ESTIMATION OF VIABLE CELL COUNT BY MODERN AND IMPROVED METHODS

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INTRODUCTION

Routine microbiological testing for the presence and number of viable microorganisms in food is usually restricted to non-pathogenic microorganisms, whose presence and numbers are used as indices for accepting or rejecting food. The microbial tests most frequently used are the aerobic plate count, which measures aerobic bacteria, yeast, and molds, and indicator counts, which measure the coliform group, Escherichia coli and Staphylococcus aureus. The aerobic plate count, also referred to as "total plate count" and "standard plate count" simply measures the number of the entire population of aerobic microorganisms present in a food. The actual count represents a mixed flora. Indicator counts are selective since specific growth media are designed to inhibit growth of all microorganisms except the desired group. For this reason, indicator counts are much lower than the aerobic plate count in a food since only part of the total population of microorganisms is recovered.

The essence of present day microbiological specifications for foods is enumeration, which is the determination of numbers of organisms per gram, per ml or per cm^2. The development of microbiological standards increasingly reflects this preoccupation with numbers.

There are many reasons why enumerative methods (standard plate count) fail to provide absolute data. One is the coherence of microorganisms. Many organisms (bacteria and molds, in particular) exist in clusters or chains of viable units, which resist complete dispersal to single cells in the blending processes used in most microbiological analyses. The colonies subsequently formed on agar plates may arise from nuclei of one, two, twenty or more cells, in a distribution dependent on the food, the microbial flora, and the blending process. There is no way of deciding the number of cells involved in forming a particular colony.

Microbiological counts are usually quoted as numbers of colony-forming-units (CFU) rather than number of cells. Unfortunately, such counts often tend to be referred to and treated as "true" counts whenever comparisons are made with alternative methods of analysis.

Another factor is the inability of microbial counting media to support the growth of every organism in the sample. Errors of viable cell count may also arise due to variations in weighing, pipetting, and counting. Despite all the short-comings involved with viable cell count, it is still the "standard" method for determining viable cell populations in foods.

While the methodologies of viable cell count have not changed for the past 50 years, physical and chemical techniques for estimating microbial populations have advanced rapidly. Many microbiologists are now looking into alternative methods to speed up microbiological procedures. Automation has touched many areas of science and technology. Its influence on microbiology has been minimal until recently.

The first area in microbiological laboratories to be affected by automation was that of routine procedures. Included in this category are automated pipettors and dilutors, automated streaking and incubation, colony counters and staining machines. These procedures only speed up the manual labor relative to viable cell count but do not represent radical departure from basic philosophy of viable cell count. The most promising rapid and automated methods for the estimation of microbial loads are those which do not involve counting. Included in this category are very sensitive analytical instruments that can detect microbial components or metabolites, bioluminescence, limulus lysate, enzymes, pyruvate, impedance changes, temperature changes (microcalorimetry), and radioactive by-products (radiometry).

This report traces the history of standard plate count, advantages,

limitations, automation and miniaturization of the method. Also a review of the new methods in food microbiology is included.

I. VIABLE CELL COUNT

A. Standard Plate Count

The development of solid media in the late 1880's opened the way for the development of methods for enumerating microorganisms by colony counts. It was Robert Koch who, between 1881 and 1883, developed simple methods for the isolation and maintenance of pure cultures of microorganisms on chemically defined solid media (Brock, 1961). Prior to that time, microorganisms were grown in liquid broths. Koch developed a way of solidifying liquid broths that could support the growth of a greater variety of microorganisms, using gelatin. The product was referred to as "nutrient gelatin" (Bullock, 1938).

Koch's technique was to melt tubes of sterile jelly at a low temperature, inoculate them with small quantities of the material containing the bacteria under investigation, shake each tube gently to distribute the organism evenly throughout the whole tube of medium, and then pour it onto a sterilized sheet of smooth glass to harden. By this means the bacteria in the original drop of inoculating material were distributed evenly in the thin layer of gelatin, and as soon as it had hardened they became fixed in their position and could no longer swim or float around. These plates were carefully covered to keep off dust, and placed at a proper temperature for growth. The bacteria then began to feed upon the culture medium, grew, multiplied and developed into visible colonies, although fixed in their position (Conn and Conn, 1923).

The essential ingredient in the nutritive fluid was 1% meat extract. The nutrient basis of the medium has been varied to an endless extent by the addition of various substances such as sugars, alcohols, albumen, serum, blood, and other substances to enhance the nutritive value or to reveal some particular fermentative property of the organism under examination. A disadvantage of the

use of gelatin is that it melts at 23°C and can be liquefied by a number of bacteria. Since 37°C is best for incubation of pathogenic bacteria, gelatin is not a good solidifying medium.

Agar--Agar soon replaced gelatin and was introduced into bacteriological culture technique by Frau Hesse, the wife of Walter Hesse, one of Koch's early co-workers (Bullock, 1938). The peculiar virtue which has established the dominance of agar in bacteriological culture technique is that a high temperature, 90°-100°C, is required to melt it, yet once melted it can be cooled down to about 42°C before it sets into a stiff and relatively transparent solid mass (Bullock, 1938).

Another drawback of Koch's technique was the difficulty in covering a flat glass plate with an even layer of gelatin and in keeping it protected from contamination until the colonies had time to develop. In 1887 R. J. Petri, an assistant of Koch, introduced what he called a "slight modification" of the method. He devised a special glass dish. These Petri dishes, as they are called today, are essentially very shallow round glass boxes, consisting of two shallow cylindrical dishes, with the larger one covering the smaller one (Brock, 1961).

The improvement permitted repeated examination of the contents in the plates without risk of aerial contamination at any stage of the incubation and preservation of cultures. Also, the plates could be stacked thus allowing a large number to be placed in a small amount of space. Breed and Dotterer (1916) critically analyzed Koch's procedures and adopted the procedure used today for growing and counting bacteria.

The Plate Count (Aerobic Plate Count or Pour Plate Method) Technique is based on the principle that each viable organism will grow into one visible colony. Furthermore, the assumption is made that the bacterial suspension is homogeneous and no aggregates of cells are present. Obviously bacteria that are

counted are only those which can grow on the medium used and under the conditions of incubation.

Since microbial population in nature is very large in number, the method usually requires dilutions of the sample. Known volumes of the diluted sample are dispensed into sterile Petri dishes after which a suitable, melted, tempered nutrient agar is poured into the dishes and the mixtures are allowed to solidify. The plates are incubated at 25° to 37°C for 24 to 48 hours to allow viable cells to multiply and form visible colonies. The plates are inspected, and those having between 30 and 300 colonies are counted. This number is multiplied by the appropriate dilution factor to provide a bacterial density of the sample. A variant of this procedure is to spread known volume of diluted sample on the surface of a prepoured agar plate and then incubating before counting.

For non-liquid foods it is necessary to treat (shaking, blending, massaging, etc.) the samples in liquid to release microorganisms from foods. The most common practice is to use an electric mixing device with cutting blades revolving at a high speeds (blender). Although this method is satisfactory in dispersing bacteria in liquid it creates aerosols, and heat during blending. Also for every sample a different sterile jar must be used. A new innovation by Sharpe and Jackson (1972) called the Stomacher was developed and will be discussed in a following section.

To dilute the sample a suitable diluent should be used. Some diluents, e.g. saline or distilled water may be lethal for some organisms (Butterfield, 1932). Diluents and methods of mixing dilutions have been compared in many reports. Diluents examined include distilled water or demineralized water--buffered and unbuffered, tap water, Ringer solution, skim milk, peptone, tryptone solutions, and sodium citrate (Anon, 1968; Huhtanen, et al., 1975; Keller et al., 1973; Straka and Stokes, 1957). Phosphate-buffered dilution water is a suitable diluent used for most foods (APHA, 1985). Because of the lethal effect of diluents, the interval between

mixing and removing the test portion should not exceed three minutes (Hartman and Huntsberger 1961). The accuracy of serial dilutions was investigated by Jennison and Wadsworth (1940). The errors involved when 1-ml pipettes were used with 9-ml volumes of diluent, are presented in table 1; the data show that errors increase enormously if serial dilution is carried out with an inaccurate measuring device or a lack of care.

Table 1. Error of the Serial Dilution Procedures as a Function of the Accuracy of Pipetting and Diluent Volume (Jennison and Wadsworth, 1940).

Diluting Volume ml	Pipette S.D. of the volume ml	Diluent volume ml	S.D. of the diluent volume ml	Error arising from the dilution expressed in per cent S.D. at dilution levels			
				10^2	10^4	10^6	10^8
1	±0·01	9	±0·1	±2·8	± 5·7	± 8·5	±11·3
1	±0·01	99	±1	±1·4	± 2·8	± 4·2	± 5·7
1	±0·03	9	±0·3	±8·4	±17·1	±25·5	±33·9
1	±0·03	99	±3	±4·2	± 8·4	±12·6	±17·1

Specific methods for mixing dilutions have been tested. Standard Methods for the Examination of Dairy Products (APHA, 1985) recommend mixing the samples by rapidly inverting the sample container (shake) 25 times in 7 seconds over a one foot arc. Wilson et al. (1935) found that vigorous shaking of dilutions increased counts by 25% due to the disintegration of bacterial clumps. However, the American Public Health Association (APHA, 1985) specifies that dilutions be shaken vigorously.

Pipette errors of up to 9% (depending of the area of the inner wall surface) are introduced when portions of either samples or dilutions are transferred, because bacteria adhere to the surface of the inner wall (Wilson et al., 1935). This is reduced by wetting the inside of the pipette with the sample or dilution several times before transferring the portion (Wilson et al., 1935). Plastic pipettes are

more consistent and accurate in their calibrations than glass pipettes (Felland and Nading, 1965). Appropriate sample size and pipette size should be considered when performing dilutions. The use of a 0.1-ml sample size results in a 10% higher variation than when a 1.0-ml quantity is plated (Marth, 1965). To have less variation in the result, samples should be measured with the proper pipette, e.g. when a 0.1-ml sample is plated a 0.1-ml glass pipette should be used or when a 1-ml sample is plated a 1-ml glass pipette should be used (Marth, 1965).

Inoculation and incubation are other factors that should be considered. Uniformity of colony distribution should be ensured when inoculum and agar medium are mixed in a Petri dish. Procedures for the Aerobic Plate Count, including conditions of plate incubation for various foods differ depending upon the nature of the food and the type of microbial flora to be enumerated (Babel et al., 1955; Hartman and Huntsberger, 1961; Randolph et al., 1973). The count obtained depends on the composition of the nutrient medium, the temperature and time of incubation, whether incubation is aerobic or anaerobic, and the skill of the observer who counts the colonies. Inaccurate counts may result when the agar used for pouring plates is too hot (Huhtanen et al., 1975). The agar temperature should be 45°$\pm$1°C (APHA, 1985). Lower counts may occur also if the agar cools too slowly as in plastic Petri dishes and stacked plates (Koburger, 1980).

The medium in general used for bacterial counts is the Plate Count Agar which contains tryptone, glucose and yeast extract. It was officially recommended for use by the American Public Health Association (1953). Plate Count Agar replaced an earlier medium of the same composition developed in the 1930's, except that beef extract was used instead of yeast extract. Thomas et al., (1966) studied the effect of the type of bacteriological peptone in the plating medium upon the enumeration of pasteurization resistant bacteria in milk. They stated that although seemingly adequate for enumeration of raw milk, the bacteriological

peptone currently recommended for the standard plate count may not be satisfactory for the determination of the maximum viable bacteria population of pasteurized milk. They noted that heat injured bacteria were more demanding in their nutritional requirements than unheated control organisms.

Accuracy of counting colonies also depends on the agar medium being free of particulate matter which could be confused with colonies. Also, tiny colonies in the agar may be missed by the analyst (Fruin and Guthertz, 1977; Fowler et al., 1978). Differences between counts by analysts on the same plate can be more than 10% in at least 9% of samples (Peeler et al., 1982). Automatic colony counters for agar plates have been developed by several manufacturers who claim that bacterial colonies can be recorded quickly and accurately. The Bactronic Colony Counter (New Brunswick Scientific Co. Inc.) is an instrument which marks the colonies as it counts automatically. An electronic probe picks up radio impulses on contact with any agar medium, actuates the counting mechanism, and leaves an identifying puncture in the agar. Fruin and Clark (1977) compared automatic colony counters versus true counts. They identified factors which caused counting difficulties with automatic colony counters and concluded, that after eliminating plates known to be difficult to count by automatic colony counters, equally accurate counts were obtained manually and by automatic colony counters.

To obtain a reliable count with minimum error only those plates whose counts fall in a certain range, usually 30 to 300 colonies, should be counted. This range, accepted widely, is based on the work of Breed and Dotterrer (1916). Postgate (1969), however, regarded the range of 200-300 colonies better because the percentage standard error is lower. Tomasiewicz et al., (1980) examined the counting range critically and developed statistical methods to derive a mean-squared-error function based on the variance function and the square of the bias. They concluded that the best counting range was 25-250 with the minimum

error being at a count of 110 colonies. APHA (1985) recommends the use of this range in standard plate count.

Attempts have been made to shorten the length of incubation of colony-counting procedures. Such modifications have included the oval tube count (Donnelly et al., 1960), plate loop count (Thompson 1960), and cylinder count method (Donnelly et al., 1970). Although these methods have been accepted in certain areas of food microbiology, they have not supplanted the standard plate count method.

B. Most Probable Number

Viable cell numbers of food and water can also be determined by the Most Probable Number (MPN) technique. The MPN is an estimate of the density of viable organisms in a sample. To obtain this estimate the sample must be diluted in such manner that a more dilute sample will result in fewer positive tubes, which are indicated by the presence of gas or microbial growth. To obtain the MPN, the theory of probability is applied to the test result. Usually a 10 ml, 1 ml, and 0.1 ml of a sample are prepared with 5 replicates of each dilution. After incubation (48-96 hrs) the numbers of positive and negative tubes determined by turbidity are obtained. The number of dilutions to be prepared should be based on the expected population of the sample. By use of a 5-tube MPN table the MPN of the sample can be obtained. The most reliable results are obtained when all tubes at the lower dilution are positive (microbial growth present) and all tubes at the higher dilution are negative (microbial growth absent). Most MPN tables include the 95% and 99% confidence limits. This technique is especially useful in situations where extremely low cell densities are encountered (less than 10 cells/g) and when certain types of organisms are to be determined. A simpler 3-tube MPN is introduced to reduce the numbers of tubes for the MPN method.

The MPN method of enumeration is used frequently for determining the number of specific types of microorganisms in samples. In this case, media selective for the specific types of microorganisms are used. Before the development of membrane filters, the most probable number (MPN) technique was used to estimate the number of coliform organisms present in a water sample. Since the use of total coliforms as indicator bacteria has been the subject of debate (Dutka, 1973), a modified Most-Probable-Number procedure was developed to document the magnitude of interferences with total coliform detection in the standard MPN technique. In this technique the same media and procedure are used as in the standard MPN method, except that EC broth is added as an additional confirmatory broth for samples that give a false result in the standard MPN method. Evans et al. (1981) found that the modified MPN procedure was superior to the standard MPN in estimating the number of coliforms in potable drinking water.

To improve the MPN method Feng and Hartman (1982) developed a rapid assay for E. coli in Most Probable Number tubes by adding 100µg/ml of MUG (4-Methyl umbelliferone glucuronide) into Lauryl sulfate tryptose broth (LST). The analysis is based on the enzymatic breakdown of MUG by a glucuronidase synthesized by E. coli producing a fluorescent moiety. Due to the presence of an interfering endogenous glucuronidase in oysters Koburger and Miller (1985) failed to detect E. coli in oysters, by the MUG test. By incorporating the MUG into EC broth rather than LST broth, Koburger and Miller (1985) eliminated the interference and made the MUG test work.

Because of the dependence on tables, requirement for 10-fold dilutions, and difficulty in determining confidence limits, computational methods suitable for hand-held calculators have been reported (Koch, 1982; MacDonell, 1983) for the MPN test.

C. Membrane Filter Method

The Standard Plate Count Method cannot satisfactorily monitor samples with very low counts. The Membrane Filtration technique was developed for samples with very low cell counts. Microbial cells can be collected on the membrane surface and stained for counting under the microscope (direct count). More frequently, the filter with microbes is placed on the surface of a solid medium where viable cells can form colonies and be counted after incubation.

In practice, at least four problems may occur when organisms are present in very low concentration: (1) greater weights of food must be used; (2) high concentration of other species may prevent observation of the species of interest; (3) inhibitory substances may occur in the food; and (4) insufficient dilution of other interfering substances may cause false-positive growth reactions. Membrane filters (MF) techniques are potentially attractive in this area of analysis (Sharpe et al., 1979). The MF may, therefore, directly improve theoretical limits of detection by permitting examination of larger quantities of food. It may also eliminate problems 3 and 4 by allowing soluble interfering materials to be completely removed. The pore size of the membrane filter is selected according to the size of the microorganisms to be counted. For bacteriological work, membranes of 0.2-2.0 μm pore diameter are usually used.

One major advantage of membrane filters is that bacteria can be concentrated on their surfaces from a relatively large volume. However, when one attempts to filter milk through a membrane filter the milk clogs the filter because of the fat globules, casein micelles and somatic cells. This problem can be avoided by acidifying and diluting the milk in a 0.1% Triton X-100 solution before filtration (Merrill, 1963). Kirkman and Hartman (1962) filtered reconstituted albumen by hydrolyzing it first with pepsin and then treating it with diatomaceous earth.

Another advantage of the membrane filter is the reduction of incubation

time in obtaining visible colonies on the filter. Frazier and Gneiser (1968) reported that by using membrane filters, incubation time can be reduced to 8 - 18 hrs. However, they concluded that only limited application of their method was feasible in routine examination of fresh and frozen vegetables. Winter et al. (1971) developed a method where results are obtained after 4 to 5 hours of incubation. They used a vital stain (Janus green) to help observe the micro-colonies developed after incubation and concluded that the method is statistically valid when compared to standard plate count.

Compared to the Standard Plate Count method, very few research studies use the membrane filtration method for food samples. Most dealt with filterable beverages such as wines, beers and some on milk, butter, ice-cream and egg albumen (Barber et al., 1954; Kirkham and Hartman, 1962; Nutting et al., 1959).

1. Hydrophobic Grid-Membrane Filters

Hydrophobic grid membrane filters (HGMF) consisting of conventional membrane filters divided into grid like patterns of growth compartments were introduced by Sharpe and Michaud (1974). This is a simple modification of conventional membrane filter, and is essentially a membrane base upon which a hydrophobic grid is applied to divide the membrane into a large number of individual growth compartments. The term "growth unit" is preferred over the conventional "colony forming units" (Sharpe and Michaud, 1975). The HGMF "count" is determined by a special most-probable-number calculation. Thus the counting range of the HGMF is expanded well beyond the actual number of observed growth units and, consequently, not only covers a much larger range than is obtainable with the traditional pour or spread plate technique but with greater precision (Sharpe and Michaud 1974).

The HGMF has a large operating range (three log cycles), and because of

this, there are many situations in which the use of the HGMF can eliminate the normal requirement of preparing dilutions. The elimination of dilution operations could be particularly attractive in the automation of quantitative food microbiology. The limitation of membrane filtration of a limited numerical range resulting from the small growth areas is avoided with the use of HGMF.

The HGMF designed by QA Laboratories Limited (ISO-GRID^{TM} membrane filter, Toronto, Canada) has a grid pattern consisting of 1600 growth compartments which provide an effective MPN counting range of three logarithmic cycles. The ISO-GRID pre-filtration system and food treatment was developed by the same laboratory for the use with the HGMF. This system has overcome the main drawback of the MF method (clogging filters with food particles). They also developed an automated counting system (ISO-GRID^{TM} sample processor) to avoid the impracticality of manually counting large number of growth units.

Enumeration of bacteria by the HGMF method is comparable to counts obtained using conventional membrane filters and pour and spread plates (Sharpe and Michaud, 1975). Brodsky et al. (1982) demostrated that HGMF produced aerobic plate counts and yeast and mold counts in foods that are equivalent to or greater than counts using traditional analytical methods. Enumeration of indicator organisms in food using the HGMF technique was made by Brodsky et al. (1982). Their results suggested that the automated HGMF system is a viable alternative to conventional most-probable-number and spread plate techniques for the isolation and enumeration of food-borne microorganisms on selective media. However, individual confirmation of presumptive colonies by the coagulase test must be done when enumerating Staphylococcus aureus. Peterkin and Sharpe (1984) developed a method based on enzyme-linked immunosorbent assay for the direct demonstration of enterotoxin B production of S. aureus. The test requires three hours to complete and yields a purple stain at the site of enterotoxin B producing

colonies, thus allowing direct enumeration of confirmed S. aureus in foods within 27 hours. Lin et al. (1984) used the ISO-GRID for enumeration of yeast and mold.

Advantages of the HGMF method for enumeration of microorganisms over the standard plate count (Brodsky et al., 1982) include:

a. HGMF have the ability to detect low numbers of organisms by filtering larger volumes.
b. The absence of temperature stress from molten agar.
c. The removal of water soluble materials that could interfere with growth.
d. The ability to recover stressed or injured cells by allowing for resuscitation on a repair medium.
e. Greater counting precision than provided by a conventional MPN.
f. Availability of more rapid results.

2. Direct Epifluorescent Filter

The direct epifluorescent filter technique (DEFT) is a rapid, sensitive method for counting bacteria and somatic cells which uses membrane filtration and epifluorescent microscopy (incident illumination) (Pettipher et al., 1980). The bacteria and somatic cells retained on membrane filters are stained with fluorochromes (acridine orange, ethidium bromide, euchrysine etc.) and counted visually with an epifluorescent microscope. The fluorochrome, acridine orange, is the most commonly used. It can be used for differentiating deoxyribonucleic acid from ribonucleic acid by orange as opposed to green fluorescence in fixed or living mammalian cells (Von-Bertalanffy and Bickis, 1956).

According to Hobbie et al. (1977), the acridine orange staining characteristic permits actively growing bacteria which fluoresce orange-red to be distinguished from inactive bacteria which fluoresce green. However, Pettipher

and Rodriguez (1981) observed considerable variation in the acridine-orange staining characteristic of the different bacterial species after heat treatment. They concluded that samples which are heat-treated or inoculated with starter cultures usually had higher DEFT counts compared with plate count. Some organisms, even though killed by heat or drying, remained intact and can be stained by fluorochrome resulting in a positive DEFT count. Better agreement between DEFT and plate count can be obtained when heat treated cells are also lysed and can not absorb fluorochromes (Pettipher and Rodriguez 1981).

Fluorochromes are used in conjunction with a black membrane background for contrast. Two types of filters are available, cellulose and polycarbonate. Because cellulose filters have a rough surface, many cells may not be counted since only one plane is analysed. Nucleopore polycarbonate membranes have flat surfaces, thus allowing all the retained cells to be counted.

The major disadvantage of the DEFT is operator fatigue. The manual DEFT count is suitable only for 30-40 samples/day/operator. Pettipher and Rodriguez (1982) devised a semiautomated DEFT utilizing closed-circuit television and computer analyses to reduce operator fatigue. The semiautomated DEFT studies on raw milk have been compared with manual DEFT (94% agreement), plate count (83% agreement) and Coulter Counter method (81% agreement).

Enumeration of microorganisms in foods by DEFT was made by Pettipher and Rodriguez (1982). They filtered stomached food suspensions through nylon filters (pore size 5μm) to remove most of the food debris without affecting the recovery of microorganisms. They concluded that with prefiltration, DEFT can be used to obtain a count of microorganisms in a variety of foods in less than 30 minutes. And as an added advantage of the technique the organisms can be tentatively identified as bacteria, spores, yeast or fungi.

D. Improved Viable Cell Count

Standard plate count method has a number of disadvantages. The uncertainty of the number of individuals cells which eventually give rise to one visible colony is a major disadvantage. Variations of culture media will greatly affect the recovery of different types of organisms in the sample. The time involved in the appearance of a visible colony differs from species to species, thus depending on the time of data collection, some species will be counted while other species will remain invisible. The number of organisms in a sample also affects the counting process if inappropriate dilutions are plated. The ideal range is to have 30-300 colonies per agar plate but such is not always the case when an analyst does not know the range of cells at the beginning of the experiment.

Perhaps the greatest drawback of the standard plate count method is the time involved in obtaining final data. This may take 24 - 48 hours, a few days and in extreme cases a week or more to collect the data. In this age of fast moving production lines such delays are unacceptable. Therefore, microbiologists are always looking for faster methods to process microbiological samples and obtain meaningful data. Ideally a rapid method should provide final data in matters of hours and even in minutes.

The following sections deal with some improvement of the standard plate count method to increase efficiency of operation of the procedure through miniaturization, mechanization, and semi-automation.

1. Miniaturization

Miniaturized techniques have been developed specifically to aid busy laboratories by speeding analysis, increasing throughput and reducing costs. These techniques use smaller quantities of materials than the standard plate count. The savings in Petri dishes, agar medium, peptone solution and incubator space are

considerable. Another important saving obtained is the reduction in support work.

A Microtiter method based on the loop dilution principle was developed by Fung and Kraft (1968) for the evaluation of viable-cell counts of bacterial cultures. The method consists of rapid serial dilution and spot plating procedures. Spots containing an arbitrary range of 10-100 colonies are counted, and then the counts are multiplied by the appropriate dilution factor. This method has potential for studying large numbers of samples for estimation of bacterial populations growing aerobically.

A direct comparison of the Microtiter and the conventional Standard Plate Count Method was made by Fung and Kraft (1968). The microtiter method was comparable to the conventional method in accuracy and precision of counting viable cells of bacterial cultures. Applications of the microtiter method were made by Baldock et al. (1968) to evaluate spore survival. Fung and LaGrange (1969) concluded that the microtiter method is comparable to the standard plate count in accuracy for estimating viable cell counts in both manufacturing grade milk and Grade A milk. A modification of the method was made by Casas et al. (1977). They overlayed the spots with four drops of the same media melted to avoid spreading of the drops Casas et al. (1977) use the technique to evaluate mesophile, psychrotroph, and coliform counts for raw and pasteurized milk samples. They also concluded that the accuracy of the microtiter method is comparable to the SPC.

The Agar Droplet technique, first described by Sharpe and Kilsby (1971), is another miniaturized method. Essentially a sample is diluted in agar, then the agar drops are deposited on sterile Petri dishes. After growth the colonies can be counted in these miniaturized agar drops. A functional instrument was developed (Sharpe et al., 1972) to use with the droplet technique and later this instrument was commercialized (Colworth Droplette). The Colworth Droplette is an instrument that combines a diluter dispenser (to dilute and pour the sample), a viewer for

incubated droplets, and a counter, in a single compact and portable unit.

2. Mechanization

Since preparation of the media and dilutions for the determination of viable microorganisms is time-consuming, there has been an increased use of established aids such as media makers, plate pourers, Spiral platers, Stomacher-type blenders and electronic colony counters. Also, several simplified viable count methods have been developed which offer savings in time and money if used in place of the standard plate count. Accurate delivery of .01 ml of sample, which was not possible with a calibrated loop (Thompson and Black, 1967) was facilitated by the use of a calibrated cylinder (Berridge, 1966). Bradshaw et. al. (1973) developed an apparatus that would further reduce the cost and labor of plating milk samples by utilizing the loop and cylinder simultaneously. Modifications of the Plate Loop count procedure have been made (Fleming and O'Connor, 1975; Olsen and Richardson, 1980). But this technique has been used only for evaluating the quality of milk.

a. Automatic Dispenser

The Colworth 2000 (Sharpe et al., 1972) is a machine that carries out all of the most time-consuming parts of the preparation and technical work involved in a pour plate count. This instrument inoculates sample suspensions into selected nutrient media, making up to eight decimal dilutions and dispenses molten agar into the petri dish from its own storage tank. It also mixes the content of the Petri dish and stacks the dishes ready for incubation. The total labor involved in a bacterial count such as sample preparation, manual pipetting, diluting and inoculating, careful coding of dishes, steaming and cooling of the agar media is avoided with the use of the Colworth 2000. This instrument is not marketed at the

present time.

b. Stomacher

One of the problems faced with food sample preparation using a blender is that since the samples come in contact with the sample jar another sterile jar must be used for every sample. In 1972 Sharpe and Jackson (1972) introduced an entirely new mixing device, called the stomacher which is particularly suitable for preparing suspensions from foods, fabrics, swabs and other fairly soft materials. In the stomacher there is a separation between the sample and the machine. This separation is achieved by enclosing the sample, plus diluent, in a flexible, sterile, high quality polyethylene bag. The bag prevents contact between the machine and sample and is discarded after use. This allows samples to be prepared one after another without sterilizing the equipment, and saves time and material compared with the conventional procedure.

The Stomacher is constructed of a sturdy cast aluminum alloy body which encloses the blending area and supports the motor and stainless steel front door and closing mechanism, and a mild steel motor housing. There are three sizes of Colworth Stomacher, identified by the maximum recommended cubic capacity of the sample: the Stomacher #80 (8-80 ml) which is the smallest, model #400 (40-400 ml), and model #3500 (400-3500 ml) being the largest.

The Stomacher can effectively extract deep-seated bacteria without completely breaking down the sample tissue. The efficient recovery of aerobic and anaerobic bacteria by the Stomacher has been demonstrated by Sharpe and Harshman (1976). Bacterial recovery using the Stomacher, is slightly depressed in foods containing higher than 20% fat. However, addition of 1% Tween 80 to the diluent improved the recovery efficiency (Sharpe and Harshman, 1976). The efficiency of the Stomacher varies depending on the type of food sample (Andrews et al., 1978). The use of the stomacher for the microbiological examination of

foods has been recommended by many workers (Andrews et al., 1978; Deibel and Banwart, 1982; Emswiler et al., 1977; Schiemann, 1977).

With rapidly increasing popularity of the stomacher, the development of accessories was needed. A balance bag holder and bench bag holder was introduced by Sharpe and Dudas (1978). Konuma et al. (1982) introduced an improved Stomacher bag with a teflon filter. This filter separates or retains particles when liquid is poured from the bag to another container, thus when a pipette is used for sampling, clotting is prevented.

Advantages of the Colworth Stomacher are numerous. In some laboratories it can replace 80 to 100 bottom-drive-type blenders. The Stomacher bag costs considerably less than the cost of labor required for recycling conventional methods. The Stomacher is mechanically safe (no rotating blades), microbiolologically safe (samples are contained within a polyethylene bag), and causes no mechanical damage to the cell. The rise in temperature is less than 1°C/minute from ambient. It also offers rapid extraction and high output in routine use.

Stomacher bag breakage in the instrument is one disadvantage, but this can be avoided by using double bags and only soft samples. Another problem stems from enumeration of bacteria clumps or chains which may not be separated as well as in a blender.

c. Automatic Diluters

Bacterial counts generally require serial 10-fold dilution in tubes. Manual dilutions are not always rigorously performed. Many factors may be involved: diluent volume can vary because of the loss of volume during sterilization; glass or plastic pipettes can be more or less hydrophilic; and finally, analyst error can be significant. Because of this problem automatic diluters were introduced to improve

the accuracy of dilutions and their standardization among laboratories (Cremer et al., 1975; Trinel, 1983; Trotman, 1967). Spiral System Instrument Inc. has a gravimetric diluter, model GD-100, that accurately performs sample dilutions, manual weighing, diluent measurement and related calculations. An analyst can program the dilution factor desired and put the sample in the instrument. This will automatically dispense the corresponding volume of sterile diluent into the vessel, thus saving considerable time in aseptic measurement of samples and diluents.

d. Automatic Streaker

Trotman (1971) has developed an automatic plate streaker with which the inoculum is placed on the surface of the agar with a pipette and a mechanically moved loop streaks the inoculum over the surface of the plates. The Autostreaker (TomTec, Orange, Conn) mechanizes the agar plate streaking process by providing storage for plates, labeling and streaking one or more plates for either isolation or enumeration, and stacking them in one of several racks for subsequent incubation. Results (Tilton and Ryan, 1978) show the Autostreaker produced agar plates with well-separated colonies and accurate colony counts.

3. Spiral Plate Count

In 1971 Campbell introduced the concept of adding inoculum to agar plates in the form of an Archimedes spiral. This concept was further developed by Gilchrist et al. (1973) and later a semi-automated instrument employing this principle, the Spiral Plater (Spiral Systems Marketing, Bethesda, Md.) was marketed. In 1977 the instrument was adopted as an official first action for foods and cosmetics by the Association of Official Analytical Chemists (Gilchrist et al., 1977). Currently this method is listed as an alternative microbiological method in the 15th edition of Standard Methods for the Examination of Dairy Products

(APHA, 1985). This technique was developed in an attempt to reduce the skilled manpower time and supply costs associated with the Standard Plate Count technique.

In contrast to conventional procedures, no serial dilutions are required in the Spiral system. The Spiral plater is a specialized dispenser that distributes 35µL of the sample onto the surface of a rotating agar plate from the center to the edge in an evenly decreasing amount resulting in a concentration range up to 10,000:1 on a single plate. Every sample is deposited in the form of an Archimedes spiral in a manner such that the volume on any portion of the plate is known and always the same. After incubation, colonies appear on the lines of the spiral, and the bacterial density is determined by counting the colonies on a countable portion of the plate and dividing this number by the volume of the sample contained in the areas counted. Spiral Systems Marketing also carries a Laser Bacteria Colony Counter Model 500A to be used with the Spiral System. An electronically guided laser beam scans the plate in a spiral from the edge toward the center. Any particle that interrupts the beam as it spirals toward the center is registered as a unit count. The laser counter was designed to enable a preset number of colonies (e.g 200 colonies) to be counted. If the plate contains less than this number, the laser counts all the colonies and the plate count is presented as a digital read out. This count is divided by the inoculum volume to give the colony count per ml from which the total count/g is calculated. If, however, the plate contains more than this number, the counter stops and displays the area in which the 200 colonies were counted. The colony count per ml is then determined by reading this area from a calibration curve. The Laser Bacteria Colony Counter can also be used with conventional pour plates. The limits of estimation using the spiral plate method are 10^3-10^6 CFU/ml suspension (Gilchrist et al., 1973) with a 9 cm Petri plate. The upper limit can obviously be extended by dilution of the sample, but the lower

limit is fixed by the amount of inoculum which can be applied to the medium. By increasing the inoculum size and/or plate dimensions the range can be extended to 10^2-10^6 CFU ml. Jarvis et al., (1977) found that at low levels of contamination (e.g. 10^3-10^4 CFU/g), slightly higher counts could be expected from the spiral plate count method.

The Spiral Plate technique has produced comparable results with the Standard Plate Count in the examination of pure cultures of bacteria (Gilchrist et al., 1973), milk (Donnelly 1976), spices, seafood, and cooked meats (Gilchrist et al., 1977). Comparative work was made to compare pour plate, surface spread plate, drop count and spiral plate count methods using samples of four types of food (Jarvis et al., 1977). They found significant correlations between the various methods, with no difference between the results from individual samples. Furthermore, Hedges et al. (1978) stated that the overall precision of the spiral plate method will usually exceed that of the surface drop and agar droplet (Colworth Droplette). Kramer et al. (1979) found that the spiral plate technique could replace the surface drop count method for the routine monitoring of a wide variety of foods without producing different results from the standpoint of practical microbiology. Konuma et al. (1982) introduced an improved Stomacher 400 bag applicable to the spiral plate system for counting bacteria.

4. Value of Standards

The utility of the aerobic plate count is derived from the fact that it provides a means of measuring the composite microbiological population in food prior to the onset of organoleptic deterioration. Generally counts must be in the millions of cells/g before organoleptic deterioration occurs. This is demonstrated in Table 2, showing the bacterial levels at odor and slime points for protein foods (Elliot and Michener, 1961). This table also shows that the microbiological

Table 2. Bacterial Level at Odor or Slime Point for Protein Foods [a]

Food	Logarithm of no. of cell/cm^2	
	Odor	Slime
Poultry meat	6.5	-
	8	8-9
	7	7.5
	6.9	-
	5.2	-
	7-8[b]	-
Beef	-	7.7-8
	-	7.7
	-	7.5
	-	6.5
	-	7-8
	7.7-8	-
	8.7[b]	-
	6.3-7	8
	8[b,c]	-
	7	7.8
Processed meats	-	7-8[b]
Frakfurters	8-8.5	8.5
Wiltshire bacon	-	6.7-8
Wiltshire bacon		7
Fish	6-6.6[b]	-
Haddock	6[b]	-
Fish	7-8.5	-
Fish	6.5[b]	-
Oysters	4-5.7[b]	-
Crabmeat	8[b]	-
Shell eggs	7[b]	-
Frozen eggs	6.7[b]	-
Liquid eggs	7[b]	-
Chicken pies	5[d]	-

[a] For original source of these data, see Elliot and Michener (1963)
[b] per gram
[c] Yeast
[d] Unacceptable flavor

spoilage point level varies with different classes of foods.

However, certain microbial groups within the microbiological flora of a food are more apt to cause spoilage than others (e.g Pseudomonas) and if these groups are given a selective growth advantage by virtue of the way the food is processed, stored or packaged, spoilage may occur at relatively low aerobic plate count levels. This was demonstrated by the variance in sensory scores indicating loss of organoleptic quality for vacuum-packed and non-vacuum packed sliced cooked ham (Silliker, 1963).

The rationale behind the adoption of microbiological standards for foods is based largely upon the ideas that foods with low microbial numbers are likely to be safer to the consumer in terms of the absence of food-borne pathogens, and to permit longer shelf-life of stored products capable of permitting growth. Since the safety and keeping quality of fresh foods are related to microbial content, microbiological standards have been proposed for a variety of foods and some of these have been adopted. Microbial standards should result in foods that have a longer shelf-life and foods that are free of microbial hazards.

A microbiological Standard is defined as a microbiological criterion that has the backing of law attached to it. An estimate of the number of microorganisms in or on foods is needed in order to determine if a product meets the microbial levels expressed in specifications, guidelines or standards. When the microbial load of a food is determined, a judgment needs to be made as to whether the food is satisfactory or not. A microbiological standard should not be established haphazardly. A standard must be meaningful and attainable by what is considered to be good manufacturing practice. It should be subject to reevaluation as new technologies are developed. Above all, to be of value, a microbiological standard must be enforceable. When enforced, a standard should reduce the public health hazard of food.

There is more justification for microbial standards for some foods than for other foods. When there is a risk of hazard, a standard for a food might be needed. There are some foods for which there is no substantive need for microbiological standards. Those foods which have demonstrated a potential health hazard should be the first ones considered for establishment of any microbial standard. Standards must be adapted to the types of food for which they are intended. They probably would be different for a food to be consumed raw than for the same food to be cooked or subjected to heating or other processing before being marketed. Foods that contain animal products are primary vehicles for organisms that cause food-borne illness.

For standards to be useful they must be based upon sampling techniques that are adequate to obtain a representative portion of the food being tested and the method of analysis must be readily reproducible so that results obtained both within a given laboratory and among many laboratories are comparable.

Deterioration of food quality as a result of microbial activity is a continuing process, terminated only when the food is consumed. Total counts (more often, aerobic plate count, APC) on food products not only reflect handling history, state of decomposition or degree of freshness; they may in some instances reflect on the sanitary quality of foods. Standards usually are based on the total number of organisms, number of an indicator organism, or number (or total absence) of pathogens; but there has been some disagreement over what counts should be considered significant, what the indicator organism should be, and whether pathogens can be demonstrated. It should be noted that low counts do not always represent safe products. In their study of commercial frozen-egg preparations, Montford and Thatcher (1961) were able to isolate Salmonella from one preparation with a total count of only 380/g and from several others with total counts below 5000/g. Even though the total counts of these preparations

were quite low, all were shown to contain coliforms. It is also possible to have low-count foods in which toxin-producing organisms have grown and produced toxins that remain stable in conditions which may not favor cell survival. The sanitary quality of foods such as sauerkraut, fermented milk, and related foods, cannot be ascertained by total plate counts, since these products are produced by the activities of microorganisms.

An excellent review of the statistical approach to sampling of food in relation to setting standards for foods was presented by the International Commission in Microbiological Specification for Foods (1974).

II. MODERN APPROACHES

A rapid and routine procedure for the quantitative detection and counting of bacteria is frequently of vital importance. Classical techniques, which have the advantage of positive identification and preservation of the bacteria for future use, are usually slow, require rather complex media, and are not suitable for enumerating the sparce populations or for assessing the relatively slow growth rates that occur in most natural environments.

Conventional methods for detecting and monitoring bacteria, yeast, and other microorganisms usually include agar plate counting techniques which generally take from one to five days. One of the main goals of microbiology is to quantitatively detect bacteria without having to rely on time-consuming cultural techniques. To eliminate this delay we need rapid, sensitive and selective techniques.

A. Cell Components

1. Limulus Amoebocyte Lysate

The limulus amoebocyte lysate (LAL) endotoxin assay was introduced by Levin and Bang (1968). This test is well established as a sensitive and rapid test for bacterial endotoxins (Sullivan and Watson, 1974). The limulus lysate test employs a lysate protein obtained from the blood cells (amoebocytes) of the Horseshoe crab (Limulus polyphernous). In the presence of purified endotoxins, which are lipopolysaccharide (LPS) components of the outer-cell wall layer of gram-negative bacteria, the lysate gels (turbid solution) and gives the appearance of a positive coagulase tube test (Jay, 1977). The biochemical mechanism involved in the lysate-endotoxin reaction is an enzymatic reaction consisting of endotoxin activation of a high-molecular weight enzyme followed by its conversion of a

low-molecular weight clottable protein to a gel (Young et al., 1972). The amount of turbidity measured photometrically minus that caused by free LPS, is linearly proportional to the amount of LPS and hence to the amount of gram-negative bacteria (Harris and Kell,1985). This method is extremely sensitive and can measure 0.000001 g of LPS.

The limulus amoebocyte lysate can be used to determine the amount of gram-negative cell wall material (endotoxin) present in a food sample. Several issues need to be considered before adopting this method. First, it measures endotoxins in both live and dead cells. Second, types of microbes present in a food, as well as their metabolism, are easily affected by storage conditions, and mere detection of the presence of particular microbes is not sufficient as an index of quality. For example, vacuum-or gas-packed meat will not develop the normal gram-negative organism flora but will develop a mainly gram-positive flora consisting principally of lactic acid bacteria which are gram-positive organisms and will not respond to the limulus test. Jay (1981) obtained comparative data between aerobic plate counts and LAL. He concluded that although the variance and confidence limits of some of the APC and gram-negative bacteria ratio values were wide, use of LAL to estimate APC in one hour was found workable. The value of the limulus test lies in the speed at which results can be obtained. Foods that have high limulus titers may be candidates for further testing by other methods, while those that have low titers may be placed immediately into categories of lower risk relative to the number of gram-negative bacteria.

The LAL showed both greater sensitivity and greater specificity than the gram-stain procedure. Moreover, the LAL is much less susceptible to errors of interpretation than methods involving microscopy (Jorgensen and Jones, 1975). The test has been shown to be useful in the detection of gram-negative bacteria, particularly coliforms, in water and waste water (Evans et al., 1978 and Jorgensen

et al., 1979), as well as in the detection of spoilage organisms in certain food products, such as ground beef (Jay, 1977), to assess the overall microbial quality of fresh meats relative to endotoxin content (Jay et al., 1979), to estimate microbial numbers in fresh ground beef (Jay, 1981) and as an index of seafood spoilage (Brown, 1979).

2. DNA Method

Classically, DNA in biological material has been determined by the diphenylamine method (Munro and Fleck, 1966). Based on the reaction of diphenylamine with deoxyribose, this method requires 17 hours for color development. Although there is interference by a variety of substances, the lower limit of detection is 5μ of DNA (Burton, 1968). Recently, dyes more specific for DNA which do not require extractions or separation of DNA from cellular constituents have been employed in DNA estimations. These include adriamycin, mithramycin, ethidium bromide, 4',6-diamidino-2-phenylindole (DAPI), and Hoechst 33258 dye.

Adriamycin combines with DNA to yield a yellow product that absorbs light at 475 nm (Hill, 1976). As a colorimetric method, the minimum sensitivity is only 1μ of DNA, and RNA interferes with the determination (Dalbow and Bartuska, 1979). The ethidium bromide technique is extremely sensitive for DNA but requires RNAase digestion since this fluorochrome reacts with both DNA and RNA (LePecq and Paoletti, 1966). The ethidium dimer technique suffers from the same mixed specificity (Markovits et al., 1979). Mithramycin, although extremely specific for guanidine-plus-cytosine portions of DNA, does not bind DNA effectively in the presence of nucleoproteins, and solubilization with heparin is required (Groyer and Robel, 1980).

Recently, two structurally related fluorochromes with similar specificities

for DNA, Hoechst 33258 dye and DAPI, have been employed for DNA determinations in crude cell homogenates (Ceasaron et al., 1979; LaBarca and Paigen, 1980). Hoechst 33258 dye, a weak fluorescent, binds DNA specifically and quantitatively with increases in fluorescence (Latt and Stetten, 1976). The greatest fluorescence occurs in portions of DNA rich in adenine plus thymine (A-T); while the guanine and cytosine regions possess only 50% of the fluorescence of the A-T regions. Hoechst 33258 dye is thought to bind without intercalation in the major groove of the double helix of A-T rich regions, perhaps by hydrophobic interactions with the methyl of thymidine (Comings, 1975). The interaction of the dye with DNA is rapid, coming to completion in the time required for the simple mixing of Hoechst 33258 dye and DNA solutions (LaBarca and Paigen, 1980). The fluorescence of Hoechst 33258-DNA is not affected by common laboratory reagents or low concentration of detergents.

The Fluorometric method that utilizes diaminobenzoic acid is quite sensitive, but the more modern methods in which DNA-specific fluorochromes like the ones cited before are faster, simpler, and more sensitive, with the Hoescht 33258 method being the most sensitive fluorometric DNA assay (McCoy and Olson, 1985). This method has been used to quantitate DNA concentration in drinking water and also shows good correlations between epifluorescence direct cell counts and colony-forming units concentration (McCoy and Olson, 1985; Paul and Myer, 1982).

3. ATP Method

The universal presence of Adenosine Triphosphate (ATP) in all life forms makes this compound an excellent indicator of the presence of bacteria. ATP is the main source of energy in every living cell. Since bacteria and yeast are living cells, they contain ATP. ATP content has been shown to be proportional to the

number of viable cells present in a living bacteria (Chapman and Atkinson, 1977). McElroy (1947) observed that the luminescence of firefly extracts could be restored with the addition of ATP. This observation was utilized to develop a sensitive and convenient analytical method for ATP. Chappelle and Levin (1968) found that measuring the bacterial population on the basis of ATP content is a simple and rapid technique with accuracy comparable to that of slower and more classical means such as plate counting and direct microscopic counting. The firefly bioluminescence assay is based upon the assumption that living cells of a given type contain a reasonably constant amount of ATP which is lost rapidly upon cell death.

The emission of light in Firefly bioluminescence results from a three-step reaction (McElroy and DeLuca, 1981).

$$(1)\quad E + ATP + LH_2 \xrightarrow{Mg^{++}} E\,LH_2\text{-}AMP + PP$$

$$(2)\quad E\,LH_2\text{-}AMP + O_2 \longrightarrow \text{oxyluciferin} + Co_2 + AMP + \text{light}$$

$$(3)\quad E + L + MgATP \longrightarrow E + L\text{-}AMP + PP$$

E= Enzyme Luciferase, LH_2= Luciferin (reduced) PP= Pyrophosphate, AMP= Adenosine monophosphate L= Dehydroluciferin.

In the first reaction, the carboxyl group of luciferin is the key site for the formation of an anhydride with adenylic acid from ATP accompanied by the formation of inorganic pyrophosphate. In the second reaction, oxygen combines with the enzyme bound luciferyl adenylate which leads to a decarboxylation of luciferin and the formation of an excited product (oxyluciferin) that subsequently decays to the ground state with light emission and the liberation

of free adenylic acid. The light produced from this chemical reaction is measured on an instrument designed for this purpose (luminometers) e.g. Packard's PICOLITE 6200 Luminometer (Packard Instrument Co.) and Dupont 760 Luminescence biometer (Dupont Instruments). The optimal conditions for the ATP reaction are 15°-25°C and pH of 7.4-8.2. This reaction is specific for ATP and no reaction will occur with other triphosphonucleotides. If the luciferase enzyme and luciferin are present in excess, the intensity of light produced is proportional to the quantity of ATP present. ATP can be measured very rapidly (1 to 2 minutes) using Firefly Luciferase and cofactors to produce light.

In a food sample which contains bacteria or yeast, the ATP from the living microbial cells can be measured. ATP is usually located within a cell. Cell walls or cell membranes are not normally permeable to luciferase. In order to measure intracellular ATP, cells must be lysed or made permeable to ATP so that luciferase can react with the ATP. This may be done in a variety of ways such as boiling buffers, acid extractions, chemical extractions, sonification and others. The turnover time of ATP in bacteria and yeast is less than one second (Chapman and Atkinson, 1977). Thus any extraction method has to stop the metabolic activity in the sample immediately, or release the ATP so quickly that the enzymes of the cells can not alter the ATP level before the ATP is quantitatively released from the cells. Most of the extraction methods introduce chemical quenchers that reduce the bioluminescence light reaction, dilute the sample or cause error due to inadequate extraction or inactivation of the cell's own enzymes. Chapelle and Levin (1968) devised a way to extract ATP from bacteria by using the solvent n-butanol, but later Lundin and Thore (1975) found that extraction with trichloroacetic acid was the method most closely reflecting actual levels of ATP in intact bacterial cells. In order to simplify cellular ATP measurements, Packard Instrument Company has developed reagents and

instrumentation for performing such luminescence assay. PICO-ZYME F is a higly purified firefly luciferase containing luciferin and other cofactors. PICO-EX is a reagent that allows ATP to be released from a cell within seconds so that it may come in contact with PICO-ZYME to produce light. The PICO-LITE is an instrument specially designed to perform luminescence measurements including cellular ATP.

Accurate detection of bacteria or other microorganisms using this technique depends on the number of microorganisms present, the amount of ATP per cell, and the presence of other living cells such as somatic or mammalian cells. Generally, lower than 10^5 bacteria cells/ml or 10^3 yeast cells/ml cannot be accurately detected without some sample preparation. The level of ATP of a cell is dependent on the size of the cell. Thus in different species of bacteria and other microbes the ATP levels vary according to the cell size of the species (Table 3). The presence of somatic cells in samples may interfere with microbial ATP measurements since a somatic cell generally has 10 to 100 times more ATP/cell than a bacterial cell and about the same as yeast or a mold cell.

Measurement of microbial ATP has been applied to enumerating bacterial populations in water (D'Eustachio et al., 1968, and Levin et al., 1967), waste water (Levin et al.,1975), milk (Bossuyt, 1981), brewing processes (Hysert et al., 1976) and various foods, including fresh meats (Baumgart et al., 1980; Jouve et al., 1981; Sharpe et al., 1970; Stannard and Wood, 1983). Interference by non-microbial sources of ATP has been found to be a difficulty in the application of the ATP method (Bossuyt, 1981; Chappelle and Levin, 1968; Sharpe et al., 1970). This non-microbial ATP must be destroyed before the microbial ATP assay begins. Selective measurement of microbial ATP in the presence of non-microbial ATP has been accomplished with reasonable success by selective extraction and enzymatic destruction of non-microbial ATP

Table 3. Quantity of ATP per Cell Reported in The Literature

Species	ATP/cell 10^{-15}	Reference
Aerobacter areogenes	2.0	Hysert et al., 1976
Bacillus coagulans	17	Chappelle and Levin, 1968
Chromobacterium marinum	6.5	Hamilton and Holm-Hansen, 1967
Flavobacillus proteus	0.01	Hysert et al., 1976
Flavobacterium divergensis	0.15	Chappelle and Levin, 1968
Klebsiella pneumoniae	0.5	Chappelle and Levin, 1968
Lactobacillus acidophilus	2.6	Sharpe et al., 1970
Lactobacillus brevis	1.3	Hysert et al., 1976
Lactobacillus casei	1.1	Hysert et al., 1976
Lactobacillus delbrueckii	1.2	Hysert et al., 1976
Micrococcus Lysodeikticus	0.13	Chappelle and Levin, 1968
Micrococcus sp.	0.5	Hamilton and Holm-Hansen, 1967
Mycobacterium phlei	0.19	Chappelle and Levin, 1968
Proteus mirabilis	0.5	Hysert et al., 1976
Pseudomonas aeruginosa	0.31	Chappelle and Levin 1968
Pseudomonas sp.	1.5	Hamilton and Holm-Hansen, 1967
Staphylococcus aureus	1.0	Sharpe et al., 1970
Vibrio sp.	3.6	Hamilton and Holm-Hansen, 1967
Species Yeast		
Bakers yeast	200-800	Sharpe et al., 1970
Saccharomyces cerevisiae	130-170	Hysert et al., 1976

(Chappelle and Levin, 1968; McWalter and Sharp 1982) or by physical separation of microorganisms from sample material followed by extraction and assay of microbial ATP (Stannard and Wood 1983). The United Technologies Packard technique to eliminate non-bacterial ATP, involves using various pore size filters to simultaneously concentrate and separate somatic cells or larger microbial cells. Filtration techniques can be approached in several ways depending on the sample types to be examined, e.g. vacuum-filtration for simple samples with no contaminating somatic cells and double filtration for products containing somatic cells and/or large debris. In some cases, a short incubation may be necessary to allow the microbial population to recover from the stress of filtration.

Bioluminescent techniques have been specifically applied to estimating microbial levels on meat surfaces. Baumgart et al. (1980) reported some interference by nonmicrobial ATP in meat-surfaces samples whereas Carlier et al. (1982) found a "linear" relationship between $\log_{10}$ relative light units (RLU) and the $\log_{10}$ colony counts of beef carcass surfaces with correlation coefficients of 0.79 to 0.80, using an enzymatic procedure for elimination of nonmicrobial ATP. Stannard and Wood (1983) used a rapid procedure for physical separation of bacteria from meat tissue homogenates for selective assay of bacterial ATP. They reported a "linear" relationship between $\log_{10}$ microbial ATP content and $\log_{10}$ colony-forming-units in various fresh meat tissues, with a correlation coefficient of 0.94. Kennedy and Oblinger (1985) studied the relationship between microbial ATP measurements and aerobic plate counts (APC's at 35°, 20° and 7°C) from ground beef samples. They found high overall correlation and linearity between bioluminescent measurements and APC (20°C) or APC (7°C) over a range of $\log_{10}$4.8 to 9.9 CFU/g but less correlation for APC at 35°C. They concluded that bioluminescent methods are applicable to rapid estimation of microbial levels in

ground beef, particularly in individual processing operations, and offer great potential for prompt assessment of the microbial condition of raw materials or products throughout the processing operations for various meat products.

Bioluminescent ATP assays of microbial populations in foods such as milk were reported capable of detecting 10^5 bacteria/g with a correlation coefficient of 0.93 (Bossuyt, 1981). This technique can also detect 10^3 yeast/ml (Hysert et al., 1976). Use of the bioluminescent assay seems to be feasible for monitoring products where active populations of 10^3 yeast/ml or 10^5 bacteria/ml or more are present, such as during processing operations at ambient temperatures or in sterility testing where samples are preincubated (Graumlich, 1985).

Errors in the estimated viable counts may arise from one or more of the following sources (Harris and Kell, 1985):

1. Incomplete extraction of ATP;
2. Quenching by extraction chemicals, buffers or other substances in the sample;
3. Use of impure, though relatively inexpensive Luciferase (Picciolo et al., 1978);
4. Stress on cells (Graumlich, 1985);
5. Activity of ATPases and other Kinases;
6. Variation of cellular ATP content with physiological conditions (Chapman et al., 1971; Atkinson, 1977; Chappelle and Levin 1968)
7. The presence of free ATP of non-microbial origin (Picciolo et al., 1977)
8. Degradation of ATP by the extraction reagents.

B. Pyruvate Method

Pyruvate is a central intermediary metabolite of most bacteria found in milk. It is produced in glycolysis through the Embden-Meyerhof Parnas, the Dickens-Hoerecker and the Entner-Doudoroff pathways, the former primarily for homofermentative lactic acid producers and the latter primarily for many psychrotrophs. Pyruvate is also produced from deaminated amino acids and from free fatty acids.

The pyruvate method is based on the principle that pyruvate can be enzymatically reduced by lactate dehydrogenase and reduced nicotinamide adenine dinucleotide ($NADH_2$). The change in concentration of $NADH_2$ is measured colorimetrically at 340 nm, or it can be measured fluorometrically (Marshall and Harmon, 1978).

Tolle et al. (1976) published a report of extensive studies in which they examined numerous tests of milk including pyruvate. They concluded that the automated test for pyruvate gave promise of being highly useful in determining the quality of milk. They also found that the enzymatic determination of pyruvate is highly specific, that samples can be preserved for several days with trichloroacetic and that pyruvate is not destroyed by pasteurization. Tolle et al. (1976) also reported that quantities of pyruvate produced in a sample are directly proportional to the number of cells. The pyruvate test has the characteristic that it can detect small numbers of psychrotrophic bacteria and give more emphasis to psychrotrophs that grow rapidly and produce large amount of degradative enzymes. It might also give certain emphasis to mastitis bacteria and somatic cells in raw milk (Marshall and Harmon, 1978). A test with this characteristic is the kind of test needed in the dairy industry.

This test has been used as a quality test for grade A milk (Marshall and Harmon, 1978) and as an indicator of quality in grading nonfat dry milk (Marshall

et al., 1982a). The test also has been compared to SPC, psychrotrophic plate count and direct microscopic counting methods in milk samples. Results obtained by these investigators do not correlate with the standards from the Federal German Republic, 2.5 mg/kg as equivalent of $3X10^6/cm^3$. This concentration of pyruvate is less than one half of what American investigators observed. Marshall et al. (1982b) attributed this to the possibility that there is a higher content of pyruvate in America milk than the German milk. The test had successful applications in West German (German Federal Republic, Budesminister fur Ernahrung, Landwirtschaft und Forsch, 1980), but still needs further investigation before being used in America.

Errors with the pyruvate test generally increase as age of the sample increase, probably because certain bacteria commence to destroy pyruvate when their numbers exceed 10^7/ml (Marshall and Harmon, 1978).

C. Microcalorimetry

Bacterial growth is accompanied by heat production. The minute amount of heat produced by metabolic processes occurring in a growing bacterial culture of any contaminated material can be measured using very sensitive calorimeters (Monk and Wadso, 1968). The principle under investigation is the measurement of the energy transfer involved during the metabolic growth cycles of bacteria under controlled conditions. With the use of a pulse microcalorimeter or a differential resistance microcalorimeter one can measure on a routine basis the amount of heat emitted by molecular interactions in minute samples. This method could detect the presence of bacteria at concentrations of 10^3-10^5 organisms/ml depending upon the bacterial strain (Beezer et al., 1978). The profile of heat production versus time, or thermogram of microorganisms has been shown to be sensitive to growth condition.

The potential of microcalorimetry as an analytical tool for determining the microflora of foods was first suggested by Insalata et al. (1967). Sacks and Menefee (1972) using thermistors, studied thermal changes in canned food undergoing bacterial spoilage. They discussed the possible use and limitations of microcalorimetry as a non-destructive technique for detecting cans which were not properly processed. Microcalorimetry was applied by Cliffe et al., (1973) for estimation of bacteria in raw milk. Their studies revealed that the heat output from incubated samples correlated closely with the bacterial counts. The sensitivity of microcalorimetry for estimation of bacterial levels in milk was studied by Berridge et al., (1974). They showed that $15X10^5$ cfu/ml produced detectable heat effects. Rowley et al., (1974) found that the minimum heat production rate (HPR) detectable with their system required the presence of about 10^4 bacteria/ml. They also demonstrated a linear relationship between initial bacterial numbers in broth cultures and the times elapsing until maximun HPRs were attained. The potential of microcalorimetry as a rapid method for the estimation of bacterial levels in ground meat was studied and significant correlations between exothermic heat production rates (HPRs) and colony counts were obtained (Gram and Sogoard, 1985).

Research has been scanty for this method in foods. The change in response due to small changes in medium composition could be a serious limitation of microcalorimetry. Another limitation could be temperature changes or heat evolved from sources other than the metabolism of microorganisms.

D. Radiometry

Since CO_2 is the most commom end product of carbohydrate metabolism, its detection should be indicative of the presence of most microorganisms. CO_2 from radiolabeled substrates such as glucose has been used to rapidly detect

the presence of microorganisms. The principle of the radiometric method is based on detecting the presence of viable organisms by measuring the $^{14}CO_2$ produced in a culture medium containing utilizable ^{14}C substrates. The time required to reliably detect radioactive CO_2 is inversely related to the initial number of organisms in the sample. The uses of the method in microbiology include sterility testing, numeration and potentially microbiological assays. Enumeration is accomplished with the aid of a calibration curve that relates the number of organisms with their instrument detection time. It can be applied to estimate total aerobic counts and using selective media, specific groups and single organism counts.

The commercially available automated system, Bactec (Johnston Laboratories, Inc., Cockeysville, Md) uses the fact that when living organisms were fed appropriate ^{14} C labeled substrates, $^{14}CO_2$ will be produced during metabolism of the substrates. This radioactive gas enters the head space above the culture liquid in a sealed test vial. The Bactec instrument samples this gas and measures the amount of radioactivity in the ionization chamber. When the activity is about a threshold value (detection level) the test will indicate the presence of living bacteria in the vial. This is the basis for a sterility test. Also, the detection time is inversely proportional to the amount of bacteria . By use of proper standard curves, number of bacteria in food can be estimated. Aerobic and anaerobic counts can be made with the radiometric method by changing the gaseous environment and the media in the vials. Radiometric enumeration works well with selective media for such groups as coliforms or fecal coliforms (Bachrach and Bachrach, 1974; Hatcher et al , 1977) and with single entities such as Staphylococcus aureus, Streptococcus faecalis and yeast.

Waters (1972) reported that initial inoculum size influenced the length of the detection time. For 10^7 cells the detection is 1.5-2.5 hours, whereas for one

cell the time increased to 6 to 8 hours. Bachrach and Bachrach (1974) obtained similar results in detecting $^{14}CO_2$ produced by E coli grown in ^{14}C lactose ; they reported a detection time of 6 hours for 1-10 cells in water samples. Rowley et al., (1976) found that 6-7 hours was sufficient time to differentiate suspect from non-contaminated food samples by radiometric procedures.

Previte (1972; 1973) applied the radiometric technique to the detection of food-borne bacteria and suggested the application of this technique for sterility testing of foods. However, he recognized the need for further studies with a variety of aerobes and anaerobes from different sources, and the importance of optimizing the media used for their detection. Evancho and Ashton (1974) optimized a medium and cultural conditions for the detection of growth of clostridial species.

Some food-borne bacteria such as strains of Pseudomonas do not readily produce $^{14}CO_2$ from D-(UL-^{14}C) glucose. However, by the incorporation of 5-^{14}C glutamate and ^{14}C formate into a broth medium, Previte et al., (1975) radiometrically detected Alcaligenes faecalis, and three strains of Pseudomonas within 4-6 hours. This medium also allowed the rapid detection of many food-borne pathogens Bacillus cereus, S. aureus, Clostridium perfringens, C. botulinum and Salmonella typhimurium. Hatcher et al . (1977) used the radiometric method for estimating total microbial population in frozen concentrated orange juice. Additional labeled nutrients were added to the test medium used (Tryptic soy broth plus labeled glucose) for certain lactic acid bacteria found in orange juice that do not produce gas from glucose. Only two false positive radiometric results were observed. They suggested that fast reliable estimates of microbial contamination can be obtained radiometrically, using a 1.0 ml inoculum of orange juice sample with test vials read after 8 and 12 hours incubation. Rowley et al. (1978) used the radiometric method for rapid screening of cooked foods for

microbial acceptability. They found that 75% of a wide variety of cooked and frozen foods were correctly classified as acceptable (smaller than 10^5bacteria/g) or unacceptable (greater than 10^5 bacteria/g) within 6 hours. A minimum of one and a maximum of five of the 404 tested foods were incorrectly classified, 23% of the tested samples were classified as suspect and required confirmation of acceptability by the SPC. Rowley et al. (1979) enumerated stressed fecal coliforms from cooked meats. They concluded that radiometric techniques show future promise as rapid screening techniques for determining if a cooked food meets the microbial criterion of 0 fecal coliform/g. The test that they used included a resuscitation period of 1-3 hours to allow recovery of cells stressed by freezing or heating, respectively. In this case they were able to determine microbial acceptability within a maximum of 18 hours.

E. Electrical Impedance

Impedance is defined as electrical resistance to the flow of an alternating current being passed through a given medium. As an organism grows, various biochemical changes take place in the medium. Metabolism converts nutrients to end products and complex uncharged particles are converted into smaller, charged particles. The conversion of one molecule of the non-ionized nutrient glucose to two molecules of the ionized metabolite lactic acid by microorganisms increase the conductivity of the growth medium. During microbial growth, then, there is a general decrease in impedance, while capacitance and conductivity increase.

The presence of organisms or their end products in a circuit will alter the voltage current relationship and this will also change the impedance. In measuring impedance or other electrical characteristics the physiologic material is placed between measuring electrodes in such a way that any changes that alter

the current density distribution between the electrodes can be manifested as a change in impedance. The measured change in impedance is a function of the type and number of microorganisms, the medium in which they are growing, the frequency of the applied signal, the surface to volume ratio, the temperature and the interelectrode distance.

In practice, a multiple-well chamber cuvette with electrodes is used. Each well can accomodate one sample. The cuvette is placed in an instrument which can automatically monitor the change of impedance. Bactomatic (Princeton, N.J.) is the most popular instrument designed to measure impedance change in foods and environmental samples.

Impedance remains relatively constant until the number of microorganisms present in the incubated sample reaches a threshold of 10^6 to 10^7 organisms/ml. When this threshold level is reached, marked impedance changes occur due to changes in the ionic constituency of the medium. Fatty acids, amino acids, and organic acids produced through breakdown of fats, proteins and carbohydrates, respectively, contribute towards this change in impedance. The time required for the initial inoculum to reach the threshold level (10^6 to 10^7 cell/ml) is designated as the impedance detection time (IDT). The instrument automatically registers an IDT.

The time required for the bacteria in the sample to reach the instrument's threshold level correlates with the original bacterial number present as determined by the SPC method. After a calibration curve relating IDT levels to SPC has been established, samples are impedimetrically tested and their IDT levels are used together with the calibration curve to determine the original microbial concentration of the sample. IDT "cut off" levels are established to designate whether or not a specific sample contains microbial concentrations above or below specifications.

The success of the impedance technique depends on the quality of curves obtained with the specific sample/medium combination tested. Figure 1 presents a characteristic impedance curve. The active segment of the impedance curve starts after the establishment of a stable baseline, where the impedance curve starts accelerating. It continues until the curve starts to decline. A portion of this segment is linear and its slope is obtained as the percent change per hour. The impedance detection time (IDT) is defined as that point (in hours) where the baseline ends and the region of acceleration begins.

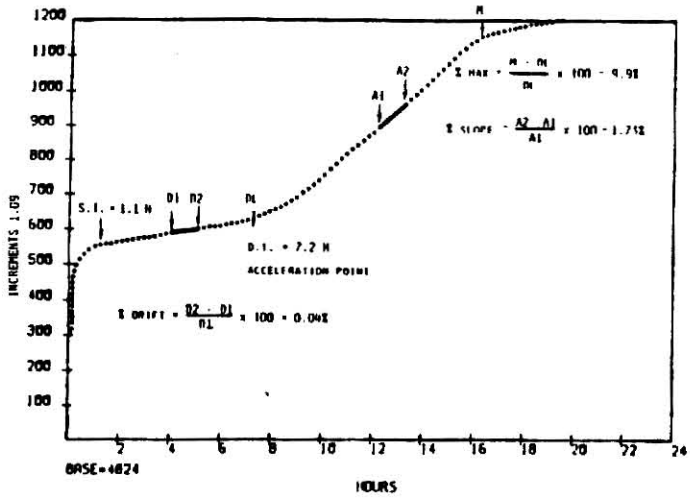
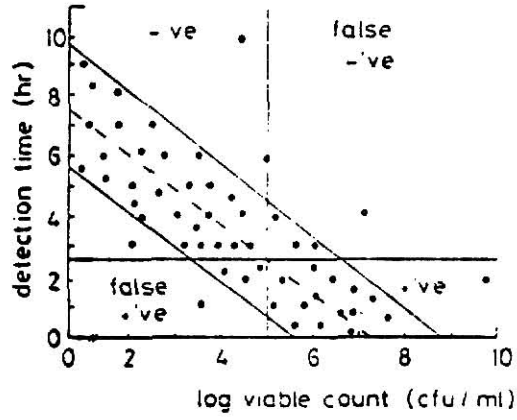


Fig 1. Parameter Definitions Associated with Impedance Curves (McElroy and DeLuca, 1981)

A characteristic scattergram (Fig 2) is produced when initial cell concentration, as measured for example by plate counts, is plotted against the impedimetric detection time. The large spread in the data comprising this scattergram may be due to error associated with the plate count, or it may arise from the impedimetric measurements. Variation in the exact composition of the

microorganisms, or types of microorganisms, within the sample , may give rise to different lag and generation times, while the impedance method assumes the same growth and lag times for all microorganisms present (Hardy et al., 1977). Furthermore, as a result of their microscopic examinations on milk flora, Gnan and Luedecke (1982) showed that different microorganisms grow to differing extents on the agar plates and the impedance media, which would also create a wide scattering of points in the scattergram. The scattergrams do not seem to possess the required accuracy for the very exact direct estimation of the number of viable cells present, but they do provide a useful quality control method, for example in food processing by choosing an appropriate cut-off time.



Fi 2. Impedimetric scattergram of detection time compared with viable cell counts. The broken line indicate an ideal response, whilst the solid diagonals indicate the limits between which, say 90% of all samples fall. The quadrants indicated give the false positive and negative regions using cut-off values of 2.6 h. and 10^5 cfu/ml (Harris and Kell, 1985).

With samples containing low numbers of microorganisms, a large volume of sample suspension must be used, to obtain large numbers of organisms. To overcome this, suspensions are preincubated, thereby allowing the organisms to multiply to concentration yielding a smaller random error in the aliquots that are subsequently transferred to wells of the instrument. The preincubation period should be chosen to permit the microbial population to reach a level to introduce

at least 30 microorganisms into each well (Firstenberg-Eden and Eden, 1984).

Hardy et al. (1977) achieved a 96% agreement between Standard Plate Counts and Bactometer in quantifying the microbial contamination of 367 frozen vegetable samples. They reported that the impedance measurements themselves did not appear to interfere with microbial growth, and found that the threshold concentration of 10^6-10^7 CFU/ml necessary for detection was attained in 5 hours for a sample initially containing 10^5 CFU/ml. Several investigators (Gnan and Luedecke, 1982; Cady et al., 1978; O'Connor, 1979) have attempted to develop an impedimetric method for the estimation of total counts (SPC) in raw milk. All workers mixed the milk (at a 1:1 ratio) with 10% yeast extract or BHI and incubated 32°C. Inconsistent results and low correlation coefficients (below 0.8) were obtained in these studies. Gnan and Luedecke (1982) observed that samples with a high SPC and low impedance detection time were associated with entirely different flora than those with low SPC counts and short IDT's. They speculated that the impedance method detected psychrotrophs and mesophiles in the former case and mesophiles in the later. Later Firstenberg-Eden and Tricarico (1983) showed that mesophiles often have generation times 1/4 to 1/5 as long as psychrotrophs do. They found that 18°C is a better temperature for the impedance measurement in milk since it allows similar generation times of psychrotrophs and mesophiles. A coliform medium for use with impedance measurement was develop (Firstenberg-Eden and Klein, 1983). The medium was evaluated in the estimation of coliforms in dairy products and the same information was obtained for confirmed Violet Red Bile agar medium counts.

Comparative studies in meat with the impedance method and standard plate count resulted in poor correlation coefficients (Firstenberg-Eden, 1982; Martins and Selby, 1980). Firstenberg-Eden (1983) noted that diluents in which meat is stomached may affect impedance curve and the impedimetric detection time data.

She noted that samples stomached directly into growth media give superior signals and earlier detection compared to those stomached into diluent and subsequently mixed with growth medium.

Impedance method studies in fish were done by Gibson and Ogden (1984). They used a temperature of 20°C for SPC and 37°C for selective counts (E. coli coliforms and vibrios) and Nutrient Broth Seawater broth and Trimethylamine Oxide Medium. The use of trimethylamine medium resulted in a better correlation (0.89). Impedance has been used for detection of yeast in fruit juices (Zindulus, 1984) and for detection of lactic acid bacteria in fruit juices (Schaertel and Firstenberg-Eden, 1985)

The Bactometer M123 Microbial Monitoring System (Bactomatic, Princeton N.J.) is the instrument that most researchers have been using. The Malthus Microbiological Growth Analysis (Malthus Instruments) is another impedimetric instrument on the market.

F. Catalase Method.

Bacteria commonly associated with foods can be divided into catalase-positive and catalase-negative bacteria. Each group contains important food-borne organisms. A bacterial culture is considered catalase-positive when bubbles are generated when a drop of 3% H_2O_2 is placed on the colony. Catalase-negative culture will not generate gas bubbles. A semi-quantitative scale using capillary tubes for catalase activity was described by Fung and Petrishko (1973). Different bacterial species have varying degrees of catalase activity and this concept has been used to monitor catalase activity in certain foods.

Gagnon et al. (1959) and Charbonneau et al. (1975) used the principle of disc flotation method to quantitatively measure catalase activity of bacterial cultures. A catalasemeter was introduced commercially by Bio-Engineering Groups

Ltd. (Westport, Conn, USA). Dodd et al. (1983) used the catalasemeter to monitor vacuum-packaged comminuted cooked turkey and found that the catalasemeter results correlated well with a specific number of organisms but it was not sensitive below 10^4 organisms/ g of meat.

Fung (1985) introduced a pasteur pipette method to measure catalase. Wang and Fung (1985) tested both the catalasemeter method and the Pasteur pipette method on laboratory cultures and cold storaged chicken. In the study of catalase producing ability of eight species of both catalase-positive and catalase-negative bacteria by both catalasemeter with the paper disc method and the gas column method, they found that different species of catalase-positive bacteria had different catalase producing ability. Catalase-negative bacteria gave no response in both the gas column method and catalasemeter with paper disc method. Among the bacteria that they tested, Micrococcus luteus had the strongest catalase producing ability. S. aureus and Pseudomonas fluorescens were second, E. coli and Bacillus cereus were next and S. typhimurium had the least catalase producing organisms. The potential exists for the use of catalase to estimate microbial loads on certain foods. More research and development are needed to be done to use the catalase methods for routine analysis.

G. Turbidity

When a beam of light (the incident ray) passes through a cell suspension at a certain angle (the incident angle) the light can be absorbed, reflected, refracted, or diffracted. The amount of light or angle of the emergent ray can be quantitatively detected by a photo multiplier tube and translated into various units. The intensity, wavelength, and angle of the incident beam can be manipulated. The Autobac Instruments, (Charles Pfizer, Groton, Conn.), the

MS2 (Abbot Laboratories Diagnostic Division, Dallas), and the AutoMicrobic System (Vitek Systems Inc., St. Louis Mo.) are examples of commercially available instruments using turbidimetric and light-scattering measurements with a fixed angle beam.

Routine photometric determinations as commonly executed in the laboratory measure, indirectly, the amount of light scattered by microorganisms in suspension. They do so by recording the amount of light that is absorbed or transmitted by the sample, rather than that which is actually scattered. This provides a relatively quick and convenient way of estimating cell concentration. Probably the most fully automated photometric system for monitoring relatively sparse microbial populations is the AutoMicrobic System (AMS, Vitek Systems Inc., St. Louis, Mo). This automated and computerized system was developed by Aldridge et al. (1977) for the detection, enumeration and identification of bacteria and yeast. The biological basis for the system resides in lyophilised, selective media enclosed in tiny wells of a disposable plastic card. The introduction of the sample into these tiny wells both rehydrates and inoculates the media. An automated optical system monitors the change in the light transmission by solid state optics. This system does not require lenses or optical condensers, and uses an array of light-emitting diodes with a peak emission of 665 nm. The computer interprets any optical changes within the sample, and provides enumeration within 8 - 13 hours. The use of selective media enhances the detection time of individual target organisms. The effect of bubbles produced by the microorganisms during growth is eliminated by scanning the entire sample-containing card.

The sensitivity of the AMS has been extensively studied using urine specimens. The AMS can detect $7X10^4$ colony-forming units (cfu)/ml, with a 92 % agreement with plate counts. The detection time varied from 4 to 13 hours,

depending on the bacteria present and its concentration (Aldridge et al., 1977). This instrument has been evaluated in the identification Enterobacteriaciae in foods (Bailey et al., 1985), but it has not been used in the enumeration of microorganisms in foods.

Another automated system for use in clinical work that measures optical density using light-emiting diodes is the ABBOT MS-2. It monitors the growth in a sample at 5 minute intervals and is able to enumerate and identify the microorganisms present with the use of specific and selective media.

III. CORRELATION BETWEEN MODERN METHODS AND STANDARD PLATE COUNT

Every new quantitative technique is eventually compared with existing standard methods. In most cases this entails regression analysis to measure appropriate correlation and calculation of confidence limits for such comparisons. The comparison becomes more difficult with non-counting methods (microcalorimetry, impedance, bioluminescence, radiometry, etc), since the different procedures are based on different assumptions. In reviewing the new microbiological technologies one must raise the question of whether the current counting methods are the best, or even appropriate for estimating the microbial quality of products. It might very well be that a new method can better estimate shelf-life, safety, and acceptability of food products than the standard counting methods.

Because of its historical status as the reference method, many microbiologists tend to regard the results of the standard plate count as absolute. The true relationship between the standard plate count and the actual number of viable microorganisms must, however, be carefully evaluated in terms of the limitations associated with this method. Those limitations are summarized below.

1. Microorganisms naturally exist in clusters or clumps of viable units which tend to resist fractionation into single cells during the blending or stomaching processes used to prepare samples. Therefore, each colony subsequently formed on agar may be actually derived from one, two or even twenty or more cells. The actual degree of separation of clumps depends on the sample type, the endogenous microflora, and the preparation process. Thus there is only a very vague relationship between numbers of microbes in the sample potentially able to form colonies and colonies actually counted.

2. To be enumerated, a specific microorganism must be capable of forming

a visible colony under conditions employed in the assay. This requires that all organisms counted thrive and reproduce under the medium and temperature conditions employed, and within the incubation period of the assay. Since there is no universally acceptable medium/temperature/time combination, no plate count method can be expected to include all microorganisms originally present in a sample.

3. Sampling errors inherent in any procedure where different analysts are involved in weighing samples, pipetting dilutions, and enumerating colonies also contribute to the inaccuracy of test results.

Despite these acknowledged limitations of the standard plate count method, it remains the reference method and any automated procedure proposed as an alternative must correlate with it. In this Chapter correlations are given only for modern methods that have been employed in a wide range of foods or new methods that have high potential to replace SPC or a high potential to be used in the food industry. Methods that require more than 24 hours to obtain results are not included because they are not classified as rapid techniques.

A. Correlation With Impedimetric Detection Times

One major advantage of the impedance method is that no pretreatment of sample is necessary. The time to complete the test is variable depending on the sample, kind of bacteria, and amount of contamination. The temperature of incubation depends on the kind of microorganisms (Table 4) for total count which ranges from 30°-37°C for mesophiles and 18°-21°C for psychrotrophs. Although comparative studies were made between impedance method and standard plate count for about 10 years, only in recent years (1980's) relevant data were published for food microbiology.

The impedance method has been successful in the enumeration of

Table 4. Correlation Between Impedimetric Detection Times and SPC

Time of Incubation	M.O.[1] Tested	Temperature	Food Tested	Results	References
4 h	Psychrotroph	21°C	milk	r=0.73	Cady et al. 1978
7-3 h	Mesophilic	32°C	milk	r=0.6	Cady et al. 1978
8.5 h	Total count	32°C	milk	r=0.68	O'Connor, 1979.
24 h	Total count	35°C	meat	r=0.68[2]	Martins and Selby, 1980
24 h	Total count	20°C	fish	r=0.89	Gibson and Ogden, 1980
-	Total count	32°C	milk	r=0.80	Gnan and Luedecke, 1982
14 h	Total count	32°C	milk	r=0.55	Martins et al., 1982
24 h	Psychrotrophic	18°C	milk	r=0.96	Firstenberg-Eden et al., 1983
~4.2	Total count	30°C	meat	r=0.97	Firstenberg-Eden, 1983
9 h	Total count	35°C	dairy products	r=0.91-0.95	Firstenberg-Eden et al., 1984
~2	Total count	30°C	meat	r=0.91[3]	Bulte and Reuter, 1984
24	Lactic acid bact.	32°C	orange juice	r=0.93	Schaertel and Firstenberg-Eden, 1985

[1] M. O. Microorganisms tested

[2] Method compared with MPN and combined MPN with impedance.

[3] Compared with drop plating method.

microorganisms in meat and beverages. Improvements of the culture media used in the impedance method (after 1983) have increase strength of correlations with standard plate count. Impedance method seems quite promising for food microbiology. Although it is slower than some of the other modern methods, such as ATP and radiometric, it does not require complicated culture and sample preparation. In summary, advantages of the impedance method include ease to use, reduction in analysis time, ability to function in opaque media, and ability to handle large numbers of samples, and a high level of agreement with conventional counting techniques.

B. Correlation with Radiometric Techniques.

Since the radiometric method was introduced by Deland and Wagner (1969), it has been used mainly in clinical microbiology. Previte (1972) used the method for the detection of some food-borne bacteria and recommended the application of the technique for sterility testing of foods. Development of suitable media and best conditions for the detection of microorganisms by the radiometric technique continued until this time (Previte et al., 1972; Previte and Rowley, 1973; Evancho and Ashton, 1974; and Previte et al., 1975). These studies concentrated on development of suitable media for the estimation of food-borne microorganisms by radiometric techniques.

Rowley et al. (1974) applied the method to the detection of indigenous flora of meat loaf. He obtained a of correlation (r=0.97) between the logarithm of the concentration of microorganisms and the radiometric detection time, and suggested the use of the radiometric method instead of the SPC. Advantages of this technique include no sample preparation and less than 18 hours to obtain final results. Stressed microorganisms need a resuscitation period of about 3 hours in this system (Rowley et al., 1979). Table 5 shows the correlation of radiometric

Table 5. Relationship Between Radiometric Method and SPC

Analysis time	Bacteria Tested	Temperature	Food tested	Results	References
11 h	Coliform	37°C	Precooked meat loaf	r=0.97	Rowley et al., 1974
6-12 h	Total count	30°C	Frozen concent. juice (600 samples)	44 (+) Radio. 42 (+) SPC	Hatcher et al., 1977
6 h	T~tal count	37°C	Cooked food	75% correct 23% suspect 2% incorrect	Rowley et al., 1974
17-18h*	Stressed colif.	42°-45°C	Meat loaf	100% agreement[1]	Rowley et al. , 1979

* Resuscitation period of 3 hours.

[1] Compared to impedance.

method results with standard plate count and in one case the impedance method.

C. Correlation with ATP Method

Early attempts to estimate the microbial content of foods by microbial ATP assay were frustated because of the interference of large amounts of non-microbial ATP in foods (Sharpe et al., 1970; Baumgart, 1980.). Sharpe et al. (1970) studied the assay of microbial ATP as a rapid method of estimating microorganisms in foods. They concluded that the method was unlikely to be successful unless microorganisms could be separated from foods. Bossuyt (1981) suggested the use of enzyme (ATPase) to destroy non-microbial ATP that opened the way for better correlation coefficients with conventional methods and stimulated research of ATP methods (Table 6) in food microbiology.

Although average ATP/bacterial cell values of 0.5 and 2.0 Fg (1 $Fg=10^{-15}$ g) have been used for enumerating mixed microbial populations in the environment (D'Eustachio et al., 1968; Stannard and Wood, 1983), values varying from 0.01 Fg from Flavobacillus proteus (Hysert et al., 1976) to 6.5 Fg for Chromobacterium marinum (Hamilton and Holm-Hansen, 1967) have been reported. When yeast cells and bacteria cells are present in a food (e. g. orange juice) the results essentially will represent yeast population because yeast has approximately 100 times more ATP than bacteria. The ATP content of bacterial cells also varies with the physiological state of the bacteria and an activation period of at least 30 minutes has been recommended for equilibration of ATP/cell levels before bioluminescence measurements (Bossuyt, 1981; Graumlich, 1985)

The accuracy of bioluminescent estimation of microbial levels in foods or other materials is generally predicated upon low variations in ATP/cfu values, particularly if conversion of ATP measurement to cfu is based simply upon dividing ATP values by an ATP/cfu value. However, some authors (Stannard and

Table 6. Correlation Between ATP Method and cfu/ml or g

Pretreatment	Food tested	Microorganism Tested/ml or g	correlation agreement	Interference Extrinsic ATP	References
no	meat, dairy prod. soup mix, dressings	total count	not reported	yes	Sharpe et al., 1970
no	meat	total count	not reported	yes	Baumgart et al., 1980
Enzymatic	milk	total count	r=0.93	no	Bossuyt, 1981
Enzymatic	meat	total count	r=0.79-0.80	no	Carlier et al., 1982
Dual treatment[1]	meat	total count	r=0.94	no	Stannard and Wood, 1983
Dual treatment	Reconstituted juice active m.o populat.	yeast	r=0.92	no	Graumlich, 1985
Dual treatment	Juice inmediately reconstituted	yeast	r=0.58	no	Graumlich, 1985
Enzymatic	ground meat	total count	r=0.86-0.99	no	Kennedy and Oblinger, 1985
Dual filtration and enzyme	meat	total count	80-100% agreement	no	Littel, 1985
Filtration	Carbonated beverages	yeast	r=0.85	no	Littel, 1985

[1] Centrifugation, stirring with cation exchange resin and filtration

Wood, 1983; Kennedy and Oblinger, 1985; and Graumlich, 1985) indicate that estimates based upon regression equations over a broad range of cfu values are very accurate despite variation in ATP/cfu.

Despite all the disadvantages of the method, good correlations had been obtained in the last years. This means that with a good pretreatment or elimination of non-microbial ATP, the method will give reliable results. An advantage of this method over the other methods is that results are obtained within one hour.

D. Future

The use of automated analytical techniques and computer handling of data will make microbiological testing more automated and less dependent on human error than it is today. Techniques are available for the extremely sensitive detection of chemical compounds (ATP and Impedance) and of energy changes (microcalorimetry).

Many of the current rapid microbiological methods are largely in the developmental phase. Many of the concepts are predominantly designed to correlate with standard plate count. The next generation of automation will address the question of detecting specific pathogen and toxins such as Salmonella and Staphylococcus aureus enterotoxins.

A new rapid microbiological technique will have to be equal to a standard method or superior to it in a) sensitivity, b) validity and c) reproducibility. Fung (1983) listed all the important attributes that an ideal automated system should have (Table 7). As the science of microbiology progresses, new exotic techniques will be developed and eventually will be placed in the applied microbiology laboratory in the future.

Present day enumeration methods play an important role in defining the

Table 7. Attributes for an Ideal Autmated Food Microbiology Assay System [a]

1. Accuracy for intended purpose
   - Sensitivity: Minimal detectable limits
   - Specificity of test system
   - Versatility: potential applications
   - Comparison to referenced methods

2. Speed - Productivity
   - in obtaining results
   - number of samples processed per run; per day

3. Cost
   - Initial
   - Per test
   - Reagents
   - Other

4. Acceptability
   - By scientific community
   - By regulatory agencies

5. Simplicity of Operation
   - Sample preparation
   - Operation of test equipment
   - Computer versatility

6. Training
   - On site; how long
   - Quality of training personnel

7. Reagents
   - Reagent preparation
   - Stability
   - Availability
   - Consistency

8. Company Reputation

9. Technical Services
   - Speed and availability
   - Scope of technical background
   - Cost

10. Utility and space requirements

[a] from Fung (1983)

wholesomeness of foods. Rapid methods are designed to help monitor microbial load of foods on a routine basis. In quality control work, rapid methods can help the food industry make corrective measures much faster than currently possible. Also, food-borne disease organisms and their metabolites may be detected much faster thus, helping prevent large scale food-borne disease outbreaks. The quest for rapid methods in microbiolology must continue. This report described the current state of viable cell count and the relationship between the conventional method with several promising methods. It is the hope of this author that this report has updated pertinent information and will help stimulate more research in this area.

CONCLUSIONS

1. The Standard Plate Count method is still the most important procedure for monitoring viable cell numbers in foods.

2. By use of improved methodologies, the Standard Plate Count method can be made easier to operate.

3. By the development of ingenious methods such as impedance measurements, radiometric determination and ATP analysis, the microbial loads of foods can be obtained in much shorter time and automatically.

4. The future of automation is bright and should be explored.

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ESTIMATION OF VIABLE CELL COUNT BY MODERN AND IMPROVED METHODS

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ABSTRACT

Limitations and improvement in automation of the standard plate count, and rapid methods that have been used to monitor microbial numbers in foods have been reviewed . Standard plate count is a technique that has been used to monitor microbial loads in foods for about 100 years. The standard plate count technique is based on the principle that each viable organism will grow into one visible colony. This method measures the number of the entire population of viable microorganisms present in a food. Since standard plate count cannot satisfactorily monitor samples with very low counts, the Most Probable Number and membrane filtration technique were developed. Hydrophobic grid membrane filters and direct epifluorescent filter are modifications of conventional membrane filters.

There are many reasons why standard plate count fails to provide absolute data. One is the coherence of microorganisms; another factor is the inability of microbial counting media to support the growth. Errors of standard plate may also arise due to variations in weighing, pipetting and counting. To avoid these problems and to improve the accuracy of the results, miniaturization and mechanization of the method have been done.

Conventional methods for detecting and monitoring bacteria, yeast, and other microorganisms usually include agar plate counting techniques which generally take from one to five days. One of the main goals of microbiology is to quantitatively detect bacteria without having to rely on time-consuming culture techniques. To eliminate this delay, rapid, sensitive and selective techniques have been developed. Those rapid methods include limulus amoebocyte lysate, DNA method, ATP method, pyruvate method, microcalorimetry, radiometry, electrical impedance, catalase method, and turbidity. This report describes each method, their limitations, and how succescful those methods have been in monitoring micoorganisms in foods.

Many of the current methods for rapid monitoring microbial loads in foods are largely in the development phase. Despite their limitations, some of them have been succescfully used to monitor microbial numbers in foods. The conclusion of this review indicates that these rapid methods have a bright future in monitoring microbial loads in foods.