recent F.A.O. monograph (98).

While the phosphatase test may be applied to freshly churned butter to determine whether or not it was made from pasteurized cream, unsatisfactory results have attended its application to storage butters (95, 128).

Recently, interest has been aroused by food-poisoning outbreaks attributed to the presence of staphylococcus enterotoxin in nonfat milk solids (4, 8) and in Cheddar cheese and its modifications (6, 159, 160). While toxin production in raw milk itself rarely presents a hazard to health (93), due to the repression of growth of staphylococci by other types, some growth may take place both before and during the cheese-making process (107, 157). Cheese with excessive numbers of coagulase-positive staphylococci must, therefore, be regarded with suspicion (160). Counts of staphylococci in cheese are much higher during the warmer months (157); greater emphasis on prompt cooling of night's milk should reduce the hazard here.

Reliance can not be placed entirely upon low plate counts, however. In the manufacture of powder or in subsequent storage, the staphylococci may be killed off or die, but the enterotoxin remain. This was the case in the Puerto Rico outbreaks (8). Faulty plant practice, rather than excessive growth of staphylococci in milk before it reaches the plant, is more likely to be responsible for the presence of toxin in the powder (4).

GENERAL OBSERVATIONS

There is an unfortunate tendency for many people to forget that bacteriological determinations on milk or milk products are far from being precise.
measurements. Making fine distinctions between samples on the basis of small differences in plate counts, for example, is entirely unwarranted, and is expressly discouraged in Standard Methods (2). It should further be emphasized that conclusions as to the quality of a milk supply should be drawn only after examining a series of samples, not on the basis of a single sample. This is recognized in the Standard Milk Ordinance (168), where either the logarithmic average of the last four samples is taken or else one high count out of four is not penalized.

Because milks may show appreciable differences in bacteriological content from day to day, it is desirable that testing be carried out as frequently as possible. The less expensive dye reduction tests have an advantage here; they also indicate substandard supplies with a minimum of delay, so that the fieldman can check at once for the source of the trouble.

It should be emphasized once again that the hygienic quality of milk embraces a number of factors. No one method can furnish all the information required in quality control. In addition to estimating the total bacterial population, tests for thermoduric organisms and for excessive leucocyte counts are essential to the control of raw milk supplies; for pasteurized milk the coliform test, or plating after refrigerated storage for several days, is more valuable than the S.P.C. in indicating the care taken to minimize recontamination.

Probably because America has been in the van of progress in respect to milk hygiene, there has been a tendency to overlook testing methods developed elsewhere. This attitude appears to be changing. It seems probable that such developments as the Aschaffenburg and Mullen (9) modification of the phosphatase test, the use of penicillin in media for coliform testing (104, 147), the roll-tube procedure (5), and the coliform test on pasteurized products after previous incubation (16, 18, 125) could be used here to advantage.

The widespread adoption of farm bulk tanks makes it necessary to reappraise standards and methods of testing developed for milk in cans. Some means of encouraging the growth of contaminants prior to testing appears necessary, if bacteriological analysis is to give a reliable indication of the care taken in producing and handling milk. Such a procedure, applied to all milks regardless of the method of handling, appears less open to objection than that of setting up two different standards for acceptable milk (36). Furthermore, the adoption of preincubation would make possible a marked improvement in many milk supplies without altering currently accepted standards for milk handled in cans. In effect, it would mean acceptance of the principle on which control of raw milk has long been based in Britain—that testing freshly taken samples, particularly if milks have been well-cooled, may yield misleading results.

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