Assessment of Dairy Product Quality and Potential Shelf-Life - A Review

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ABSTRACT

Over the years, many tests and assays have been developed to estimate the quality and potential shelf-life of dairy products. These have ranged from simple, standard bacterial enumerations to more complex metabolite detections. This paper is a review of the parameters that have been used to estimate, or indicate the inherent quality of dairy products.

Milk has been shown to be ideal for the growth of microorganisms due to its water, protein, carbohydrate, mineral and vitamin content, all of which are important to many forms of bacteria (62). In the days before adequate refrigeration, bacterial spoilage ran rampant, and economic losses were staggering (5). Today, farm bulk tanks, every-other-day pick-up at farms, 5-d and 4-d-a-week plant operations, discontinuing of home delivery, and purchasing of milk only on shopping days have increased the age of milk before consumption (149).

These methods and a more critical consuming public have caused the food industries to re-evaluate their quality standards. Quality means different things to different people and standards are ever changing. It has been pointed out that concepts of milk quality depend on many factors: (a) educational background of the individual, (b) economic and social status of the individual, (c) scientific information available, and (d) the ordinances, laws, regulations, etc., under which the dairy industry operates (62). The milk industry has been a leader in establishing programs to give the consumer a product that is pure, of good flavor, of attractive appearance and of desirable keeping quality. These programs emphasize rigorous laboratory examination of milk and dairy products to ensure that this quality is maintained (62).

It is a frequently heard axiom that production of such superior quality dairy products possessing extended shelf-life requires high quality milk. Milk and other ingredients used should be free of off-odors and -flavors, abnormal chemical and physical properties, and undesirable microorganisms and their metabolites, especially those that occur from post-pasteurization contamination (134).

In determining the presence of post-pasteurization contaminants, two major problems are encountered. First, time is critical. Rapid results are needed so that the sources of contamination can be found and eliminated. Second, the number of contaminants in a freshly pasteurized sample may not be sufficient to detect contamination. The problem of detecting post-pasteurization contamination at a very early stage is serious. There may never be a perfect test for a specific plant, but any improvement would be helpful, especially if results can be obtained sooner than with some of the presently used methods (19).

The major goal of any test or assay used to assess the quality of a perishable dairy product must be to provide reliable and accurate results within a test period (less than 48 h) that would allow for effective corrective measures. These measures may mean removal of product, selling quicker or adjusting the next batch. As a result of this need, the objective of this review is to compare methods that have been used, and are currently being used to assess dairy product quality.

KEEPING QUALITY

The period between processing/packaging and when milk becomes unacceptable to consumers is referred to as the “shelf-life” or “consumable life” of milk. Although it reflects the “keeping quality” of milk, Baker (7) states that there is no adequate working definition of shelf-life. Consumers of dairy products must have some indication of the expected or potential shelf-life of the products they buy. Therefore, to allow consumers to assess the age of products at the time of purchase, a date is placed on the container that indicates either the date of packaging or the last date that the product may be sold or offered for sale. The consumer then expects that a product purchased at any time up to that date is of acceptable quality. Also, if properly treated, it should remain acceptable beyond the last date of sale (78).
On a daily basis, the consumer uses taste and smell to judge how long milk, kept under refrigeration maintains an acceptable flavor. This, in effect, is the consumer's own test of keeping quality (75); therefore, flavor and keeping quality are of paramount importance to maintaining consumption of fluid milk (8). The most common descriptions given to unacceptable milk flavors by consumers are staleness, putrid, and/or curdled, bitter and fruity (7).

When dairy products leave the plant and are displayed in supermarket showcases and, finally, reach the home refrigerator, low temperature refrigeration is the exception rather than the rule for each of these (149). Temperatures below 7°C are one of the key factors to extending shelf-life (10). It has been generally accepted that an increase in storage temperature of 5°F will decrease the shelf-life by approximately one-half (94). Hankin et al. (79) concluded that keeping quality at any storage temperature was unrelated to the manufacturer's code date (last day product is to be sold). There was a significant correlation between keeping quality at 10°C and the other two storage temperatures of 1.7 and 5°C, suggesting a practical test to measure keeping quality at the lower temperatures.

Commercially processed milk stored at 0°C averaged 31 d of shelf-life but only 6 d at 7°C, with summer milk possessing twice the shelf-life of winter milk, as reported by Finely et al. (55). Grosskopf and Harper (69) found that aseptically packaged milk stored at 4°C had a shelf-life of 28 d. Hankin et al. (79) reported that keeping quality of commercially filled milk cartons stored at 1.7, 5.6 and 10°C remained organoleptically acceptable, on the average 17.5, 12.1 and 6.9 d, respectively. Harmon et al. (82) found that the shelf-life of commercial samples of milk was 21 to 63 d when stored at 5°C, 7 to 17 d at 6°C, 3 to 7 d at 12°C, and only 1 d at 20°C. They found keeping quality to be directly related to rate of development of bacterial populations. They added that the maximum number of microorganisms ultimately attained at spoilage was the same regardless of storage temperature. Janzen et al. (94) noted a whole milk shelf-life of 9 d at 7°C. Langlois et al. (110) indicated an average shelf-life of skim milk at 4.5°C was 13.3 d. Randolph et al. (160) found that the average keeping quality of market milk purchased at retail outlets was 7.7 d at 7°C, and 6.2 d at 10°C. The samples were from 0 to 14 d old when purchased. Most of the samples possessed psychrotrophic (now known as psychrotrophic) type of defects at the time the flavor quality was judged unacceptable. Sherman et al. (181) reported that pasteurized milk had a shelf-life of 8 to 12 weeks at 0°C and spoilage marked by extensive proteolysis, was of bacterial origin, predominantly gram-negative pseudomonads. Shipe et al. (182) reported that the average keeping quality of commercial milk at 7°C was 13 d.

Shelf-life was affected by days held raw (95), pasteurization temperature, storage temperature and season as determined by taste panel and bacteriological tests (55). Other considerations regarding shelf-life were reported by Gillis and Custer (66), who found that repasteurization did not significantly extend the shelf-life beyond that of the same milk that was not repasteurized. In addition, Mistry and Kosikowski (138) reported that sorbate levels of .15 and .20% in milk at 7°C effectively prolonged quality and reduced the rate of psychrotrophic bacteria growth, 19-20 d vs. 12-14 d, respectively.

There exist opinions that the consumer may exercise the most efficient indicator of keeping quality - flavor (7,8,75,76). These researchers report that the flavor score of freshly pasteurized milk, although subjective, is much better than standard microbiological and chemical tests as a predictor of keeping quality. Also, several other researchers (13,27,68,75,208) have concluded that the keeping quality of milk is generally unrelated to standard microbiological and chemical tests.

Differences of opinion concerning efficient methods of shelf-life estimation are evident. Patel and Blankenagel (151) state that there is no doubt that in commercially pasteurized and packaged milk, post-pasteurization contamination is by far the most common cause of flavor defects of microbial origin. However, they found that even in the absence of post-pasteurization contaminants, off-flavors may be encountered if the raw milk contained large populations of psychrophilic genera of bacteria most frequently involved in keeping quality problems in pasteurized milk include Pseudomonas, Achromobacter, Chromobacterium, Alcaligenes, Proteus, Escherichia and Enterobacter (49). The gelatinous curd and flavor defects of cottage cheese produced by Pseudomonas viscosa, Pseudomonas fragi and Alcaligenes metalcaligenes are well known. In milk as well as cottage cheese, P. viscosa produces bitter, rancid and unclean flavors while that produced by P. fragi is a fairly common fruity defect (49). Elliker (50) found the major source of post-pasteurization contamination by bacteria to be the filler.

The occurrence of off-flavors in raw milk, market milk, laboratory pasteurized milk and milk inoculated with Pseudomonas fluorescens coincided with psychrotrophic counts of 107/ml. It was apparent that deterioration of pasteurized milk during storage is of microbial origin, but may not be due to the growth of bacteria per se (7). Matoba et al. (126) reported that the occurrence of a bitter flavor in milk appears to be related to the presence of a heat-stable protease. Hydrolysis of casein and lactalbumin yields bitter peptides. Therefore, keeping quality depends both on numbers of bacteria and their biochemical activities (121). This fact is illustrated by the research of Patel and Blankenagel (151) where milk with counts of 109/ml before heating frequently developed objectionable flavors after pasteurization and subsequent storage. The most common defect was a bitter flavor which developed in spite of small numbers of organisms in the pasteurized products and in the absence of post-pasteurization contamination.

**BACTERIAL ENUMERATION**

Microbiological tests done on milk have two main pur-
poses: (a) to determine whether sanitary practices are adequate, and (b) to gain information that will aid in predicting keeping quality (121). The Standard Plate Count (124) is a widely used method for determining the bacterial count of raw and pasteurized milk for regulatory purposes. The method was originally described in the first edition of Standard Methods, but has undergone several modifications since then (166). The basic underlying assumption of the Standard Plate Count - as well as other plate count procedures - is that a single viable cell, when placed at an appropriate temperature, will multiply to the point where a visible colony is produced (190). However, in some instances colonies arise from cells which were originally aggregated in pairs, small chains, or small clusters and cannot be differentiated from colonies which arise from single cells (98,124). Therefore, the basic assumption of plate count procedures is to some degree invalid (172).

Even though the Standard Plate Count has historically been the main cultural procedure used to determine viable bacterial populations in dairy products (124), literature suggests that the scientific community is not entirely satisfied with this method of microbial enumeration. Aside from being inaccurate, the Standard Plate Count has been criticized as being time consuming and expensive (12,64,65,98,154), not indicative of raw milk quality (12,43,84,85,101,102,111,162,170,215,216), and slow as a means of generating bacterial population estimates (65,67). Regardless of the drawbacks or problems associated with the Standard Plate Count, it is officially considered as a suitable method for measuring bacterial populations in most types of dairy products (124). It is not a suitable method for detection of post-pasteurization contamination or indication of keeping quality as it does not differentiate between those organisms that survived the heat treatment and those that gained entry into the product after pasteurization (19).

The coliform count (124) is also used as an indication of contamination of dairy products. If coliform bacteria are present in adequately pasteurized products, one can be sure post-pasteurization contamination has occurred. However, the absence of coliforms is no guarantee that the product is free of contaminants. This method is limited because only a relatively small group of all possible contaminants are coliforms (19).

Although all contaminants are important, the main concern of the dairy processor is the group of organisms that can grow at refrigeration temperatures - the psychrophiles. Zall et al. (223) found significant correlation (r = .878) between Standard Plate Count and psychrotrophic bacteria count values.

Determination of the pyruvate content of milk is a means of measuring bacteriological quality. One advantage of this approach is that the analysis can be automated to test 80-120 samples/h. The analysis involves the breakdown of pyruvate catalyzed by lactic dehydrogenase in the presence of reduced nicotinamide-adenine dinucleotide (NADH), and it is the concentration of this cofactor which is measured spectrophotometrically and related to pyruvate concentration. This method relies essentially on two factors. First, that pyruvate production by all bacteria in milk is in some way equal. This is not true because some organisms do not produce pyruvate while others produce various amounts depending on their physiological state. Second, the method uses the colony count procedure as its reference point (148). Ledford et al. (113) reported a correlation between pyruvate and Standard Plate Count of .72, while Marshall and Harmon (121) found no association between Standard Plate Count and pyruvate concentration.

Adenosine-5'-triphosphate (ATP) detection has been greatly simplified by the discovery of the luciferin-luciferase bioluminescent reaction, which contains two key properties that render it applicable to the quantitative measure of ATP. First, the amount of light produced during the reaction is directly proportional to the concentration of luciferin and ATP in the reaction mixture (130). Second, ATP is the primary high-energy nucleotide that participates in the bioluminescent reaction (172).

The luciferin-luciferase assay has previously been used to measure viable organisms in many diverse environments including water (117), soils (47,97), rumen fluids (51,52,60,220) and foods (24,180). Several dairy applications of the luciferin-luciferase assay have been reported, including estimation of somatic cell and bacterial contents of raw milk (21). Correlation of the ATP content of 48 tank truck samples and Standard Plate Count was .93 (21). Correlation between the ATP content of 209 farm bulk tank samples and Standard Plate Count was also .93 (172). Measurement of milk sample ATP content was considered to be an acceptable method of detecting high bacterial count milk (21,172).

Another measure of bacterial content was suggested by O'Toole (148) who stated that cell numbers are no more than a crude measure of cell mass. The dry mass of bacterial cells in a sample is probably the best measure of the bacterial content, if its determination could be achieved.

**PSYCHROTROPHIC BACTERIA**

When considering the shelf-life of refrigerated milk and milk products, the concern is almost exclusively with those microorganisms which grow rapidly at storage temperatures. These bacteria were referred to as "psychrophiles", a term which means "cold loving". This is a misnomer as they are not "cold-loving" but rather "cold-enduring"; while they are able to grow below 10°C, they grow much better at ambient or higher temperatures. A more appropriate name is "psychrotrophs" - "cold-enduring" (100). These psychrotrophs are defined in essentially four major ways based on (a) optimum growth temperature, (b) ability to grow at low temperatures, (c) method of enumeration and (d) criteria which are independent of the incubation temperature (219).

Psychrotrophic microflora consist of microorganisms that can grow relatively rapidly at commercial refrigeration.
tion temperatures. This does not imply that the optimum
temperature of growth of this group of organisms lies in
the temperature range encountered in normal commercial
refrigeration of foods. Optimum growth temperature of
many psychrotrophs is in the mesophilic range (20-45°C).
The term psychrotrophic was coined to accommodate the
heterogeneous group that did not fall within the strict
physiological definition of the psychrophilic group. Cur-
rently, the term psychophilic is reserved for microor-
organisms that optimally grow at temperatures below 10°C
(obligate psychrophiles) (203).

Psychrotrophs are mostly gram-negative, non-spore
forming rods; they are usually aerobic; nearly all of them
produce heat-resistant metabolites; most of them are fairly
resistant to penicillin and some other compounds. This
physiological definition of the psychrophilic group. Cur-

pseudomonads are the most common contaminants of
bacteria, enterococci, micrococci and certain species of ther-
moduric Bacillus and Clostridium are important
(134,207). Additionally, certain molds and yeasts are
able of rapid growth in refrigerated foods (203).
Among psychrotrophs producing heat-resistant proteases,
pseudomonads are the most common contaminants of
milk (152).

Psychrotrophs are generally found in water and soil
and are introduced into milk through these sources and
become established on milk contact surfaces, equipment,
flooring and drains in the processing plant. They may be
introduced via wash water (203). Milk produced under
sanitary conditions usually contains less than 10% of the
total microbial flora as psychrotrophs, but milk produced
under unsanitary conditions can contain more than 75%
psychrotraphs (96).

While there have been a few reports of psychrotrophs
surviving laboratory pasteurization in very small num-
bers, there is abundant evidence that in commercial oper-
ations this rarely happens. Consequently, there is a high
certainty that their presence in a pasteurized product rep-
resents recontamination at or beyond the pasteurizer
through leaking gaskets, hairline or pinhole cracks in
plates, etc. (100). With conventionally pasteurized milk,
psychrotrophs increased from 1/ml to 10⁶/ml after 10 d
at 7.2°C (134). El-Farekh (48) calculated the generation
time of P. fluorescens to be 7.2 h at 7.2°C.

The standard enumeration method for psychrotrophic
bacteria involves pour-plating a sample in tryptone yeast
extract agar (TGE), and incubation for 10 d at 7°C (124).
A more rapid technique was developed by Oliveria and
Parmalee (146), who reported that enumeration of psy-
chrotrophic bacteria at 21°C for 25 h (mPBC) produced
counts in very good agreement with those obtained by
standard psychrotrophic count. Correlation coefficients in
raw milk were .992 and in pasteurized milk .996. More
specific detection and enumeration of gram-negative bac-
teria is accomplished by the use of inhibitors. Crystal
violet and neotetrazolium chloride inhibits growth of
gram-positive bacteria without causing inhibition of gram-

negative bacteria (43,187). Oehlerich and McKellar (144)
evaluated an 18°C/45 h plate count technique for enumera-
tion of psychrotrophs with correlation values to the 7°C,
10 d method of .866 in raw milk and .936 in pasteurized
milk.

Many psychrotrophs produce lipases which can degrade
milkfat and cause flavor problems in dairy products (59).
Common thermal processing may leave heat-stable lipases
almost intact (3). The major producer of heat-stable
lipases in milk appears to be P. fluorescens (59). Frieden
(63) stated the lipase from P. fluorescens is activated
after a short heat treatment. Frieden also stated that en-
zymes and enzymatic activities are connected with associ-
ation to other molecules, e.g. proteins, and this activation
is probably from dissociation of an enzyme-inhibitor
complex as a result of the heat treatment. The lipase from
P. fluorescens was heat stable enough to be active in
foods which were subjected to high-temperature-short-
time (HTST) sterilizing processes. As a consequence,
lipases can cause quality changes in foods and reduce
their shelf-life (3,168).

During psychrotrophic growth in milk, certain gross
changes in the protein occur which include: (a) a de-
crease in the total protein content, (b) changes in the rela-
tive amounts of the protein fractions and (c) the appear-
ance of two different atypical protein fractions (149).
Psychrotrophic microorganisms may have an indirect as
well as a direct effect on the quality of dairy products.

Indirectly, psychrotrophs produce off-flavors and -odors
during growth in stored refrigerated raw milk which may
carry over into the finished product even though the or-

ganisms fail to survive pasteurization. Directly, or-

ganisms surviving pasteurization or resulting from post-
pasteurization contamination may multiply in sufficient
numbers during manufacture and storage of dairy prod-

ucts so as to reduce the shelf-life, the quality and the
yield of the finished product (134). These bacteria cause
bitter, fruity, rancid or yeasty flavors (36). Psychrot-
rophic levels in excess of 10⁶-10⁷/ml are usually required
before organoleptic changes are detected in milk
(114,134,151,159).

Cottage cheese manufactured from milk in which psy-
chrotrophs had multiplied to levels of 10⁴/ml and then pas-
teurized at 62.8°C for 30 min was found to have a firmer
curd and to require less manufacturing time than control
milk. However, the cottage cheese was organoleptically
unacceptable (88). Mohamed and Bassette (140) attrib-
uted cottage cheese vat failure to milk heavily contami-
nated with psychrotrophs whether the cheese was man-
ufactured by the direct-acid-set method or by the conven-
tional starter culture procedure. The formed curd disinteg-
rated and shattered during the manufacturing procedure.
It was found that average cottage cheese yields decrease
(6,86). Principal psychrotrophic defects in cottage cheese
are slime formation, surface discoloration, off-odors and
off-flavors (123). Stone and Naff (191) found increases
in soluble nitrogen and bitter flavor to be associated with
increases in psychrotrophic bacteria in cottage cheese.

JOURNAL OF FOOD PROTECTION. VOL. 49, SEPTEMBER 1986
Contaminated water has been shown to be a source of cottage cheese spoilage organisms (142). Survival of psychrotrophic coliforms in a cultured product was found to be limited, and it was concluded that they have little effect on shelf-life (174).

PROTEOLYSIS

The first indication of naturally occurring proteolytic enzymes in milk was published as early as 1879 (164). Storr and Hull (192) also contributed substantially to the belief that the protease enzyme is a natural constituent of milk. Harper (83) stated that raw milk usually (but not always) contains a small and variable amount of protease enzyme, and that proteolysis is slight. The most frequently represented protease-producing psychrotrophic gram-negative bacterial genus is Pseudomonas, with P. fluorescens as the most common species (112).

Shahani (197) reported that at least 19 enzymes have been found in normal cow’s milk. Enzymes occurring naturally in milk could be classified as follows: (a) enzymes acting as a hydrolytic group, (b) enzymes having a physiological role, (c) enzymes associated with the microsomal particles of milk and (d) enzymes having an unknown role. Proteinases and peptidases constitute the primary enzyme forms in bacteria responsible for proteolysis of milk proteins (209).

Most of the work on identification of native milk proteinases has been with trypsin-like enzymes (30,46,109,199). These enzymes belong to the group of serine proteinases and predominantly hydrolyze beta-casein (70). Results indicate that the trypsin-like enzyme system consists of two serine proteinases and, in addition, a chymotrypsin-like enzyme. Gamma-caseins and related phosphoproteins are fragments of beta-casein. However, gamma-casein formation during storage of milk could be expected to be slow as the enzyme reaction would be limited by association of both substrate (beta-casein) and enzyme with casein micelles.

An entirely different situation exists during cold-storage of bulk milk at 2 to 6°C. Large quantities of beta-casein dissociate from micelles into milk serum primarily because of its hydrophobicity and changes in the salt equilibrium (163). Behavior has been similar for proteinases associated with micelles. Proteolytic activity brings about a modification of the electrophoretic pattern of dairy products (90).

Pennington et al. (153) made the observation in 1913 that when milk was held at 0°C, the following phenomena were noted: (a) proteolysis of casein was primarily of bacterial origin, (b) proteolysis of lactalbumin was primarily due to native enzymes of the milk and (c) both enzyme systems combined gave rise to more rapid proteolytic changes than either system alone.

Warner and Polis (206) stated that proteolysis which occurred in casein solutions was attributed to the presence of an enzyme on the basis of the following evidence: (a) the activity was affected by heat, (b) the activity had a definite optimum pH, (c) the activity could be concen-
trated and (d) the proteolysis proceeded in sterile solutions containing enzymes but no living organisms. Peterson and Gunderson (155), in studying certain characteristics of proteolytic enzymes from P. fluorescens, reported that extracellular, proteolytic enzyme elaboration was inversely proportional to the temperature, at least from 0 to 30°C. It would appear temperature plays an important role in the effect of proteolytic activity on dairy quality.

Sandvik (176) concluded that the interpretation of the food spoilage potential of psychrotrophic bacteria should include the possible residual effect of the enzyme after pasteurization or sterilization. Extracellular enzymes, produced by microbes in refrigerated foods before heat treatment, may not be completely inactivated by the heating and may be active in the stored product (129). Protease enzymes survived 70°C for 30 min; however, when heating was at 40°C for 30 min, proteolytic activity decreased significantly (33). Several researchers have shown protease inactivation at 63°C for 15 h, 71°C for 8 h and 121°C for 9 min (1,122,129, respectively). Adams (i) compared inactivation of MC 60 protease at 149°C to inactivation of bacterial spores to establish the ultra-high-temperature (UHT) sterilization parameters. The protease was more than 4,000 times more resistant than Bacillus stearothermophilus spores. The resistance of psychrotrophic proteases suggests that their destruction by extreme heat is impractical.

The destruction of heat-resistant bacterial proteases at sub-sterilization temperature may be feasible. Maximum low temperature inactivation occurred at 55°C. For low temperature inactivation to be beneficial, it is essential that inactivation occur at all protease concentrations. The extent of protease inactivation appeared to be independent of protease concentration and, therefore, could occur at the low protease levels which might be in raw milk (9). The practicality of this procedure in a dairy plant is questionable but it is possible.

Optimum temperature and pH ranges for protease activity are 5 to 50°C and pH 6 to 8 (1,32,122). Half (46%) of the variability of protease activity was explained by milk pH, by psychrotrophic bacteria and by the stage of lactation. The second half (54%) of this variability in activity could be only due to variations of the native milk proteinase system (91).

The degree of measurable proteolysis is directly correlated with the incidence of the naturally occurring “bitter” flavor (16,17,39,87,108,210,212,213,214). Janzen et al. (96) reported a significant relationship between protease activity and flavor score of whole and skim milk (r= -0.936 and -0.917, respectively) at 7°C. Also concluded in the study was that, because the shelf-life of skim milk was significantly less than that of whole milk, and protease activity was significantly higher in skim milk as compared to whole milk, the increased protease activity in the skim may be partially responsible for its decreased shelf-life.

The successful use of UHT treatment of milk can be hindered by heat-stable enzymes in milk (141). White
and Marshall (211), and Thomas and Mills (198) showed a reduction in shelf-life of Cheddar and cottage cheese due to the addition of a heat-stable protease enzyme.

Speck and Adams (189) introduced the following methods for controlling heat-stable proteases in milk: (a) prevent contamination by psychrotrophs, (b) prevent growth and metabolism by psychrotrophs by lowering temperature and decreasing aeration, addition of antibiotics and other inhibitors, and addition of starter organisms, (c) UHT-inactivation, and (d) inactivation at sub-pasteurization temperatures.

Many methods exist for detection of proteolysis. The trichloroacetic acid (TCA) method of Hull (89) is the most widely used to date, but not necessarily the best. Samples et al. (175) and McKellar (131) found the trinitrobenzene sulfonic acid (TNSB) method to be more suitable for detecting proteolysis than the Hull procedure. A sensitive assay for protease activity based on the reaction of primary amino groups of trichloroacetic acid-soluble peptides and amino acids with fluorescamine was found to be suitable for determining protease activity in general (31). Halambeck et al. (72) stated that because the Hull procedure is specific for the release of aromatic amino acids, it lacks sufficient sensitivity to detect small differences in the release of other amino acids and peptides. More sensitive methods are based on amine reaction with TNBS or fluorescamine. Church et al. (34) introduced a spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. Because all hydrolytic products are assayed, the method is more accurate than procedures that depend upon properties of aromatic residues. Furthermore, the o-phthaldialdehyde assay is more rapid and convenient than methods using ninhydrin, TNBS or fluorescamine. An immunological detection procedure (92) and a dialysis method (139) for measuring protease activity have not had apparent widespread use.

PRELIMINARY INCUBATION

Preliminary incubation (PI) is based upon the theory that as the holding temperature of milk is lowered, a point is reached where the udder flora no longer multiplies while many of the psychrotrophic contaminants grow actively. Preliminary incubation encourages growth of bacterial contaminants, and aids in detecting milk in which cooling masks unsanitary production and handling practices. Experimental evidence has indicated that the ratio of bacteria detected before and after PI is closely related to the cleanliness of milking equipment, especially milking machines (99).

The PI count is simply a Standard Plate Count (SPC) following incubation of raw milk for 18 h at 12.8°C (124). The test is designed to detect sanitation problems (172). Chalmers (29) in Scotland had suspected that efficient cooling in farm bulk tanks might cover up insanitary practices. To check this, he held samples at 15°C for 12 h, then ran a second set of plate counts at 37°C. He concluded that 24% of the 103 samples gave initial counts (before PI) under 10,000/ml, which did not reflect the probable true hygienic conditions of milk production. Other Scottish workers (184), following Chalmers’ (29) lead, repeated the analysis on similar samples after holding for 24 h at 12.8-15.6°C. Because certain samples with initial counts under 10,000/ml showed a 100-fold increase following PI, they concluded that there would seem to be little correlation between production methods and the relative increase in count. Meany (132) applied PI at 15.6°C for 24 h to a series of samples from selected farms in the Chicago area. Differences in the degree of increase were related to conditions of production; when milking equipment was thoroughly cleaned, much smaller increases in counts were noted. Davis and Killmeier (44) reported excellent results using PI conditions of 12.8°C for 18 h.

Several workers (84,99,102,165) have reported that the PI count is a better indicator of raw milk microbiological quality than the SPC. A large magnitude of change in total bacterial count following PI indicates psychrotrophic contamination (102,161,215). Using the PI count (18 h at 12.8°C), 100,000-200,000/ml is suggested as a desirable standard. This count would serve to emphasize thorough cleaning along with efficient cooling in the production of quality milk (11,99,124,173,200).

Additionally, the PI count is considered to provide a good indication of potential keeping quality of milk and potential shelf-life of dairy foods processed from raw milk (11,215,216,217,218). Phillips et al. (157) introduced a test involving preincubation of samples at 21°C for 25 h in the presence of a mixture of nisin:penicillin:crystal violet to prevent growth of gram-positive organisms to identify post-pasteurization contamination of milk and single cream (16-18%). The test (P-INC test) could successfully predict the level of contamination after storage at 6°C for 7 d in 85% of the milk samples. Bishop and White (16,17) found that PI at 21°C for 14 h followed by plating for mPBC produced the highest correlation of those tested (none, 21°C-7 h, 13°C-18 h, and 18°C-18 h) to potential shelf-life of pasteurized fluid milk and cottage cheese, r = -.814 and -.586, respectively.

Ledford (115,116) examined the practice of PI before coliform enumeration in violet red bile agar. He found that PI for 14 d at 6.7°C indicated coliforms in 75% of the samples. Unsatisfactory flavor scores were observed in 91% of the samples positive for coliforms. Shorter PI periods resulted in much lower percentages of related flavor scores. Preliminary incubation of milk samples at 25°C for 20 h resulted in no significant increase in tyrosine values, as an indication of proteolysis, compared to unincubated samples (107).

Johns (99) stated that it is not contended that PI will detect every type of unsanitary condition or practice. Nevertheless, Johns believed that PI furnished more information regarding the care taken in production and handling than can be obtained from methods that excluded PI.
MOSELEY TEST

Over 25 years ago, personnel at the W. K. Moseley Laboratory in Indianapolis began conducting a second SPC on processed samples after holding them at 7.2°C for 5 d (100). The basis of the Moseley Keeping Quality Test is that practically all of the bacteria that grow well in milk in 5-7 d at 7°C are destroyed by pasteurization. By conducting this test, two important types of information can be obtained. First, if the bacterial numbers increase appreciably during storage, the evidence is strong that post-pasteurization contamination has occurred. Second, if the plate count on the fresh sample is high, thermoduric bacteria are probably in the raw product, a situation that now occurs less frequently (49) than in earlier times.

Application of the 5 d at 7.2°C keeping quality test followed by careful study of contamination sources has greatly improved a shelf-life of pasteurized fluid milk and has represented a real economic advantage to plants adopting the program (49). Several researchers (54,110,160,186) have reported highly significant correlations between the keeping quality of pasteurized fluid milk and the Moseley test. Bishop et al. (15) obtained a correlation of -.770 between log Moseley count vs. potential shelf-life of pasteurized whole milk.

SHELF-LIFE TESTS

If there would be such a thing as an ideal test for determining post-pasteurization contamination, and therefore, determining shelf-life, it would have the following characteristics: (a) The ideal test would be accurate, i.e. it would indicate exactly the number of organisms which contaminated the product after pasteurization. It would also differentiate between thermoduric bacteria that survived the heat treatment and post-pasteurization contaminants, regardless of how few bacteria there may be. (b) The ideal test would provide results within the shortest time possible. A little accuracy could be sacrificed to get results the morning after processing. (c) The test would be simple to do and it would be economical (19).

Examples of attempts to develop less time-consuming tests are: (a) use of preincubation to build up relatively large populations that are easier to enumerate, (b) use of selective media that permit growth of contaminants but inhibit growth of other bacteria, (c) testing for certain end-products of microbial metabolism or changes in the substrate, (d) applying the sample on the surface of agar media to accelerate growth of aerobes, (e) flavor tests, and (f) use of a combination of two or more of these (19,216).

The entire concept of shelf-life is based on the well-documented supposition that the primary cause of shelf-life deterioration is gram-negative psychrotrophic bacteria. Most of these bacteria are members of the genus Pseudomonas. Consequently, most of the tests which have been designed to predict or estimate the shelf-life of fluid dairy products are geared to measure these pseudomonads (216).

The chief objection to Mosley's storage quality test (124) has been that a week or more elapses before the results are available. Various workers have suggested different ways of shortening the waiting period. Gyllenberg (71) used ammonium lactate agar and ammonium lactate crystal violet agar, and incubated plates 2 to 4 d at 28°C, for determining the incidence of organisms responsible for spoilage of refrigerated milk. Freeman et al. (62) investigated the inhibitory action of various chemicals on gram-positive organisms. They reported that .5% sodium desoxycysteolate (SDC) was the most effective of all those tested.

Olson (147) recommended the CVT test, in which 2 ppm of crystal violet plus 50 ppm of 2,3,5-triphenyltetrazolium chloride (TTC) are incorporated into the plate count agar to repress gram-positive bacteria and also make gram-negative colonies more distinctive. Sing et al. (184) reported poor correlation between CVT counts and SPC values after 7 d of storage at 7.2°C, with only 15% of 90 samples agreeing. Lightbody (120) used 10 IU of penicillin plus TTC to suppress gram-positive organisms and found a highly significant correlation between counts and samples after holding 24 h at 20°C, 4 d at 5°C, or 4 d at 10°C with plates incubated 3 d at 30-32°C. Blankenagel and Humbert (18) described a surface disc method using crystal violet as an inhibitor.

Taking advantage of the fact that gram-negative contaminants are catalase-positive, strongly aerobic, and not repressed by surface-active agents, Maxcy (127) developed the following procedure: (a) plates are poured with nutrient agar containing .5% alkyl aryl sulfonate, (b) plates are dried 48 h at 32°C, then .5 ml milk is carefully spotted onto the surface in separate droplets, (c) plates are allowed to stand undisturbed for 30 min while the milk is absorbed before being incubated in an upright position for 16-20 h at 32°C, plus 1 h at 7°C with covers removed, and (d) plates are flooded with 5% solution of hydrogen peroxide. Catalase-positive colonies decompose the hydrogen peroxide and are recognized by the gas bubbles released; only these colonies are counted. This count is related to post-pasteurization contamination. Hankin and Dillman (73) used the fact that pseudomonads are strongly oxidase-positive to correlate the oxidase test with keeping quality. A gram-negative psychrotrophic bacteria count has been highly correlated (77,217,218) and not correlated at all (133) to keeping quality. Hankin and others (74,79,80) found an inverse correlation between flavor score and SPC and the oxidase count.

Janzen (93) suggested that shelf-life of pasteurized milk is influenced by somatic cell concentration in raw milk. High somatic cell concentrations are indicative of abnormalities that frequently involve increased microbial flora.

Boyko and Blankenagel (23) utilized the findings of Freeman et al. (62) that .5% SDC was the best inhibitor of gram-positive organisms. They found that all 45 test
organisms which grew in milk containing SDC were killed by laboratory pasteurization and, therefore, assumed that organisms present in processed milk which grew in the presence of SDC were contaminants. Catchick and Gibon (28) sought a way to shorten the SDC test to 16 h so that results would be available the next day. They modified the SDC test by adding resazurin. The advantages of this modified resazurin reduction test are: (a) use of a large (9 ml) sample, (b) use of PI to build up small numbers, (c) detection of only gram-negative organisms, and (d) providing results the next morning (19). The methylene blue test after pre-incubation at 18°C for 18 h was a good index of keeping quality, as determined by Jooste and Groeneveld (103). Custer and Knight (41) found that Early Detection (SDC + resazurin) test to be as accurate as the Moseley test in measuring the shelf-life and the results can be obtained 6 d sooner. Wae and Bossuyt (204) reported that the benzaldehyde crystal violet-ATP method was useful for predicting keeping quality.

Marshall and Harmon (121) found pyruvate concentration to be of limited value as a quality test for milk due to great variability of results. Senyk et al. (178) and Shipe et al. (182) obtained correlation values of flavor score to pyruvate content of -.81 and -.78, respectively. Asher and Sargent (4) produced the best fitted regression equation (r² = .615) with data on changes of acidity, pH, acid degree value and free fatty acids as related to keeping quality. Shipe and Hsu (183) measured fluorescent compounds in milk to provide an index of milk quality.

Rodrigues and Pettipher (169) reported that the keeping quality of pasteurized milk stored at 5 and 11°C was predicted within 24 h by pre-incubating samples and counting bacteria by the Direct Epifluorescent Filter Technique (DEFT).

For any of the aforementioned shelf-life tests to be effective, there must exist a control of heterogeneity of samples. Maxcy and Wallen (128) stated that a single package provides low probability for predicting behavior of the entire production lot. The extreme differences in spoilage rates of individual units with sample sets indicated sensory evaluation of multiple samples to be the most logical, simple criterion for evaluating shelf-life.

The aim of shelf-life tests should be to detect and eliminate all sources of recontamination. When this has been accomplished, shelf-life determination and detection should no longer be a problem (100).

ENDOTOXIN (LIPOPOLYSACCHARIDE) DETECTION

Levin and Bang (118) originally described the ability of amoebocyte lysate from Limulus polyphemus, the horseshoe crab, to form a gel in the presence of minute amounts of endotoxin. Since that time, the Limulus assay has been used as a method of detecting endotoxin in patients with gram-negative septicemia (26,119,167), for the study of experimental endotoxemia and shock in animals (4), and as a method of detecting pyrogen in parenteral pharmaceuticals (37). This method has been shown to be the most sensitive available for detection of endotoxin (38,104).

According to the reported mechanism of the Limulus Amoebocyte Lysate (LAL) assay, endotoxin activates a proenzyme-enzyme cascading reaction resulting in activation of coagulase (145,195). Coagulase hydrolyzes coagulin, a clottable heat-stable protein (194,196), at arginine-glycine and arginine-threonine linkages, resulting in removal of the peptide-C chain to form a gel (coagulin) (201).

This particular assay included the following "grading of lysate gelation": 4+ = firm gel with considerable opacity; 3+ = soft gel with moderate to considerable opacity; 2+ = weak gel with slight to moderate opacity and adhesion of starch-like floccules to sides of the tube when slanted; 1+ = very weak gel with slight opacity and with some starch-like floccules adhering to sides of the tube; negative = no visible increase in viscosity or opacity. As little as 100 pg of certain endotoxins/ml may be reliably detected (103).

DiLuzio and Friedman (45) suggested the Limulus assay might be used for detection of bacterial endotoxin in drinking water and other surface waters. Jorgensen et al. (105) reported that the Limulus assay procedure was easily adapted to the testing of water samples for endotoxin. Measured endotoxin concentrations varied from .78 ng/ml to 1,250 ng/ml. The Limulus assay is currently permitted by the Food and Drug Administration to determine possible endotoxin contamination of ingredients used to prepare parenteral and biological products in the pharmaceutical industry. The great advantage of the assay is its rapidity - a total test time of less than 2 h.

The Limulus endotoxin assay was compared to the SPC and coliform count for assessment of the bacteriological quality of reclaimed waste water by Jorgensen et al. (106). LAL assays were technically simpler to perform and provided results much sooner than conventional culture methods. However, the endotoxin values did not correlate extremely well with determinations of viable bacterial numbers. This lack of correlation may have been due to alterations in the normal ratio of viable gram-negative cells to endotoxin caused by water reclamation procedures. Seiter and Jay (177) determined endotoxins in ground beef by the LAL assay.

Clark (35) evaluated various methods to enumerate psychrotrophic bacteria in fluid milk and concluded that the LAL test could be used as a simple shelf-life projection test. Mikolajcik and Brucker (135) studied the LAL assay's potential for detection of psychrotrophic spoilage organisms in foods. They stated that the LAL assay is a rapid and highly sensitive method to detect endotoxins in biological systems. Endotoxin activity is associated with the Lipid A segment of lipopolysaccharides constituting the outer cell membrane of gram-negative bacteria (GNB). Both viable and non-viable GNB are detected which makes this test particularly useful in tracing...
the history of the milk supply. The LAL assay is finding increased use as a rapid indirect indicator of gram-negative spoilage organisms in refrigerated meat and dairy products. The LAL assay will detect $10^2\text{ to }10^4$ GNB/ml of milk. Mikolajcik and Brucker (137) also found that for each log increment in GNB population, the lipopolysaccharide (LPS) titers also increased one log cycle with a correlation coefficient of .88. Average LPS values increased from .86 ng/ml to 4.350 ng/ml as log increments of GNB/ml increased from 1.0 to 8.0 with correlation coefficient of .95. Currently used GNB enumeration procedures can detect viable GNB in the product resulting from post-pasteurization contamination but not GNB which were present before pasteurization. Pasteurization did not affect the LPS titer as it is heat-stable. When the mean GNB counts of commercial pasteurized milk were plotted for each LPS value, a correlation coefficient of .99 was obtained. For 186 individual trials, a correlation coefficient of .98 was obtained.

Mikolajcik (136) revealed that the LAL assay will detect within 1 h as few as 100 GNB/ml of milk. The accuracy of the test improves where counts exceed 10,000 GNB/ml of milk. The outstanding feature of the procedure is its ability to detect the quality of the raw milk supply even after pasteurization. Thus it furnishes information about the number of GNB which were present in the raw milk supply and the possibilities that proteases and lipases might be present in the milk which would affect the keeping quality of the finished product.

Sudi (193) discovered that, because of the heat stability of gram-negative lipopolysaccharides, the evidence of bacterial growth in raw milk cannot be destroyed by UHT treatment, but can be detected by the LAL assay. In fact, there was an apparent 3.6-fold increase of GN-LPS concentration following UHT treatment. The reason for this increase was not explained. It is proposed that the main advantage of the Limulus assay lies in the assessment of the bacteriological quality of heat-treated dairy products, like UHT milk, where cultural methods are negative.

Evans et al. (53) modified the Limulus lysate assay so that the reaction of lysate and endotoxin formed a turbid suspension instead of a firm clot. Absorbency at 360 nm is measured. The firm-clot method was found to be less sensitive and reproducible for detection of endotoxin than the spectrophotometric modification of the Limulus lysate assay. Tsuji and Steindler (201) found the LAL sensitivity to endotoxin increased approximately 20- to 30-fold when lyophilized LAL was reconstituted with de-pyrogenated natural seawater. Magnesium was identified as the component in natural seawater and commercial sea salts responsible for the increased sensitivity. The greatest endotoxin sensitivity for the LAL reagent was achieved with solutions of magnesium ranging from 50 to 60 nM. Magnesium may increase the LAL sensitivity by: (a) acting at the initial endotoxin recognition phase by modifying the size of endotoxin for easier recognition by LAL, (b) accelerating the rate of proenzyme-enzyme cascading reaction, (c) contributing to formation of a firmer gel or increased turbidity by strongly bridging gel micelles, or all three.

Bodyfeldt et al. (20) reported the LAL test included the following limitations: expensive reagents, lack of sufficient sensitivity to microquantities of endotoxins, and complexity of the procedure. This lack of sensitivity was alleviated by Bishop et al. (14) by removing the problem of varied background turbidity by “blanking” each sample with an unincubated duplicate. They also detected significant linear relationships between LPS content of milk and psychrotrophic bacteria count ($r = .715$), flavor score ($r = .918$) and days of storage at 4.5°C ($r = .939$). Bishop and White (16, 17) evaluated the LAL assay as related to potential shelf-life of pasteurized fluid milk and cottage cheese. The spectrophotometric LAL method was used for milk and a correlation value of -0.913 resulted. With cottage cheese shelf-life, the LAL gelatin method gave a correlation value of -0.811.

Sudi (193) pointed out that the Limulus test may easily develop into an entirely new double-purpose method of food analysis. The test may not only prove to be suitable for detection of manipulations of raw materials, but it may also provide means for demonstrating that good manufacturing practices have been followed as closely as possible. Mikolajcik (136) feels the Limulus test, because of its sensitivity, accuracy and speed, will be of tremendous value to the dairy industry as a rapid screening test for raw milk and as a guide for predicting the shelf-life of pasteurized and UHT milks.

**IMPEDANCE DETECTION**

Several rapid automated methods have been proposed to overcome the disadvantages inherent in plate counts. These include: ATP photometry (222), fluorescence microscopy (156), radiometric measurements (158) and impedance detection (25). The impedance method has been considered to be the most promising of the instrumental methods (221).

Impedance is the resistance to the flow of an alternating electrical current through a conducting material, and has been shown to be a complex entity composed of a resistive (or conductive) component and a reactive component (capacitance). The impedance technique relies on the fact that metabolizing microorganisms alter the chemical composition of the growth medium and that these chemical changes cause a change in the impedance of the medium (2150). Total impedance change represents the vector sum of changes in the resistance and capacitance components of the growth medium. Impedance changes are detectable when the concentration of microorganisms exceeds a threshold level of $10^6$ to $10^7$ cells per milliliter. The time required for the initial inoculum to reach the threshold level is designated as the detection time and is a function of both the initial concentration and the specific growth kinetics of the organism in the given medium. By comparing the detection time obtained to the results of a standard calibration curve, an estimate of the
initial concentration of microorganisms can be made. The impedance detection time (IDT) is defined as the accelerating change of impedance associated with microbial growth and metabolism. The computer uses a complex algorithm that determines the onset of acceleration in the impedance curve. The calculation of the IDT from impedance curves is a critical part of the impedimetric method. To obtain consistent interpretations of IDT by the computer algorithm, it is important to generate impedance curves with smooth baselines and sharp accelerations. It is important to realize that the basis of the impedimetric estimation is metabolic change, while that of a colony count estimation is biomass production. The consequences of this difference must be carefully considered in the preparation of samples for impedimetric estimation of the number of microorganisms.

Impedance measurements are particularly useful for rapidly screening various foods to determine whether they meet the desired microbiological criterion. Rowly et al. (171) found the impedance method to be promising as a rapid screening technique to determine if cooked meats have less than one coliform per gram. Hardy et al. (18) used the impedance method to rapidly assess whether a sample of frozen vegetable contains greater or less than $10^5$ organisms per gram. Firstenberg-Eden and Klein (57) obtained a correlation coefficient of .90 between the impedimetric test and violet red bile agar (VRBA) plate counts for coliform using a total of 91 meat samples.

O'Connor (143) used an impedance method for determination of bacteriological quality of raw milk, and found that an IDT of 8.5 h differentiated samples with less than or greater than $10^5$ bacteria per milliliter. Gnan and Luedecke (67) stated that impedance detection offers a definite time advantage, approximately 7 h vs. 48 h for the SPC, when examining raw milk samples. A classification system that selects a cut-off detection time of approximately 7 h is helpful in screening out samples likely to have a SPC exceeding $10^5$/ml.

Waes and Bossuyt (205) described a simple impedimetric method to detect, within 2 h, complete failure of a starter due to bacteriophages in the manufacture of Cheddar cheese. This method is based on the observation that about $10^5$ disturbing bacteriophages per milliliter will cause complete failure of the starter and prevent the normal change of impedance due to lactic starter bacteria.

Firstenberg-Eden and Tricarico (38) revealed that a rapid impedimetric determination for total, mesophilic and psychrotrophic counts in raw milk showed correlations between IDT and bacteria counts of -.96, -.95 and -.96, respectively. Mesophiles were most often seen as the predominant population. The impedimetric method allowed for these samples containing above $10^5$ cfu/ml to be screened out within 4 h. Psychrotrophic levels of $10^5$/ml and above were screened within 21 h, while total concentration of samples containing above $10^5$ cfu/ml were screened within 16 h.

Cadey et al. (25) stated that present keeping quality tests, which try to predict spoilage based on presence of psychrotrophic organisms, have two severe limitations. First, spoilage is not always directly related to the number of organisms present (159). Second, it appears that psychrotrophs are only part of the milk spoilage problem. Poor flavor and keeping quality can also be attributed to the presence of microbial enzymes and metabolic products (149,151) from organisms present before pasteurization. Thus, present methods, although offering some useful information, are too slow and often too inaccurate to meet the needs of milk producers and processors. An optimal microbiological test would provide counting and keeping quality estimates within a time allowing for effective corrective measures. Cady et al. (25) also found that detection times correlate better with the shelf-life than do the SPC and psychrotrophic count. Cady's early data show promise as a 9-14 h impedance-based keeping quality prediction. Bossuyt and Waes (22) determined that impedance measurements are useful to trace post-pasteurization contamination in pasteurized milk. Martins (125) evaluated a rapid impedimetric method for determining the keeping quality of milk and concluded that positive correlation coefficients indicate that samples with short shelf-life tended to produce early impedance response detection times and samples with long shelf-life tended to produce late detection times. Bishop et al. (15) and Bishop and White (16,17) evaluated the value of impedance detection as related to potential shelf-life of pasteurized fluid milk and cottage cheese. It proved the most valuable on a combined time and relationship basis as compared to standard microbiological procedures, and proteolysis and endotoxin detection. Resultant correlation values for milk and cottage cheese shelf-life and impedance detection were .930 and .897, respectively.

CONCLUSION

Each method used for shelf-life estimation has its advantages and its drawbacks. Standard microbiological methods, including the associated Moseley keeping quality test, are familiar to laboratory personnel in the dairy industry, and offer some useful information, though their estimative value is limited. Bacterial metabolite detection is of increasing interest as emphasis is decreasing with bacterial numbers. The most commonly investigated metabolites include protease and lipase enzymes, and endotoxins produced by gram-negative bacteria.

Automated microbiology has made its way into the dairy industry with the introduction of ATP measurement and impedance detection. ATP concentration has not proven very useful for shelf-life estimation. Impedance detection has been shown to offer relatively rapid results with high correlation values to shelf-life, which result in fairly accurate estimation.

The method ultimately used for shelf-life estimation will depend on many factors. Each situation evaluated will require an analysis of need based on product type and volume, information needed, time available, cost of test method/instrument, laboratory set-up and personnel.
and top management support of the quality assurance program. Thus there is no clear-cut method for shelf-life estimation for each and every situation.

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